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Attorney Docket **P1093P1D1**
PATENT

CERTIFICATION UNDER 37 CFR 1.10

EL599584388US Express Mail Number **November 27, 2000 Date of Deposit**

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Eileen Ly

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

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Title: **ANTI-VEGF ANTIBODIES**

1. Type of Application

[ ] This application is for an original, non-provisional application.

[ ] This is a non-provisional application claiming priority to provisional application no. __ , filed ___, the entire disclosure of which is hereby incorporated by reference.

[X] This is a [ ] continuation-in-part [ ] continuation [X] divisional application claiming priority to application Serial Number 08/908,469 , filed August 6, 1997 , the entire disclosure of which is hereby incorporated by reference.

2. **Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)**

80 pages of specification
5 pages of claims
1 page(s) of abstract
16 sheet(s) of drawings
[] formal [X] informal

#83589

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)
___ An executed declaration of the inventor(s) [] is enclosed [] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)
__X__ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)
___ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)
___ An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [] will follow.

(for cont./div.)
__X__ The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

__X__ Cancel in this application original claims 1-38 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

_____ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

__X__ Amend the specification by inserting before the first line the sentence:

--This is a

_____ non-provisional application
_____ continuation
__X__ divisional
_____ continuation-in-part

of co-pending application(s)

__X__ Serial No. 08/908,469 filed on August 6, 1997, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --
_____ International Application _ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

#83589

_____ provisional application No. _ filed _, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--.

6. Payment of Fees

__X__ Applicants request deferral of payment of the filing fee until submission of the missing parts of application. **DO NOT CHARGE THE FILING FEE AT THIS TIME.**

7. Additional Papers Enclosed

[] Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
[] Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
[X] Associate Power of Attorney.
[] Other:

8. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

_____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
_____ A copy of the petition for extension of time in the ***prior*** application is attached.

9. Correspondence Address:

__X__ Address all future communications to:

Attn: Steven X. Cui
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Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000

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PATENT TRADEMARK OFFICE

#83589

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ANTI-VEGF ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of co-pending U.S. Application Serial No. 08/833,504, filed April 7, 1997, which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to anti-VEGF antibodies and, in particular, to humanized anti-VEGF antibodies and variant anti-VEGF antibodies.

Description of Related Art

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun

et al). The negative regulators so far identified include thrombospondin (Good *et al. Proc. Natl. Acad. Sci. USA.* 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039(1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

SUMMARY OF THE INVENTION

This application describes humanized anti-VEGF antibodies and anti-VEGF antibody variants with desirable properties from a therapeutic perspective, including strong binding affinity for VEGF; the ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and the ability

to inhibit VEGF-induced angiogenesis *in vivo*.

The preferred humanized anti-VEGF antibody or variant anti-VEGF antibody herein binds human VEGF with a K_d value of no more than about 1×10^{-8}M and preferably no more than about 5×10^{-9}M. In addition, the humanized or variant anti-VEGF antibody may have an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*. The humanized or variant anti-VEGF antibodies of particular interest herein are those which inhibit at least about 50% of tumor growth in an A673 *in vivo* tumor model, at an antibody dose of 5mg/kg.

In one embodiment, the anti-VEGF antibody has a heavy and light chain variable domain, wherein the heavy chain variable domain comprises hypervariable regions with the following amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129). For example, the heavy chain variable domain may comprise the amino acid sequences of CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR;SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV;SEQ ID NO:3). Preferably, the three heavy chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4.

The invention further provides an anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYX_1FTX_2YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSK STAYLQMNSLRAEDTAVYYCAKYPX_3YYGX_4SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T. One particularly useful heavy chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the heavy chain variable domain sequence of SEQ ID NO:7. Such preferred heavy chain variable domain sequences may be combined with the following preferred light chain variable domain sequences or with other light chain variable domain sequences, provided that the antibody so produced binds human VEGF.

The invention also provides preferred light chain variable domain sequences which may be combined with the above-identified heavy chain variable domain sequences or with other heavy

chain variable domain sequences, provided that the antibody so produced retains the ability to bind to human VEGF. For example, the light chain variable domain may comprise hypervariable regions with the following amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6). Preferably, the three light chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4.

In one embodiment, the invention provides a humanized anti-VEGF antibody light chain variable domain comprising the amino acid sequence:
DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L. One particularly useful light chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the light chain variable domain sequence of SEQ ID NO:8.

The invention also provides a variant of a parent anti-VEGF antibody (which parent antibody is preferably a humanized or human anti-VEGF antibody), wherein the variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of the heavy or light chain variable domain of the parent anti-VEGF antibody. The variant preferably has one or more substitution(s) in one or more hypervariable region(s) of the anti-VEGF antibody. Preferably, the substitution(s) are in the heavy chain variable domain of the parent antibody. For example, the amino acid subsition(s) may be in the CDRH1 and/or CDRH3 of the heavy chain variable domain. Preferably, there are substitutions in both these hypervariable regions. Such "affinity matured" variants are demonstrated herein to bind human VEGF more strongly than the parent anti-VEGF antibody from which they are generated, *i.e.*, they have a K_d value which is significantly less than that of the parent anti-VEGF antibody. Preferably, the variant has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower, preferably at least about 20 fold lower, and most preferably at least about 50 fold lower, than that of the parent anti-VEGF antibody. One particularly prefered variant is the Y0317 variant of Example 3, which has a CDRH1 comprising the amino acid sequence:GYDFTHYGMN (SEQ ID NO:126) and a CDRH3 comprising the amino acid sequence:YPYYYGTSHWYFDV (SEQ ID

NO:127). These hypervariable regions and CDRH2 are generally provided in a human framework region, *e.g.*, resulting in a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:116. Such heavy chain variable domain sequences are optionally combined with a light chain variable domain comprising the amino acid sequence of SEQ ID NO:124, and preferably the light chain variable domain amino acid sequence of SEQ ID NO:115.

Various forms of the antibody are contemplated herein. For example, the anti-VEGF antibody may be a full length antibody (*e.g.* having an intact human Fc region) or an antibody fragment (*e.g.* a Fab, Fab' or $F(ab')_2$). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of VEGF protein comprising exposing a sample suspected of containing the VEGF protein to the anti-VEGF antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the VEGF protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.* from the host cell culture medium). The invention also provides a composition comprising the anti-VEGF antibody and a pharmaceutically acceptable carrier or diluent. The composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a tumor or retinal disorder, comprising administering a therapeutically effective amount of the anti-VEGF antibody to the mammal.

Brief Description of the Drawings

Figs. 1A and 1B depict the amino acid sequences of variable heavy domain (SEQ ID NO:9) and light domain (SEQ ID NO:10) of muMAbVEGF A.4.6.1, variable heavy domain (SEQ ID NO:7)

and light domain (SEQ ID NO:8) of humanized F(ab) (F(ab)-12) and human consensus frameworks (hum III for heavy subgroup III (SEQ ID NO:11); humκ1 for light κ subgroup I (SEQ ID NO:12)). Fig. 1A aligns variable heavy domain sequences and Fig. 1B aligns variable light domain sequences. Asterisks indicate differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. Complementarity Determining Regions (CDRs) are underlined.

Fig. 2 is a ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in brown with CDRs in tan. The sidechain of residue L46 is shown in yellow. VH domain is shown in purple with CDRs in pink. Sidechains of VH residues changed from human to murine are shown in yellow.

Fig. 3 depicts inhibition of VEGF-induced mitogenesis by humanized anti-VEGF F(ab)-12 from Example 1. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6 X 10^3 cells/well in six well plates, as described in Example 1. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1; F(ab)-12) was added at the indicated concentrations. After 2-3 hours, rhVEGF165 was added at the final concentration of 3 ng/ml. After five or six days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

Fig. 4 shows inhibition of tumor growth *in vivo* by humanized anti-VEGF F(ab)-12 from Example 1. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2 x 10^6 per mouse. Starting 24 hours after tumor cell inoculation, animals were injected with a control MAb, muMAb VEGF A4.6.1 or rhuVEGF MAb (IgG1; F(ab)-12) twice weekly, intra peritoneally. The dose of the control Mab was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated (n = 10). Four weeks after tumor cell injection, animals were euthanized and tumors were removed and weighed. *: significant difference when compared to the control group by ANOVA ($p < 0.05$).

Figs. 5A and 5B show the acid sequences of the light and heavy variable domains respectively of murine antibody A4.6.1 (SEQ ID NO:10 for the VL and SEQ ID NO:9 for the VH) and humanized A4.6.1 variants hu2.0 (SEQ ID NO:13 for the VL and SEQ ID NO:14 for the VH) and hu2.10 (SEQ ID NO:15 for the VL and SEQ ID NO:16 for the VH) from Example 2. Sequence numbering is according to Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed.

Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain κ subgroup I consensus framework (SEQ ID NO:12) and heavy chain subgroup III consensus framework (SEQ ID NO:11). hu2.10 was the consensus humanized clone obtained from phage sorting experiments described herein.

Fig. 6 depicts framework residues targeted for randomization in Example 2.

Fig. 7 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in *supE E. coli)*, then the C-terminal region of the gene III protein (residues 249-406). Transformation into F^+ *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequence encoded thereby (SEQ ID NO:100).

Figs. 9A and 9B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants in Example 3, compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. Residues are numbered sequentially in the VL and VH domains, as opposed to the Kabat numbering scheme. The template molecule, MB1.6 (SEQ ID NO's 101 and 102 for light and heavy variable domains, respectively) is shown, along with variants: H2305.6 (SEQ ID NO's 103 and 104 for light and heavy variable domains, respectively), Y0101 (SEQ ID NO's 105 and 106 for light and heavy variable domains, respectively), and Y0192 (SEQ ID NO's 107 and 108 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Figs. 10A and 10B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants from Example 3 compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains,

respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. The variants are designated Y0243-1 (SEQ ID NO's 109 and 110 for light and heavy variable domains, respectively), Y0238-3 (SEQ ID NO's 111 and 112 for light and heavy variable domains, respectively), Y0313-1 (SEQ ID NO's 113 and 114 for light and heavy variable domains, respectively), and Y0317 (SEQ ID NO's 115 and 116 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Fig. 11 depicts the results of the HuVEC activity assay in Example 3 for variants Y0238-3, Y0192 and Y0313-1 as well as full length F(ab)-12 from Example 1.

Fig. 12 depicts inhibition of VEGF-induced mitogenesis by full length F(ab)-12 from Example 1 (rhuMAb VEGF), a Fab fragment of F(ab)-12 from Example 1 (rhuFab VEGF), and a Fab fragment of affinity matured variant Y0317 from Example 3 (rhuFab VEGF (affinity matured)).

Detailed Description of the Preferred Embodiments

I. Definitions

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an

extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the

constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof . Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci.*

USA 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences (*e.g.* as in SEQ ID NO:7 or 8), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for

the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Mol. Cell. Biol.* 5(12):3610-3616(1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.*, *Protein Engineering* 3(6):547-553(1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the

function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containingprodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed

partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.,* agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by

ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. Modes for Carrying out the Invention

The examples hereinbelow describe the production of humanized and variant anti-VEGF antibodies with desirable properties from a therapeutic perspective including: (a) strong binding affinity for the VEGF antigen; (b) an ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro;* and (c) the ability to inhibit VEGF-induced angiogenesis *in vivo*.

Antibody affinities may be determined as described in the examples hereinbelow. Preferred humanized or variant antibodies are those which bind human VEGF with a K_d value of no more than about 1×10^{-7}M; preferably no more than about 1×10^{-8}M; and most preferably no more than about 5×10^{-9}M.

Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select humanized or variant antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits endothelial cell growth in response to VEGF. In one embodiment, the antibody may be able to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). Preferably, the antibody has an effective dose 50 (ED50) value of no more than about 5nM, preferably no more than about 1nM, and most preferably no more than about 0.5nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", *i.e.*, at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by 50%. A preferred "endothelial cell growth assay" involves culturing bovine adrenal cortex-derived capillary endothelial cells in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described in Example 1 below. These endothelial cells are seeded at a density of 6×10^3 cells per well, in 6-well plates in growth medium. Either parent anti-VEGF antibody (control), humanized or variant anti-VEGF antibody is then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified VEGF was added to a final concentration of 3 ng/ml. For specificity control, each antibody may be added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells are dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Data may be analyzed by a four-parameter curve fitting program (KaleidaGraph).

The preferred humanized or variant anti-VEGF antibody may also be one which has *in vivo* tumor suppression activity. For example, the antibody may suppress the growth of human A673 rhabdomyosarcoma cells or breast carcinoma MDA-MB-435 cells in nude mice. For *in vivo* tumor studies, human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) or MDA-MB-435 cells (available from the ATCC) are cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics as described in Example 1 below. Female BALB/c nude mice, 6-10 weeks old, are injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals are then treated with the humanized or variant antibody and a control antibody with no activity in this assay. The humanized or variant anti-VEGF MAb is administered at a dose of 0.5 and/or 5 mg/kg. Each MAb is administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Tumor size is determined at weekly intervals. Four weeks after tumor cell inoculation, animals are euthanized and the tumors are removed and weighed. Statistical analysis may be performed by ANOVA. Preferably, the antibody in this "*in vivo* tumor assay" inhibits about 50-100%, preferably about 70-100% and most preferably about 80-100% human A673 tumor cell growth at a dose of 5mg/kg.

In the preferred embodiment, the humanized or variant antibody fails to elicit an immunogenic response upon administration of a therapeutically effective amount of the antibody to a human patient. If an immunogenic response is elicited, preferably the response will be such that the antibody still provides a therapeutic benefit to the patient treated therewith.

The humanized or variant antibody is also preferably one which is able to inhibit VEGF-induced angiogenesis in a human, *e.g.* to inhibit human tumor growth and/or inhibit intraocular angiogenesis in retinal disorders.

Preferred antibodies bind the "epitope A4.6.1" as herein defined. To screen for antibodies which bind to the epitope on human VEGF bound by an antibody of interest (*e.g.*, those which block binding of the A4.6.1 antibody to human VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

The antibodies of the preferred embodiment herein have a heavy chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein "FR1-4" represent the four framework regions and "CDRH1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. Many human antibody framework region sequences are compiled in Kabat *et al.*, *supra*, for example. In one preferred embodiment, the variable heavy FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human heavy chains subgroup III (*e.g.* as in SEQ ID NO:11).

The human variable heavy FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding nonhuman residue (by "corresponding nonhuman residue" is meant the nonhuman residue with the same Kabat positional numbering as the human residue of interest when the human and nonhuman sequences are aligned), but replacement with the nonhuman residue is not necessary. For example, a replacement FR residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable heavy FR residues which may be substituted include any one or more of FR residue numbers: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H, 94H (Kabat residue numbering employed here). Preferably at least two, or at least three, or at least four of these residues are substituted. A particularly preferred combination of FR substitutions is: 49H, 69H, 71H, 73H, 76H, 78H, and 94H.

With respect to the heavy chain hypervariable regions, these preferably have amino acid sequences as follows:

CDRH1

$GYX_1X_2X_3X_4YGX_5N$ (SEQ ID NO:117), wherein X_1 is D, T or E, but preferably is D or T; X_2 is F, W, or Y, but preferably is F; X_3 is T, Q, G or S, but preferably is T; X_4 is H or N; and X_5 is M or I, but preferably is M.

CDRH2

WINTX$_1$TGEPTYAADFKR (SEQ ID NO:118), wherein X$_1$ is Y or W, but preferably is Y.

CDRH3

YPX$_1$YX$_2$X$_3$X$_4$X$_5$HWYFDV (SEQ ID NO:119), wherein X$_1$ is H or Y; X$_2$ is Y, R, K, I, T, E, or W, but preferably is Y; X$_3$ is G, N, A, D, Q, E, T, K, or S, but preferably is G; X$_4$ is S, T, K, Q, N, R, A, E, or G, but preferably is S or T; and X$_5$ is S or G, but preferably is S.

The heavy chain variable domain optionally comprises what has been designated "CDR7" herein within (*i.e.* forming part of) FR3 (see Figs. 9B and 10B), wherein CDR7 may have the following amino acid sequence:

CDR7

X$_1$SX$_2$DX$_3$X$_4$X$_5$X$_6$TX$_7$ (SEQ ID NO:120), wherein X$_1$ is F, I , V, L, or A, but preferably is F; X$_2$ is A, L, V, or I, but preferably is L; X$_3$ is T, V or K, but preferably is T; X$_4$ is S or W, but preferably is S; X$_5$ is S, or K, but preferably is K; X$_6$ is N, or S, but preferably is S; and X$_7$ is V, A, L or I, but preferably is A.

The antibodies of the preferred embodiment herein have a light chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein "FR1-4" represent the four framework regions and "CDRL1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. In one preferred embodiment, the variable light FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human kappa light chains subgroup I (*e.g.* as in SEQ ID NO:12).

The human variable light FR sequence preferably has substitutions therein, *e.g.* wherein the

human FR residue is replaced by a corresponding mouse residue, but replacement with the nonhuman residue is not necessary. For example, a replacement residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable light FR residues which may be substituted include any one or more of FR residue numbers: 4L, 46L and 71L (Kabat residue numbering employed here). Preferably only 46L is substituted. In another embodiment, both 4L and 46L are substituted.

With respect to the CDRs, these preferably have amino acid sequences as follows:

CDRL1

$X_1AX_2X_3X_4X_5SNYLN$ (SEQ ID NO:121), wherein X_1 is R or S, but preferably is S; X_2 is S or N, but preferably is S; X_3 is Q or E, but preferably is Q; X_4 is Q or D, but preferably is D; and X_5 is I or L, but preferably is I.

CDRL2

FTSSLHS (SEQ ID NO:122).

CDRL3

$QQYSX_1X_2PWT$ (SEQ ID NO:123), wherein X_1 is T, A or N, but preferably is T; and X_2 is V or T, but preferably is V.

Preferred humanized anti-VEGF antibodies are those having the heavy and/or light variable domain sequences of F(ab)-12 in Example 1 and variants thereof such as affinity matured forms including variants Y0317, Y0313-1 and Y0238-3 in Example 3, with Y0317 being the preferred variant. Methods for generating humanized anti-VEGF antibodies of interest herein are elaborated in more detail below.

A. Antibody Preparation

Methods for humanizing nonhuman VEGF antibodies and generating variants of anti-VEGF antibodies are described in the examples below. In order to humanize an anti-VEGF antibody, the nonhuman antibody starting material is prepared. Where a variant is to be generated, the parent antibody is prepared. Exemplary techniques for generating such nonhuman antibody

starting material and parent antibodies will be described in the following sections.

(i) Antigen preparation

The VEGF antigen to be used for production of antibodies may be, *e.g.*, intact VEGF or a fragment of VEGF (*e.g.* a VEGF fragment comprising "epitope A4.6.1"). Other forms of VEGF useful for generating antibodies will be apparent to those skilled in the art. The VEGF antigen used to generate the antibody, is preferably human VEGF, *e.g.* as described in Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991).

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard

methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) Humanization and amino acid sequence variants

Examples 1-2 below describe procedures for humanization of an anti-VEGF antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of these humanized antibodies, particularly where these improve the binding affinity or other biological properties of the humanized antibody. Example 3 describes methodologies for generating amino acid sequence variants of an anti-VEGF antibody with enhanced affinity relative to the parent antibody.

Amino acid sequence variants of the anti-VEGF antibody are prepared by introducing appropriate nucleotide changes into the anti-VEGF antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-VEGF antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-VEGF antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-VEGF antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with VEGF antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-VEGF antibody variants are screened for the desired activity. Alanine scanning mutagenesis is described in Example 3.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-VEGF antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-VEGF antibody molecule include the fusion to the N- or C-terminus of the anti-VEGF antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-VEGF antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-VEGF antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display (see Example 3 herein). Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis (see Example 3) can be performed

to identified hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-VEGF antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-VEGF antibody.

(v) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and US Patents 5,591,669, 5,589,369 and 5,545,807. Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); and US Patents 5,565,332 and 5,573,905). As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275)

(vi) Antibody fragments

In certain embodiments, the humanized or variant anti-VEGF antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al., Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vii) Multispecific antibodies

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized or variant anti-VEGF antibodies having binding specificities for at least two different

epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the VEGF protein. Alternatively, an anti-VEGF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the VEGF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express VEGF. These antibodies possess an VEGF-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to

thionitrobenzoate(TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from *E. coli* can be chemically coupled *in vitro* to form bispecific antibodies. Shalaby *et al., J. Exp. Med.* 175:217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al., J. Immunol.* 147:60 (1991).

(viii) Other modifications

Other modifications of the humanized or variant anti-VEGF antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain

disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al., Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al., Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.,* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.,* a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-VEGF antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al., Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an

exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.,* avidin) which is conjugated to a cytotoxic agent (*e.g.,* a radionuclide).

The anti-VEGF antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al., J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al., J. National Cancer Inst.*81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.,* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases,

such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-VEGF antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.,* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.,* by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain

of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence: PKNSSMISNTP (SEQ ID NO:17), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:18), HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s): HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21) and the sequence: PKNSSMISNTP (SEQ ID NO:17).

Covalent modifications of the humanized or variant anti-VEGF antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Exemplary covalent modifications of polypeptides are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized or variant anti-VEGF antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. In another embodiment, the antibody may be produced by homologous recombination, *e.g.* as described in US Patent 5,204,244, specifically incorporated herein by reference. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components

generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, *e.g.*, as described in US Patent 5,534,615 issued July 9, 1996 and specifically incorporated herein by reference.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-VEGF antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated anti-VEGF antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains

for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-VEGF antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the anti-VEGF antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and

phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzeneallow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin

(J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*,from about 0-0.25M salt).

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other (see Section F below). Such molecules are suitably present in

combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

D. Non-therapeutic Uses for the Antibody

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing

the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish

peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-VEGF antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc.

1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.,* US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulinantibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

E. Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.,* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.,* a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided

as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

F. Therapeutic Uses for the Antibody

For therapeutic applications, the anti-VEGF antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies also are suitably administered by intra tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodys of the present invention are expected to be especially useful in reducing the severity of AMD.

Depending on the type and severity of the disease, about 1 µg/kg to about 50 mg/kg (*e.g.*, 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 µg/kg to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak *et al.*, PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics,

pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy. The antibody and one or more other anti-VEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of anti-VEGF antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF antibody. Treatment with anti-VEGF antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

G. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials,

syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

EXAMPLE 1

This example describes the production of humanized anti-VEGF antibodies with desirable properties from a therapeutic standpoint.

MATERIALS AND METHODS

Cloning of Murine A4.6.1 MAb and Construction of Mouse-Human Chimeric Fab: The murine anti-VEGF mAb A4.6.1 has been previously described by Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993). Total RNA was isolated from hybridoma cells producing the anti-VEGF Mab A.4.6.1 using RNAsol (TEL-TEST) and reverse-transcribedto cDNA using Oligo-dT primer and the SuperScript II system (GIBCO BRL, Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the N-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup kV and heavy chain subgroup II (Kabat *et al. Sequences of Proteins of Immunological Interest.* 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). After polymerase chain reaction (PCR) amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned respectively into the pEMX1 vector containing the human CL and CH1 domains (Werther *et al. J. Immunol.* 157:4986-4995 (1996)), thus generating a

mouse-human chimera. This chimeric F(ab) consisted of the entire murine A4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113 and the entire murine A4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to that of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

Computer Graphics Models of Murine and Humanized F(ab): Sequences of the VL and VH domains (Figs.1A and 1B) were used to construct a computer graphics model of the murine A4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (Carter *et al. Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992) and Eigenbrot *et al. J.Mol. Biol.* 229:969-995 (1993)).

Construction of Humanized F(ab)s: The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *E. coli* has been described previously (Werther *et al., supra*). Briefly, the plasmid contains a DNA fragment encoding a consensus human k subgroup I light chain (VLkI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (Carter *et al., supra*).

To construct the first F(ab) variant of humanized A4.6.1, F(ab)-1, site-directed mutagenesis (Kunkel *et al., Proc. Natl. Acad. Sci. USA* 82:488-492 (1985)) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs according to Kabat *et al., supra*, were changed to the murine A4.6.1 sequence . F(ab)-1 therefore consisted of a complete human framework (VL k subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml Luria broth-100 mg/ml carbenicillin for

5-8 h at 37° C. The 5 ml culture was added to 500 ml AP5-50 µg/ml carbenicillin and allcwed to grow for 20 h in a 4 L baffled shake flask at 30° C. AP5 media consists of: 1.5 g glucose, 11.0 g Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO4 (anhydrous), 1.07 g NH4Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 mm Sealkeen filter. Cells were harvested by centrifugation in a 1 L centrifuge bottle at 3000x*g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM Tris-1 mM EDTA-20% sucrose, pH 8.0. 250 ml of 0.1 M benzamidine (Sigma, St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000x*g* for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (0.5 ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

Construction and Expression of Chimeric and Humanized IgG: For generation of human IgG1 variants of chimeric (chIgG1) and humanized (huIgG1) A4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12, Table 2) domains were subcloned into separate, previously described, pRK vectors (Eaton *et al.*, *Biochemistry* 25:8343-8347 (1986)). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were co-transfected into human 293 cells (Graham *et al.*, *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media was changed to serum-free and harvested daily for up to five days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore, Bedford, MA) and stored at 4° C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (Lucas *et al.*, *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line developed by L. Chasin (Columbia University), via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover,DM, Hames, BD. *DNA Cloning 4. Mammalian systems.* Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening. One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after this step was ~99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was < 0.10 eu/mg.

***F(ab) and IgG Quantitation*:** For quantitating F(ab) molecules, ELISA plates were coated with 2 µg/ml goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% bovine serum albumin (blocking buffer) at room temperature for 1 h. Standards (0.78 - 50 ng/ml human F(ab)) were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% bovine serum albumin-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a

Vmax plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program. Data points which fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples. The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

VEGF Binding Assay: For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 µg/ml rabbit $F(ab')_2$ to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (Park *et al., J. Biol. Chem.* 269:25646-25645 (1994)) in blocking buffer were incubated on the plate for 1 h. Standards (6.9 - 440 ng/ml chimeric F(ab)) and two-fold serial of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma, St. Louis, MO) followed by 3,3',5,5'-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

BIAcore™ Biosensor Assay: VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore™ biosensor (Karlsson *et al. Methods: A Comparison to Methods in Enzymology* 6:97-108 (1994)). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) (35 µl of 2 µM F(ab) at a flow rate of 20 µl/min) and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0 - 4500 sec were used for off-rate kinetic analysis. The dissociation rate constant (k_{off}) was obtained from the slope of the plot of ln(R0/R) versus time, where R0 is the signal at t=0 and R is the signal at each time point.

On-rate kinetics were measured using two-fold serial dilutions of F(ab) (0.0625 - 2 mM). The slope, K_s, was obtained from the plot of ln(-dR/dt) versus time for each F(ab) concentration using the BIAcore™ kinetics evaluation software as described in the Pharmacia Biosensor manual. R is the signal at time t. Data between 80 and 168, 148, 128, 114, 102, and 92 sec were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant (k_{on}) was obtained from the slope of the plot of K_s versus F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5 µl of 50 mM HCl at a flow rate of 20 µl/min to regenerate the chip.

Endothelial Cell Growth Assay: Bovine adrenal cortex-derived capillary endothelial cells were cultured in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as previously described (Leung *et al. Science* 246:1306-1309 (1989)). For mitogenic assays, endothelial cells were seeded at a density of 6 x 10^3 cells per well, in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF was then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified *E.coli*-expressed rhVEGF165 was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells were dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

In Vivo Tumor Studies: Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female BALB/c nude mice, 6-10 weeks old, were injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF or a control MAb directed against the gp120 protein (Kim *et al. Nature* 362:841-844 (1993)). Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks

after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

RESULTS

Humanization: The consensus sequence for the human heavy chain subgroup III and the light chain subgroup k I were used as the framework for the humanization (Kabat *et al.*, *supra*) (Figs. 1A and 1B). This framework has been successfully used in the humanization of other murine antibodies (Werther *et al., supra*; Carter *et al., supra*; Presta *et al. J. Immunol.* 151:2623-2632 (1993); and Eigenbrot *et al. Proteins* 18:49-62 (1994)). CDR-H1 included residues H26-H35. The other CDRs were according to Kabat *et al., supra*. All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli*. Typical yields from 500 ml shake flasks were 0.1-0.4 mg F(ab).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in human) was changed to the murine Gly. In addition, F(ab)s which consisted of the chimeric heavy chain/F(ab)-1 light chain (F(ab)-2) and F(ab)-1 heavy chain/chimeric light chain (F(ab)-3) were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 2). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered in order to increase binding.

Table 2: Binding of Humanized Anti-VEGF F(ab) Variants to VEGF[a]

Variant	Template	Changes[b]	Purpose	EC50 F(ab)-X		
				EC50 chimeric F(ab)[c]		
				Mean	S.D.	N
chim-F(ab)	Chimeric F(ab)			1.0		
F(ab)-1	Human FR		Straight CDR swap AlaH49Gly	>1350		2
F(ab)-2			Chimera Light Chain F(ab)-1 Heavy Chain	>145		3
F(ab)-3			F(ab)-1 Light Chain Chimera Heavy Chain	2.6	0.1	2
F(ab)-4	F(ab)-1	ArgH71Leu AsnH73Thr	CDR-H2 conformation Framework	>295		3
F(ab)-5	F(ab)-4	LeuL46Val	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78Ala	CDR-H1 conformation	36.4	4.2	2
F(ab)-7	F(ab)-5	IleH69Phe	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69Phe LeuH78Ala	CDR-H2 conformation CDR-H1 conformation	9.6	0.9	4
F(ab)-9	F(ab)-8	GlyH49Ala	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76Ser	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75Ala	Framework	3.3	0.4	2
F(ab)-12	F(ab)-10	ArgH94Lys	CDR-H3 conformation	1.6	0.6	4

[a]Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, *supra*).

[b]Murine residues are underlined; residue numbers are according to Kabat *et al.*, *supra*.

[c]Mean and standard deviation are the average of the ratios calculated for each of the independent assays; the EC50 for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

Changing human residues H71 and H73 to their murine counterparts in F(ab)-4 improved binding by 4-fold (Table 2). Inspection of the models of the murine and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 (F(ab)-5), the binding affinity increased by almost 4-fold (Table 2). Three other buried framework residues were evaluated based on the molecular models: H49, H69 and H78. Position H69 may affect the conformation of CDR-H2 while position H78 may affect the conformation of CDR-H1 (Figure 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case (F(ab)-6 and F(ab)-7, Table 2). When both were simultaneously changed, the improvement in binding was 8-fold (F(ab)-8, Table 2). Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala the binding was reduced by 15-fold (F(ab)-9, Table 2).

In F(ab)-10 and F(ab)-11 two residues in framework loop 3, FR-3, were changed to their murine counterparts: AsnH76 to murine Ser (F(ab)-10) and LysH75 to murine Ala (F(ab)-11). Both effected a relatively small improvement in binding (Table 2). Finally, at position H94 human and murine sequences most often have an Arg (Kabat *et al., supra*). In F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig.1A) and this resulted in binding which was less than 2-fold from the chimeric F(ab) (Table 2). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore™ system (Pharmacia). Using this technique the K_d of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower k_{on} and faster k_{off} (Table 3).

Table 3: Binding of Anti-VEGF F(ab) Variants to VEGF Using the BIAcore™ System[a]

Variant	Amount of (Fab) bound (RU)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
chim-F(ab)[b]	4250	$5.9x10^{-5}$	$6.5x10^{4}$	0.91
F(ab)-12	3740	$6.3x10^{-5}$	$3.5x10^{4}$	1.8

[a] The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore™ system when 2 µg F(ab) was injected onto a chip containing 2480 RU immobilized VEGF. Off-rate kinetics (k_{off}) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics (k_{on}) were measured using two-fold serial dilutions of F(ab). K_d, the equilibrium dissociation constant, was calculated as k_{off}/k_{on}.
b chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

Full length mAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human k light chain and human IgG1 heavy chain. The full length 12-IgG1 (F(ab)-12 fused to human IgG1) exhibited binding which was 1.7-fold weaker than the chimeric IgG1 (Table 4). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original murine mAb A4.6.1 (Table 4).

Table 4: Binding of Anti-VEGF IgG Variants to VEGF[a]

IgG1/chIgG1[b]			
Variant	**Mean**	**S.D.**	**N**
chIgG1	1.0		2
murIgG1[c]	0.759	0.001	2
12-IgG1[d]	1.71	0.03	2

[a] Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, (1994), *supra*).
[b] chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC50 for chIgG1 was 0.113 ± 0.013 µg/ml (0.75 nM).
[c] murIgG1 is muMAbVEGF A461 purified from ascites.
[d] 12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

Biological Studies: rhuMAb VEGF and muMAb VEGF A.4.6.1. were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). As illustrated in Figure 3, the two MAbs were essentially equivalent, both in potency and efficacy. The ED50 values were respectively 50 ± 5 ng/ml and 48 ± 8 ng/ml (~0.3 nM). In both cases 90% inhibition was achieved at the concentration of 500 ng/ml (~3 nM). Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any

effect on basal or bFGF-stimulated proliferation of capillary endothelial cells, confirming that the inhibition is specific for VEGF.

To determine whether such equivalency applies also to an *in vivo* system, the two antibodies were compared for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res* 56:4032-4039 (1996)). As shown in Figure 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements four weeks after cell inoculation. The decreases in tumor weight compared to the control group were respectively 85% and 93% at each dose in the animals treated with muMAb VEGF A.4.6.1. versus 90% and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435.

EXAMPLE 2

In this example, the murine anti-VEGF antibody A4.6.1 discussed above was humanized by randomizing a small set of framework residues and by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage in order to identify high affinity framework sequences via affinity-based selection.

MATERIALS AND METHODS

Construction of Anti-VEGF Phagemid Vector, pMB4-19: The murine anti-VEGF mAb A4.6.1 is discussed above in Example 1. The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 (Carter *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:4285-4289 (1992)) which codes for a human V_LkI-Cκ_1 light chain and human V_HIII-$C_H1\gamma_1$ heavy chain Fd fragment The transplanted A4.6.1 CDR sequences were chosen according to the sequence definition of Kabat *et al.*, *supra*, except for CDR-H1 which included residues 26-35. The Fab encoding sequence was subcloned into the phagemid vector phGHamg3 (Bass *et al. Proteins* 8:309-314 (1990) and Lowman *et al. Biochemistry* 30:10832-10838 (1991)). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion

of the M13 gene III coat protein. pMB4-19 is similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments (Garrard *et al. Biotechnology* 9:1373-1377 (1991)). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

Expression and Purification of Humanized A4.6.1 Fab Fragment: *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB4-19, or variants thereof. Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium (Chang *et al. Gene* 55:189-196 (1987)) containing 20 µg/mL carbenicillin and incubated for 26 hr at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 h. Cell pellets were then resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 min and centrifuged at 10,000 x g for 15 min. The supernatant was applied to a 1 mL streptococcal protein G-sepharose column (Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M Tris-HCl, pH 8.0. Fab preparations were buffer-exchanged into PBS and concentrated using Centricon-30 concentrators (Amicon). Typical yields of Fab were ~1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

Construction of the Anti-VEGF Fab Phagemid Library: The humanized A4.6.1 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel *et al. Methods Enzymol.* 204:125-139 (1991)). A derivative of pMB4-19 containing TAA stop triplets at V_H codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat *et al.*, *supra*). This modification was to prevent subsequent background contamination by wild type sequences. The codons targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

In order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60

and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol of 5' phosphorylated oligonucleotide 503-1 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:22)) or 503-2 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:23)) were combined with 1.5 nmol of 503-3 (5'-AGC CTG CGC GCT GAG GAC ACT GCC GTC TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG-3' (SEQ ID NO:24)) (randomized codons underlined; N=A/G/T/C; W=A/T; B=G/T/C; D=G/A/T; R=A/G; Y=C/T). Then, 1.5 nmol of template oligonucleotide (5'-CTC AGC GCG CAG GCT GTT CAT CTG CAG GTA-3' (SEQ ID NO:25)), with complementary sequence to the 5' ends of 503-1/2 and the 3' end of 503-3, was added to hybridize to each end of the ligation junction. *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95° C 1.25 min; 50° C for 5 min) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10mM Tris-HCl, 1mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons (V_L 4, 71; V_H 24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was effected in two steps. Firstly, V_L randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides 5'-GCT GAT ATC CAG TTG ACC CAG TCC CCG-3' (SEQ ID NO:26) 5'-and TCT GGG ACG GAT TAC ACT CTG ACC ATC-3' (SEQ ID NO:27). Deoxyuridine-containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (Table 5).

Oligonucleotides 504-1, a mixture of two 126-mer oligonucleotides (see above), and 5'-CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG-3' (SEQ ID NO:28) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 Blue cells (Stratagene) and combined. The total number of

independent transformants was estimated at >1.2 x 10^8, approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter *et al.*, *Ann. Rev. Immunol.* 12,433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard *et al.*, *Biotechn*, 9,1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 7). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8:309 (1990) and Lowman *et al.*, *Biochemistry* 30:10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37° C in 25 mL 2YT medium containing 50µg/mL carbenicillin and approximately 10^{10} M13KO7 helper phage (Vieira & Messing *Methods Enzymol.* 153:3-11 (1987)). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 µL PBS (~10^{14} phagemid/mL)

Selection of Humanized A4.6.1 Fab Variants: Purified $VEGF_{121}$ (100 µL at 10µg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well, in addition to an uncoated well, were blocked with 6% skim milk for 1 h and washed with PBS containing 0.05% TWEEN 20™ (detergent). Then, 10 µL of phagemid stock, diluted to 100 µL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05%TWEEN 20™, was added to each well. After 2 hours the wells were washed and the bound phage eluted with 100 µL of 0.1 M glycine (pH 2.0), and neutralized with 25 µL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger *et al. Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467 (1977)).

Determination of VEGF Binding Affinities: Association (k_{on}) and dissociation (k_{off}) rate constants for binding of humanized A4.6.1 Fab variants to $VEGF_{121}$ were measured by surface plasmon resonance (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)) on a Pharmacia BIAcore instrument. $VEGF_{121}$ was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN 20™ over the chip at a flow rate of 20 µL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 µL of 50 mM aqueous HCl at 3 µL/min. Binding profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

RESULTS

Construction of Humanized A4.6.1: An initial humanized A4.6.1 Fab fragment was constructed (hu2.0, Figs. 5A and 5B), in which the CDRs from A4.6.1 were grafted onto a human V_LKI-V_HIII framework. All other residues in hu2.0 were maintained as the human sequence. Binding of this variant to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants, the K_D for binding of hu2.0 was estimated at >7 µM. This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact V_L and V_H domains from murine A4.6.1 and human constant domains. Thus binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

Design of Antibody Library: The group of framework changes to the human framework sequence herein is shown in Table 5 and Fig. 6.

Table 5: Key Framework Residues Important for Antigen Binding and Targeted for Randomization

Framework residue		Human $V\kappa_L I$, $V_H III$ consensus residue	Murine A4.6.1 residue	Randomization[a]
V_L:	4	Met	Met	Met, Leu
	71	Phe	Tyr	Phe, Tyr
V_H:	24	Ala	Ala	Ala, Val, Thr
	37	Val	Val	Val, Ile

	67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
	69	Ile	Phe	Ile, Phe
	71	Arg	Leu	Arg[b], Leu[b]
	73	Asp	Thr	Asp[b], Thr[b]
	75	Lys	Ala	Lys[b], Ala[b]
	76	Asn	Ser	Asn[b], Ser[b]
	78	Leu	Ala	Leu, Ala, Val, Phe
	93	Ala	Ala	Ala, Val, Leu, Ser, Thr
	94	Arg	Lys	Arg, Lys

[a]Amino acid diversity in phagemid library
[b]V_H71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) V_HIII tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V_L and V_H sequences. Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V_L framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126-base oligonucleotides were preassembled from smaller synthetic fragments. This made possible randomization of V_H codons 67, 69, 71, 73, 75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

Selection of Tight Binding Humanized A4.6.1 Fab's: Variants from the humanized A4.6.1 Fab phagemid library were selected based on binding to VEGF. Enrichment of functional

phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results, summarized in Table 6, revealed strong consensus amongst the clones selected. Ten out of the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 (V_L 71; V_H 37, 71, 73, 75, 76, 78 and 94). Interestingly, residues V_H 37 (Ile) and 78 (Val) were selected neither as the human V $_H$III or murine A4.6.1 sequence. This result suggests that some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

Table 6: Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library

Variant	**Residue substitutions**												
	V_L		V_H										
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	F	A	V	F	I	R	N	K	N	L	A	R
Phage-selected clones:													
hu2.1(2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.2(2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.6(1)	L	-	-	I	T	-	L	T	A	S	V	-	K
hu2.7(1)	L	-	-	I	-	-	-	-	-	-	V	-	K
hu2.10(10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human V_LKI-V_HIII framework sequence (*i.e.* as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human V_HIII consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA assay (Cunningham *et al. EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

Expression and Binding Affinity of Humanized A4.6.1 Variants: Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes (K_D >7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

Table 7: VEGF Binding Affinity of Humanized A4.6.1 Fab Variants

Variant	k_{on} $M^{-1}s^{-1}/10^4$	k_{off} 10^4s^{-1}	K_D nM	$\frac{K_D(A4.6.1)}{K_D(mut)}$
A4.6.1 chimera	5.4	0.85	1.6	›4000
hu2.0	ND	ND	›7000**	
Phage selected clones:				
hu2.1	0.70	18	260	170

hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

*hu2.10V = hu2.10 with mutation V_L Leu->Val
Estimated errors in the Biacore binding measurements are +/-25%.
**Too weak to measure; estimate of lower bound

Additional Improvement of Humanized Variant hu2.1: Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human V_LκI-V_HIII framework (Figs. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V_L 46 sits at the V_L-V_H interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always leucine in most V_Lκ frameworks (Kabat *et al.*, *supra*), but is valine in A4.6.1. Accordingly, a Leu -> Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding, demonstrating the importance of valine at position V_L 46 in antibody A4.6.1. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

EXAMPLE 3

In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

Construction of Humanized Antibody pY0101: Phage-displayed antibody vector phMB4-19-1.6 (see Figs. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity for VEGF with oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

Table 8: Oligos for Directed Mutations

Oligo Number	Region	Substitution/ Comments	Sequence
HL-242	VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
HL-243	VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
HL-245	VH	CDR-7	5'-CGTCGTTTCACTTTTTCTGCAGACACCT CCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
HL-246	VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
HL-254	VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
HL-256	VH	I37V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
HL-257	VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTTCTTTAGACACCT CCAAAAGCACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

The resulting variant was termed Y0101 (Figs. 9A and 9B).

Construction of the First Generation of Antibody-Phage Libraries: To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis

with oligonucleotides using the degenerate NNS codon (where N is an equal mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement (Figs. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.

Table 9: Template Oligos for Mutagenesis

Oligo Number	Region Comments	Sequence
HL-248	VL1	5'-GGGTCACCATCACCTGCTAAGCATAATAATAATAAAGCAACT ATTTAAACTGG-3' (SEQ ID NO:36)
HL-249	VL1	5'-GCGCAAGTCAGGATATTTAATAATAATAATAATGGTATCAAC AGAAACCAGG-3' (SEQ ID NO:37)
HL-250	VH3	5'-GTCTATTACTGTGCAAAGTAATAACACTAATAAGGGAGCAG CCACTGG-3' (SEQ ID NO:38)
HL-251	VH3	5'-GGTACCCCCACTATTATTAATAATAATAATGGTATTTCGACG TCTGGGG-3' (SEQ ID NO:39)
HL-252	VH3	5'-CACTATTATGGGAGCAGCCACTAATAATAATAAGTCTGGGT CAAGGAACCCTG-3' (SEQ ID NO:40)
HL-263	VH1	5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATAATAAGGTATG AACTGGGTCCG-3' (SEQ ID NO:41)
HL-264	VH2	5'-GAATGGGTTGGATGGATTAACTAATAATAAGGTTAACCGAC CTATGCTGCGG-3' (SEQ ID NO:42)
YC-80	VH3	5'-CTGTGCAAAGTACCCGTAATATTAATAATAATAACACTGGTA TTTCGAC-3' (SEQ ID NO:43)
YC-100	CDR7	5'-CGTTTCACTTTTTCTTAAGACTAATCCAAATAAACAGCATAC CTGCAG-3' (SEQ ID NO:44)
YC-102	VH2	5'-GAATGGGTTGGATGGATTTAATAATAATAAGGTGAACCGAC CTATG-3' (SEQ ID NO:45)

Table 10: Random Oligos for Library Construction

Oligo Number	Region Comment	Sequence
HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNSNNSNNSNNSAGC AACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNSNNSNNSNNSTGGTATCAACA GAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSGGGAGCAGC CACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-GGTACCCCCACTATTATNNSNNSNNSNNSTGGTATTTCGACGT CTGGGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNSNNSNNSGTCTGGGGT CAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNNSNNSTTCNNSNNSNNSGGTATGA ACTGGGTCCG-3' (SEQ ID NO:51)
HL-266	VH2	5'-GAATGGGTTGGATGGATTAACNNSNNSNNSGGTNNSCCGACC TATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNSNNSNNSCACTGGTAT TTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTTCTNNSGACNNSTCCAAANNSACAGCATACCT GCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNSNNSNNSGGTGAACCGACC TATG-3' (SEQ ID NO:55)

The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from 2 x 10^7 to 1.5 x 10^8, was estimated based upon plating of the initial transformation onto carbenicillin plates.

Initial Affinity Selections: For each round of selection, approximately 10^9-10^{10} phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 µg/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEEN™ (0.05% TWEEN 20™

in PBS buffer). Typically, to select for enhanced affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEEN™ buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEEN™ buffer was removed, the remained phages were eluted with 0.1 M HCl and immediately neutralized with 1/3 volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

Phage-ELISA Assay of Binding Affinities: From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 µg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, *o*-phenylenediamine (Sigma). The reaction was stopped with addition of ½ volume of 2.5 M H_2SO_4. Optical density at 492nm was measured on a spectrophotometric plate reader.

Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R, S26N, Q27E, D28Q, and I29L in the VL region (Fig. 9A). In addition, this variant was found to have a spurious

mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

Ala-Scanning the CDRs of Anti-VEGF: To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to > 150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

Table 11: Relative VEGF Affinities of Ala-Scan Fab Variants

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
R24A	1	G26A	2
A25S	1	Y27A	34
N26A	1	T28A	1
E27A	1	F29A	16
Q28A	1	T30A	1
L29A	1	N31A	>150
S30A	2	Y32A	>150
N31A	2	G33A	6
Y32A	2	I34A	6
L33A	2	N35A	66
N34A	4		
		W50A	>150
F50A	1	I51A	4
T51A	1	N52A	>150

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
S52A	1	T53A	9
S53A	1	Y54A	9
L54A	1	T55A	4
H55A	1	G56A	1
S56A	1	E57A	2
		P58A	1
Q89A	4	T59A	3
Q90A	3	Y60A	2
Y91A	14	A61S	1
S92A	1	A62S	1
T93A	1	D63A	1
V94A	2	F64A	1
P95A	3	K65A	1
W96A	>150	R66A	1
T97A	1		
		Y99A	>150
		P100A	38
		H101A	4
		Y102A	4
		Y103A	5
		G104A	2
		S105A	1
		S106A	>150
		H107A	2
		W108A	>150
		Y109A	19
		F110A	25
		D111A	2

All variants are in the background of pY0192 ("wt"; see Figs. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction) and F110A (25-fold reduction). In contrast, only one VL substitution had a large impact on binding affinity, W96A

(>150-fold reduction). These results point to the three VH CDRs as the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

Design of Second-Generation CDR Mutation Libraries: Two additional libraries which randomized existing residues in anti-VEGF version Y0192 were designed based upon inspection of the crystal structure. In VH2, residues 52-55 were randomized because they lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see Fig. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

Second-Generation Selections of Anti-VEGF Libraries: Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from 6×10^7 to 5×10^8 which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

Table 12: Conditions for Secondary Selections of Fab Variants

Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (°C)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 µM VEGF/ELISA	room temp.

4	18	1 μM VEGF/ELISA	room temp.
5	37	1 μM VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37°C	1 μM VEGF/ELISA	room temp./37°C
7	63	1 μM VEGF/ELISA	37°C
8	121	1 μM VEGF/ELISA	37°C

ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

Phage-ELISA Assays of Second Generation Clones: After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

Table 13: Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries

Variants from library YC-81	
Name	**VH3 sequence (residues 99-111)**
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO:56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO:57)
Y0238-3	YPYYYGTSHWYFD (SEQ ID NO:58)
Y0238-4	YPYYYNQSHWYFD (SEQ ID NO:59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO:60)
Y0238-6	YPYYRDNSHWYFD (SEQ ID NO:61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO:62)

Y0238-8	YPYYRQNSHWYFD (SEQ ID NO:63)
Y0238-9	YPYYRQSSHWYFD (SEQ ID NO:64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO:65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO:66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO:67)
Y0228-21	YPYYRNASHWYFD (SEQ ID NO:68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO:69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO:70)
Y0228-24	YPYYRQRGHWYFD (SEQ ID NO:71)
Y0228-26	YPYYTGRSHWYFD (SEQ ID NO:72)
Y0228-27	YPYYTNTSHWYFD (SEQ ID NO:73)
Y0228-28	YPYYRKGSHWYFD (SEQ ID NO:74)
Y0228-29	YPYYTGSSHWYFD (SEQ ID NO:75)
Y0228-30	YPYYRSGSHWYFD (SEQ ID NO:76)
Y0229-20	YPYYTNRSHWYFD (SEQ ID NO:77)
Y0229-21	YPYYRNSSHWYFD (SEQ ID NO:78)
Y0229-22	YPYYKESSHWYFD (SEQ ID NO:79)
Y0229-23	YPYYRDASHWYFD (SEQ ID NO:80)
Y0229-24	YPYYRQKGHWYFD (SEQ ID NO:81)
Y0229-25	YPYYKGGSHWYFD (SEQ ID NO:82)
Y0229-26	YPYYYGASHWYFD (SEQ ID NO:83)
Y0229-27	YPYYRGESHWYFD (SEQ ID NO:84)
Y0229-28	YPYYRSTSHWYFD (SEQ ID NO:85)
Variants from library HL-265	
Name	**VH1 sequence (residue 26-35)**
Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO:86)
Y0243-2	GYEFQHYGMN (SEQ ID NO:87)

Y0243-3	GYEFTHYGMN (SEQ ID NO:88)
Y0243-4	GYDFGHYGMN (SEQ ID NO:89)
Y0243-5	GYDFSHYGMN (SEQ ID NO:90)
Y0243-6	GYEFSHYGMN (SEQ ID NO:91)
Variants from library YC-101	
Name	**VH "CDR7" sequence (residues 70-79)**
Y0244-1	FSVDVSKSTA (SEQ ID NO:92)
Y0244-2	FSLDKSKSTA (SEQ ID NO:93)
Y0244-3	FSLDVWKSTA (SEQ ID NO:94)
Y0244-4	FSIDKSKSTA (:95)

The sequence of the randomized region only is shown as deduced from DNA sequencing.

When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could be explained by the time-scale on which the assay was performed (< 3 hours).

In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants' DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

CDR Combination Variants: To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

Table 14: Combination CDR Anti-VEGF Variants

Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTACTATTATGGGAC GAGCCACTGGTATTTC-3' (SEQ ID NO:96)
Y0317	Y0313-1	YC-108 (revert VL1 back to wild type)	5'-GTCACCATCACCTGCAGCGCAAGTCA GGATATTAGCAACTATTTAAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCAGCCACTG GTATTTC-3' (SEQ ID NO:98)

Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

BIAcore Analysis: The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25° C at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, Kd's from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements				
Variant	**Kon (10^4/M/s)**	**koff (10^{-4}/s)**	**Kd (nM)**	**Kd (wt) / Kd (mut)**
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*
Y0313-3	1.2	0.081	0.65	4.5

* The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T28D and N31H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.15nM to 0.65 nM (see Table 15). The greater affinity enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

Cell-Based Assay of VEGF Inhibition: Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% diafiltered fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration),

and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

The results (Fig. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC50 for the mutant. For example, if the binding affinity (Kd) of the mutant is in fact < 0.2 nM, the IC50 in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*. Since the variant Y0317 differs from Y0313-1 only in the reversion of the VL1 sequence to wild-type (Fig. 10A), it is predicted that Y0317 will have similar activity to Y0313-1.

Variant Y0317 (Fab) and humanized variant F(ab)-12 from Example 1 (full length and Fab) were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF using the assay described in Example 1. As illustrated in Figure 12, Y0317 was markedly more effective at inhibiting bovine capillary endothelial cell proliferation than the full length and Fab forms of F(ab)-12 in this assay. The Y0317 affinity matured Fab demonstrated an ED50 value in this assay which was at least about 20 fold lower than F(ab)-12 Fab.

WHAT IS CLAIMED IS:

1. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M.

2. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 5 x 10^{-9}M.

3. A humanized anti-VEGF antibody which has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*.

4. A humanized anti-VEGF antibody which inhibits VEGF-induced angiogenesis *in vivo*.

5. The humanized anti-VEGF antibody of claim 4 wherein 5mg/kg of the antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

6. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129).

7. The humanized anti-VEGF antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:7.

8. The humanized anti-VEGF antibody of claim 6 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

10. The humanized anti-VEGF antibody of claim 9 comprising the amino acid sequence of SEQ ID NO:8.

11. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.

12. An anti-VEGF antibody light chain variable domain comprising the amino acid sequence: DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ KPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L.

13. An anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGYX $_1$FTX$_2$YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX $_3$YYGX$_4$SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T.

14. A variant of a parent anti-VEGF antibody, wherein said variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of a heavy chain variable domain of said parent antibody.

15. The variant of claim 14 wherein said parent antibody is a human or humanized antibody.

16. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 1×10^{-8}M.

17. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 5×10^{-9}M.

18. The variant of claim 14 wherein the substitution is in CDRH1 of the heavy chain variable domain.

19. The variant of claim 14 wherein the substitution is in CDRH3 of the heavy chain variable domain.

20. The variant of claim 14 which has amino acid substitutions in both CDRH1 and CDRH3.

21. The variant of claim 14 which binds human VEGF with a K_d value less than that of said parent antibody.

22. The variant of claim 14 which has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower than that of said parent antibody.

23. The variant of claim 18 wherein the CDRH1 comprises the amino acid sequence: GYDFTHYGMN (SEQ ID NO:126)

24. The variant of claim 19 wherein the CDRH3 comprises the amino acid sequence: YPYYYGTSHWYFDV (SEQ ID NO:127).

25. The variant of claim 14 wherein the heavy chain variable domain comprises the amino acid sequence of SEQ ID NO:116.

26. The variant of claim 25 further comprising the light chain variable domain amino acid sequence of SEQ ID NO:124.

27. The variant of claim 26 comprising the light chain variable domain amino acid sequence of SEQ ID NO:115.

28. The humanized anti-VEGF antibody of claim 1 which is a full length antibody.

29. The humanized anti-VEGF antibody of claim 28 which is a human IgG.

30. The humanized anti-VEGF antibody of claim 1 which is an antibody fragment.

31. The antibody fragment of claim 30 which is a Fab.

32. A composition comprising the humanized anti-VEGF antibody of claim 1 and a pharmaceutically acceptable carrier.

33. A composition comprising the variant anti-VEGF antibody of claim 14 and a pharmaceutically acceptable carrier.

34. Isolated nucleic acid encoding the antibody of claim 1.

35. A vector comprising the nucleic acid of claim 34.

36. A host cell comprising the vector of claim 35.

37. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 36 so that the nucleic acid is expressed.

38. The process of claim 37 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

39. A method for inhibiting VEGF-induced angiogenesis in a mammal comprising administering a therapeutically effective amount of the humanized anti-VEGF antibody of claim 1 to the mammal.

40. The method of claim 39 wherein the mammal is a human.

41. The method of claim 39 wherein the mammal has a tumor.

42. The method of claim 39 wherein the mammal has a retinal disorder.

ANTI-VEGF ANTIBODIES

Abstract of the Disclosure

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and inhibit tumor growth *in vivo*.

Variable Heavy

```
A4.6.1     EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQAPGKGLKWMG
            *   *  ** *   *** *  *                 *       * *
F(ab)-12   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG
                                      *  ** * *              *
humIII     EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS
           1        10        20        30        40

A4.6.1     WINTYTGEPTYAADFKRRFTFSLETSASTAYLQISNLKNDDTATYFCAK
                                   *  *      *** ***   * *
F(ab)-12   WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAK
           * **** ***  *** *   * * *  * *                   *
humIII     VISGDGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
          50  a      60        70        80  abc      90

A4.6.1     YPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
                          *  *
F(ab)-12   YPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:7)
           *           *
humIII     G----------FDYWGQGTLVTVSS (SEQ ID NO:11)
                      110
```

Fig. 1A

Variable Light

```
A4.6.1     DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKPDGTVKVLIY
                 **      *    * *                  ****
F(ab)-12   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIY
                                  *   *     *          *
humKI      DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKPGKAPKLLIY
           1        10        20        30        40

A4.6.1     FTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSTVPWTF
                                **    * *   *
F(ab)-12   FTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTF
           **   *                                      ***
humKI      AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPWTF
          50        60        70        80        90

A4.6.1     GGGTKLEIKR (SEQ ID NO:10)
            *   *
F(ab)-12   GQGTKVEIKR (SEQ ID NO:8)

humKI      GQGTKVEIKR (SEQ ID NO:12)
          100
```

Fig. 1B

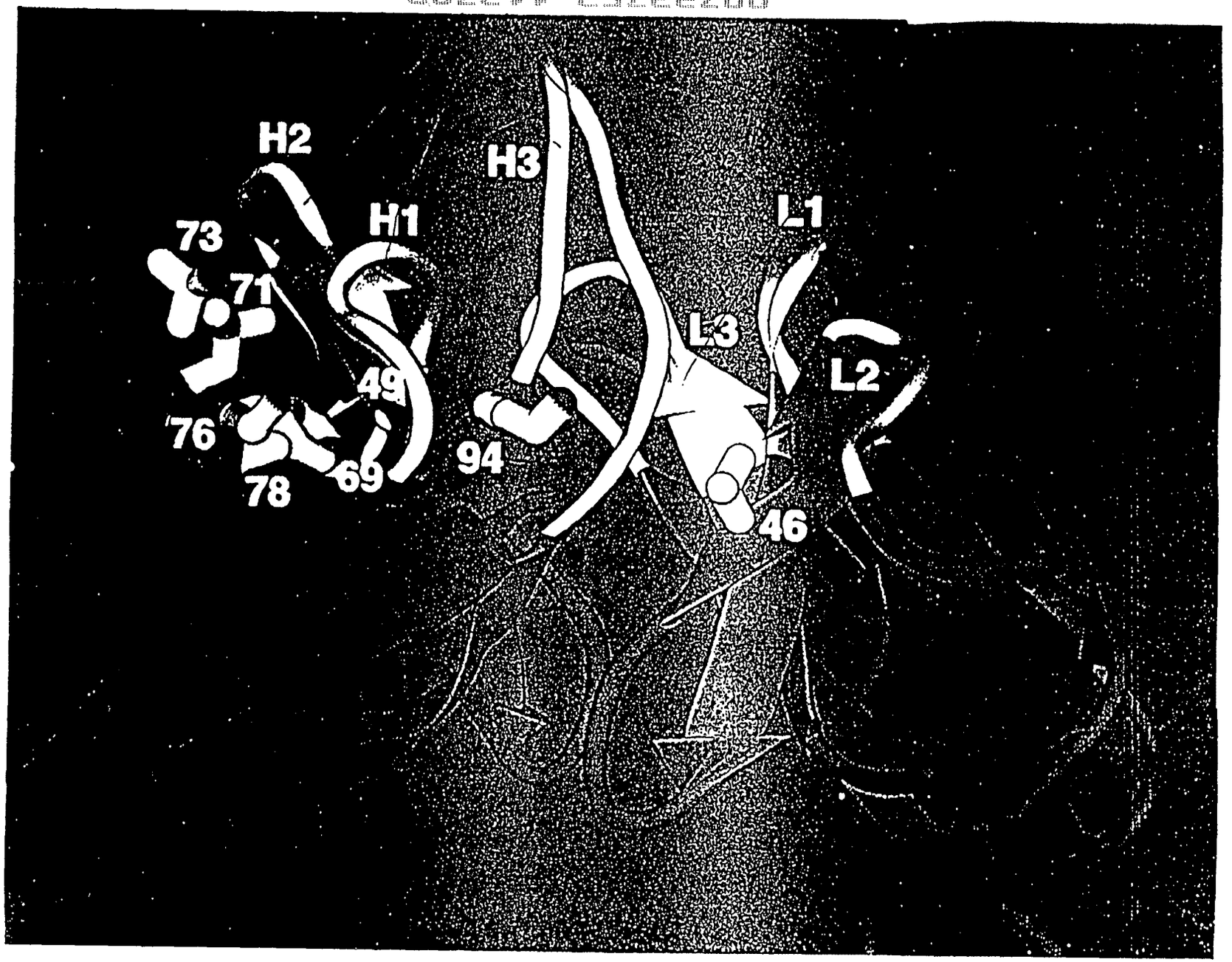

Fig. 2

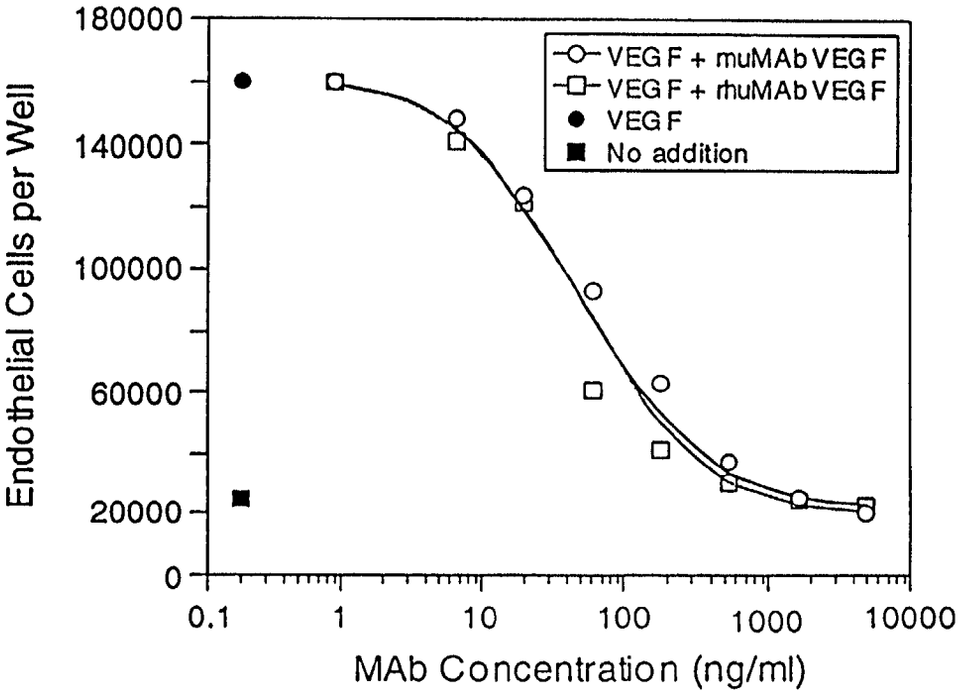

Fig. 3

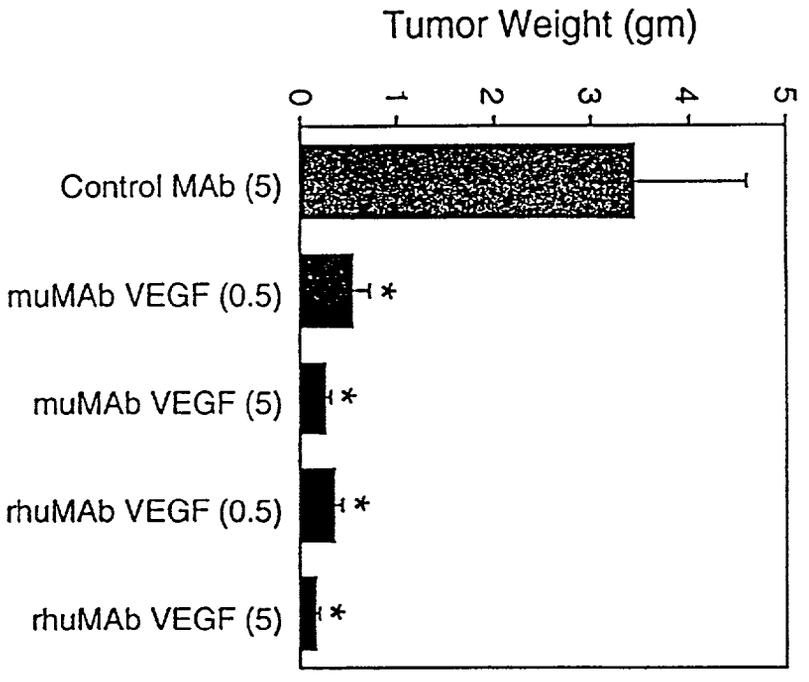

Fig. 4

V_L domain

```
                10        20        30        40
A4.6.1  DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
              **      *    * *
hu2.0   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP

hu2.10  DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP
```

Fig. 5A

```
                50        60        70        80
A4.6.1  DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
        **** *                          **    * *
hu2.0   GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
                                        .
hu2.10  GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTISSLQP


                90       100
A4.6.1  EDIATYYCQQYSTVPWTFGGGTKLEIK (SEQ ID NO:10)
          *                 *   *
hu2.0   EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:13)

hu2.10  EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:15)
```

V_H domain

```
                10        20        30        40
A4.6.1  EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
          *   *  ** *   *** * .*                *
hu2.0   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                              .
hu2.10  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIRQA


                50 a      60        70        80
A4.6.1  PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
              * *                   * *** ** *
hu2.0   PGKGLEWVGWINTYTGEPTYAADFKRRFTISRDNSKNTLYL
                                      . . .. .
hu2.10  PGKGLEWVGWINTYTGEPTYAADFKRRFTISLDTSASTVYL


          abc      90       100abcdef       110
A4.6.1  QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
          *** ***   * *  *                  *  *
hu2.0   QMNSLRAEDTAVYYCARYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:14)
                    .
hu2.10  QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:16)
```

Fig. 5B

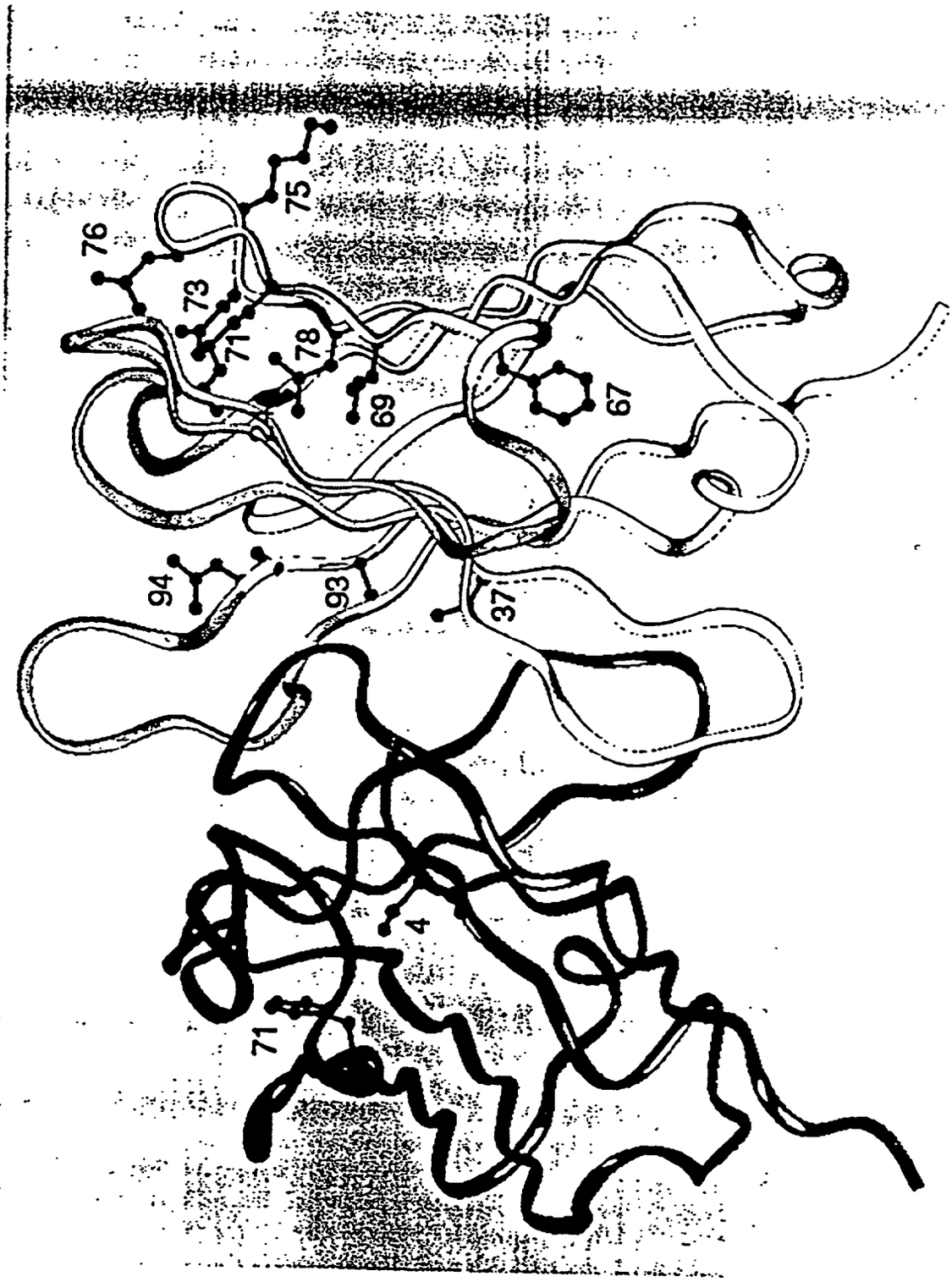

Fig. 6

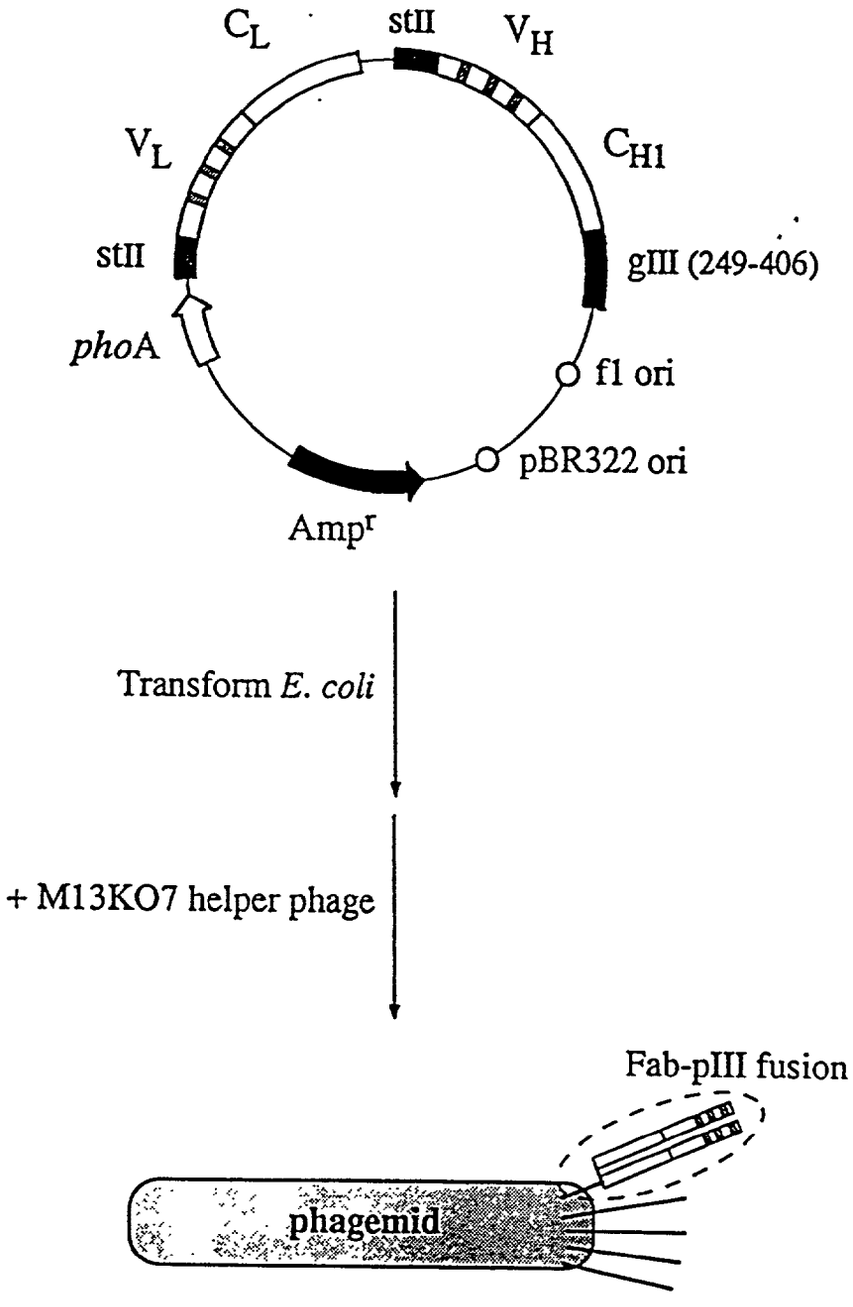

Fig. 7

```
  1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC
    CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAAACCT CTAATAGCAG TGACGTTACG

101 TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC
    AAGCGTTATA CCGCGTTTTA CTGGTTGTCG CCAACTAACT AGTCCATCTC CCCCGCGACA TGCTCCATTT CGGGCTACGG TCGTAAGGAC TGCTGCTATG

201 GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT
    CCTCGACGAC GCGCTAATGC ATTTCTTCAA TAACTTCGTA GGAGCAGTCA TTTTTCAATT AGAAAAGTTG TCGACAGTAT TTCAACAGTG CCGGCTCTGA

301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGGTACCC GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA
    ATATCAGCGA AACAAAAATA AAAAATTACA TAAACATTGA TCTTAAGCTC GAGCCATGGG CCCCTAGGAG ATCTCCAACT CCACTAAAAT ACTTTTTCTT
-23                                                                                                   M etLysLysAsn
                                                                                          Begin stII signal sequence
401 TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG CGTACGCTGA TATCCAGTTG ACCCAGTCCC CGAGCTCCCT GTCCGCCTCT
    ATAGCGTAAA GAAGAACGTA GATACAAGCA AAAAAGATAA CGATGTTTGC GCATGCGACT ATAGGTCAAC TGGGTCAGGG GCTCGAGGGA CAGGCGGAGA
-19  IleAlaPhe LeuLeuAlaS erMetPheVa lPheSerIle AlaThrAsnA laTyrAlaAs pIleGlnLeu ThrGlnSerP roSerSerLe uSerAlaSer
                                                                  Begin light chain
501 GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG ATATTAGCAA CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAACTACTGA
    CACCCGCTAT CCCAGTGGTA GTGGACGTCG CGTTCAGTCC TATAATCGTT GATAAATTTG ACCATAGTTG TCTTTGGTCC TTTTCGAGGC TTTGATGACT
 15 ValGlyAspA rgValThrIl eThrCysSer AlaSerGlnA spIleSerAs nTyrLeuAsn TrpTyrGlnG lnLysProGl yLysAlaPro LysLeuLeuIle

601 TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA TCCGGTTCTG GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA
    AAATGAAGTG GAGGAGAGAG GTGAGACCTC AGGGAAGAGC GAAGAGACCT AGGCCAAGAC CCTGCCTAAT GTGAGACTGG TAGTCGTCAG ACGTCGGTCT
 49   TyrPheTh rSerSerLeu HisSerGlyV alProSerAr gPheSerGly SerGlySerG lyThrAspTy rThrLeuThr IleSerSerL euGlnProGlu

701 AGACTTCGCA ACTTATTACT GTCAACAGTA TAGCACCGTG CCGTGGACGT TTGGACAGGG TACCAAGGTG GAGATCAAAC GAACTGTGGC TGCACCATCT
    TCTGAAGCGT TGAATAATGA CAGTTGTCAT ATCGTGGCAC GGCACCTGCA AACCTGTCCC ATGGTTCCAC CTCTAGTTTG CTTGACACCG ACGTGGTAGA
 82  AspPheAla ThrTyrTyrC ysGlnGlnTy rSerThrVal ProTrpThrP heGlyGlnGl yThrLysVal GluIleLysA rgThrValAl aAlaProSer

801 GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG GAACTGCTTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT
    CAGAAGTAGA AGGGCGGTAG ACTACTCGTC AACTTTAGAC CTTGACGAAG ACAACACACG GACGACTTAT TGAAGATAGG GTCTCTCCGG TTTCATGTCA
115 ValPheIleP heProProSe rAspGluGln LeuLysSerG lyThrAlaSe rValValCys LeuLeuAsnA snPheTyrPr oArgGluAla LysValGlnTrp

901 GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA GAGCAGGACA GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT
    CCTTCCACCT ATTGCGGGAG GTTAGCCCAT TGAGGGTCCT CTCACAGTGT CTCGTCCTGT CGTTCCTGTC GTGGATGTCG GAGTCGTCGT GGGACTGCGA
149   LysValAs pAsnAlaLeu GlnSerGlyA snSerGlnGl uSerValThr GluGlnAspS erLysAspSe rThrTyrSer LeuSerSerT hrLeuThrLeu

1001 GAGCAAAGCA GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT GAGCTCGCCC GTCACAAAGA GCTTCAACAG GGGAGAGTGT
     CTCGTTTCGT CTGATGCTCT TTGTGTTTCA GATGCGGACG CTTCAGTGGG TAGTCCCGGA CTCGAGCGGG CAGTGTTTCT CGAAGTTGTC CCCTCTCACA
182  SerLysAla AspTyrGluL ysHisLysVa lTyrAlaCys GluValThrH isGlnGlyLe uSerSerPro ValThrLysS erPheAsnAr gGlyGluCys
```

Fig. 8A

```
1101 TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA CGGAACTAGT GGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG
     ATTCGACTAG GAGATGCGGC CTGCGTAGCA CCGGGATCAT GCGTTGATCA GCATTTTTCC CATAGATCTC CAACTCCACT AAAATACTTT TTCTTATAGC
 215 OC*                                                                                                -23 MetLys LysAsnIleAla
                  Begin stII signal sequence
1201 CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC GCTGAGGTTC AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG
     GTAAAGAAGA ACGTAGATAC AAGCAAAAAA GATAACGATG TTTGCGCATG CGACTCCAAG TCGACCACCT CAGACCGCCA CCGGACCACG TCGGTCCCCC
  -17   PheLeuLe uAlaSerMet PheValPheS erIleAlaTh rAsnAlaTyr AlaGluValG lnLeuValGl uSerGlyGly GlyLeuValG lnProGlyGly
                                                                Begin heavy chain
1301 CTCACTCCGT TTGTCCTGTG CAGCTTCTGG CTATACCTTC ACCAACTATG GTATGAACTG GATCCGTCAG GCCCCGGGTA AGGGCCTGGA ATGGGTTGGA
     GAGTGAGGCA AACAGGACAC GTCGAAGACC GATATGGAAG TGGTTGATAC CATACTTGAC CTAGGCAGTC CGGGGCCCAT TCCCGGACCT TACCCAACCT
   17  SerLeuArg LeuSerCysA laAlaSerGl yTyrThrPhe ThrAsnTyrG lyMetAsnTr pIleArgGln AlaProGlyL ysGlyLeuGl uTrpValGly

1401 TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTTACT ATATCTGCAG ACACCTCCAG CAACACAGTT TACCTGCAGA
     ACCTAATTGT GGATATGGCC ACTTGGCTGG ATACGACGCC TAAAGTTTGC AGCAAAATGA TATAGACGTC TGTGGAGGTC GTTGTGTCAA ATGGACGTCT
   50 TrpIleAsnT hrTyrThrGl yGluProThr TyrAlaAlaA spPheLysAr gArgPheThr IleSerAlaA spThrSerSe rAsnThrVal TyrLeuGlnMet

1501 TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC CCGCACTATT ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG
     ACTTGTCGGA CGCGCGACTC CTGTGACGGC AGATAATGAC ACGTTTCATG GGCGTGATAA TACCCTCGTC GGTGACCATA AAGCTGCAGA CCCCAGTTCC
   84   AsnSerLe uArgAlaGlu AspThrAlaV alTyrTyrCy sAlaLysTyr ProHisTyrT yrGlySerSe rHisTrpTyr PheAspValT rpGlyGlnGly

1601 AACCCTGGTC ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC
     TTGGGACCAG TGGCAGAGGA GCCGGAGGTG GTTCCCGGGT AGCCAGAAGG GGGACCGTGG GAGGAGGTTC TCGTGGAGAC CCCCGTGTCG CCGGGACCCG
  117  ThrLeuVal ThrValSerS erAlaSerTh rLysGlyPro SerValPheP roLeuAlaPr oSerSerLys SerThrSerG lyGlyThrAl aAlaLeuGly

1701 TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT
     ACGGACCAGT TCCTGATGAA GGGGCTTGGC CACTGCCACA GCACCTTGAG TCCGCGGGAC TGGTCGCCGC ACGTGTGGAA GGGCCGACAG GATGTCAGGA
  150 CysLeuValL ysAspTyrPh eProGluPro ValThrValS erTrpAsnSe rGlyAlaLeu ThrSerGlyV alHisThrPh eProAlaVal LeuGlnSerSer

1801 CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA
     GTCCTGAGAT GAGGGAGTCG TCGCACCACT GGCACGGGAG GTCGTCGAAC CCGTGGGTCT GGATGTAGAC GTTGCACTTA GTGTTCGGGT CGTTGTGGTT
  184   GlyLeuTy rSerLeuSer SerValValT hrValProSe rSerSerLeu GlyThrGlnT hrTyrIleCy sAsnValAsn HisLysProS erAsnThrLys

1901 GGTCGACAAG AAAGTTGAGC CCAAATCTTG TGACAAAACT CACCTCTAGA GTGGCGGTGG CTCTGGTTCC GGTGATTTTG ATTATGAAAA GATGGCAAAC
     CCAGCTGTTC TTTCAACTCG GGTTTAGAAC ACTGTTTTGA GTGGAGATCT CACCGCCACC GAGACCAAGG CCACTAAAAC TAATACTTTT CTACCGTTTG
  217  ValAspLys LysValGluP roLysSerCy sAspLysThr HisLeuAM*S erGlyGlyGl ySerGlySer GlyAspPheA spTyrGluLy sMetAlaAsn
                                        end light chain   Begin g3p domain
2001 GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG CGCTACAGTC TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA
     CGATTATTCC CCCGATACTG GCTTTTACGG CTACTTTTGC GCGATGTCAG ACTGCGATTT CCGTTTGAAC TAAGACAGCG ATGACTAATG CCACGACGAT
  250 AlaAsnLysG lyAlaMetTh rGluAsnAla AspGluAsnA laLeuGlnSe rAspAlaLys GlyLysLeuA spSerValAl aThrAspTyr GlyAlaAlaIle

2101 TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG
     AGCTACCAAA GTAACCACTG CAAAGGCCGG AACGATTACC ATTACCACGA TGACCACTAA AACGACCGAG ATTAAGGGTT TACCGAGTTC AGCCACTGCC
  284   AspGlyPh eIleGlyAsp ValSerGlyL euAlaAsnGl yAsnGlyAla ThrGlyAspP heAlaGlySe rAsnSerGln MetAlaGlnV alGlyAspGly

2201 TGATAATTCA CCTTTAATGA ATAATTTCCG TCAATATTTA CCTTCCCTCC CTCAATCGGT TGAATGTCGC CCTTTTGTCT TTAGCGCTGG TAAACCATAT
     ACTATTAAGT GGAAATTACT TATTAAAGGC AGTTATAAAT GGAAGGGAGG GAGTTAGCCA ACTTACAGCG GGAAAACAGA AATCGCGACC ATTTGGTATA
  317  AspAsnSer ProLeuMetA snAsnPheAr gGlnTyrLeu ProSerLeuP roGlnServa lGluCysArg ProPheValP heSerAlaGl yLysProTyr
```

Fig. 8B

```
2301 GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCCGTG GTGTCTTTGC GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA
     CTTAAAAGAT AACTAACACT GTTTTATTTG AATAAGGCAC CACAGAAACG CAAAGAAAAT ATACAACGGT GGAAATACAT ACATAAAAGA TGCAAACGAT
 350 GluPheSerI leAspCysAs pLysIleAsn LeuPheArgG lyValPheAl aPheLeuLeu TyrValAlaT hrPheMetTy rValPheSer ThrPheAlaAsn

2401 ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC
     TGTATGACGC ATTATTCCTC AGAATTAGTA CGGTCAAGAA AACCGATCGC GGCGGGATAT GGAACAGACG GAGGGGCGCA ACGCAGCGCC ACGTACCTCG
 384   IleLeuAr gAsnLysGlu SerOC* (SEQ ID NO: 100)
                 end g3 protein
2501 CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC
     GCCCGGTGGA GCTGGACTTA CCTTCGGCCG CCGTGGAGCG ATTGCCTAAG TGGTGAGGTT CTTAACCTCG GTTAGTTAAG AACGCCTCTT GACACTTACG

2601 GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC
     CGTTTGGTTG GGAACCGTCT TGTATAGGTA GCGCAGGCGG TAGAGGTCGT CGGCGTGCGC CGCGTAGAGC CCGTCGCAAC CCAGGACCGG TGCCCACGCG

2701 ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT TACTGGTTAG CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT
     TACTAGCACG AGGACAGCAA CTCCTGGGCC GATCCGACCG CCCCAACGGA ATGACCAATC GTCTTACTTA GTGGCTATGC GCTCGCTTGC ACTTCGCTGA

2801 GCTGCTGCAA AACGTCTGCG ACCTGAGCAA CAACATGAAT GGTCTTCGGT TTCCGTGTTT CGTAAAGTCT GGAAACGCGG AAGTCAGCGC CCTGCACCAT
     CGACGACGTT TTGCAGACGC TGGACTCGTT GTTGTACTTA CCAGAAGCCA AAGGCACAAA GCATTTCAGA CCTTTGCGCC TTCAGTCGCG GGACGTGGTA

2901 TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC
     ATACAAGGCC TAGACGTAGC GTCCTACGAC GACCGATGGG ACACCTTGTG GATGTAGACA TAATTGCTTC GCGACCGTAA CTGGGACTCA CTAAAAAGAG

3001 TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG TAACCGGGCA TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC
     ACCAGGGCGG CGTAGGTATG GCGGTCAACA AATGGGAGTG TTGCAAGGTC ATTGGCCCGT ACAAGTAGTA GTCATTGGGC ATAGCACTCG TAGGAGAGAG

3101 GTTTCATCGG TATCATTACC CCCATGAACA GAAATTCCCC CTTACACGGA GGCATCAAGT GACCAAACAG GAAAAAACCG CCCTTAACAT GGCCCGCTTT
     CAAAGTAGCC ATAGTAATGG GGGTACTTGT CTTTAAGGGG GAATGTGCCT CCGTAGTTCA CTGGTTTGTC CTTTTTTGGC GGGAATTGTA CCGGGCGAAA

3201 ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG AGCTGGACGC GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC
     TAGTCTTCGG TCTGTAATTG CGAAGACCTC TTTGAGTTGC TCGACCTGCG CCTACTTGTC CGTCTGTAGA CACTTAGCGA AGTGCTGGTG CGACTACTCG

3301 TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC
     AAATGGCGTC CTAGGCCTTT AACATTTGCA ATTATAAAAC AATTTTAAGC GCAATTTAAA AACAATTTAG TCGAGTAAAA AATTGGTTAT CCGGCTTTAG

3401 GGCAAAATCC CTTATAAATC AAAAGAATAG ACCGAGATAG GGTTGAGTGT TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG
     CCGTTTTAGG GAATATTTAG TTTTCTTATC TGGCTCTATC CCAACTCACA ACAAGGTCAA ACCTTGTTCT CAGGTGATAA TTTCTTGCAC CTGAGGTTGC

3501 TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG
     AGTTTCCCGC TTTTTGGCAG ATAGTCCCGA TACCGGGTGA TGCACTTGGT AGTGGGATTA GTTCAAAAAA CCCCAGCTCC ACGGCATTTC GTGATTTAGC

3601 GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG
     CTTGGGATTT CCCTCGGGGG CTAAATCTCG AACTGCCCCT TTCGGCCGCT TGCACCGCTC TTTCCTTCCC TTCTTTCGCT TTCCTCGCCC GCGATCCCGC

3701 CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC CTGCCTCGCG CGTTTCGGTG
     GACCGTTCAC ATCGCCAGTG CGACGCGCAT TGGTGGTGTG GGCGGCGCGA ATTACGCGGC GATGTCCCGC GCAGGCCTAG GACGGAGCGC GCAAAGCCAC

3801 ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC
     TACTGCCACT TTTGGAGACT GTGTACGTCG AGGGCCTCTG CCAGTGTCGA ACAGACATTC GCCTACGGCC CTCGTCTGTT CGGGCAGTCC CGCGCAGTCG
```

Fig. 8C

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3901 GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA GCGGAGTGTA TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA
     CCCACAACCG CCCACAGCCC CGCGTCGGTA CTGGGTCAGT GCATCGCTAT CGCCTCACAT ATGACCGAAT TGATACGCCG TAGTCTCGTC TAACATGACT

4001 GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA GGCGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC
     CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT ATGGCGTAGT CCGCGAGAAG GCGAAGGAGC GAGTGACTGA GCGACGCGAG

4101 GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA
     CCAGCAAGCC GACGCCGCTC GCCATAGTCG AGTGAGTTTC CGCCATTATG CCAATAGGTG TCTTAGTCCC CTATTGCGTC CTTTCTTGTA CACTCGTTTT

4201 GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC
     CCGGTCGTTT TCCGGTCCTT GGCATTTTTC CGGCGCAACG ACCGCAAAAA GGTATCCGAG GCGGGGGGAC TGCTCGTAGT GTTTTTAGCT GCGAGTTCAG

4301 AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA
     TCTCCACCGC TTTGGGCTGT CCTGATATTT CTATGGTCCG CAAAGGGGGA CCTTCGAGGG AGCACGCGAG AGGACAAGGC TGGGACGGCG AATGGCCTAT

4401 CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT
     GGACAGGCGG AAAGAGGGAA GCCCTTCGCA CCGCGAAAGA GTATCGAGTG CGACATCCAT AGAGTCAAGC CACATCCAGC AAGCGAGGTT CGACCCGACA

4501 GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG
     CACGTGCTTG GGGGGCAAGT CGGGCTGGCG ACGCGGAATA GGCCATTGAT AGCAGAACTC AGGTTGGGCC ATTCTGTGCT GAATAGCGGT GACCGTCGTC

4601 CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG
     GGTGACCATT GTCCTAATCG TCTCGCTCCA TACATCCGCC ACGATGTCTC AAGAACTTCA CCACCGGATT GATGCCGATG TGATCTTCCT GTCATAAACC

4701 TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC
     ATAGACGCGA GACGACTTCG GTCAATGGAA GCCTTTTTCT CAACCATCGA GAACTAGGCC GTTTGTTTGG TGGCGACCAT CGCCACCAAA AAAACAAACG

4801 AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA
     TTCGTCGTCT AATGCGCGTC TTTTTTTCCT AGAGTTCTTC TAGGAAACTA GAAAAGATGC CCCAGACTGC GAGTCACCTT GCTTTTGAGT GCAATTCCCT

4901 TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC
     AAAACCAGTA CTCTAATAGT TTTTCCTAGA AGTGGATCTA GGAAAATTTA ATTTTTACTT CAAAATTTAG TTAGATTTCA TATATACTCA TTTGAACCAG

5001 TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG
     ACTGTCAATG GTTACGAATT AGTCACTCCG TGGATAGAGT CGCTAGACAG ATAAAGCAAG TAGGTATCAA CGGACTGAGG GGCAGCACAT CTATTGATGC

5101 ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA
     TATGCCCTCC CGAATGGTAG ACCGGGGTCA CGACGTTACT ATGGCGCTCT GGGTGCGAGT GGCCGAGGTC TAAATAGTCG TTATTTGGTC GGTCGGCCTT

5201 GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT
     CCCGGCTCGC GTCTTCACCA GGACGTTGAA ATAGGCGGAG GTAGGTCAGA TAATTAACAA CGGCCCTTCG ATCTCATTCA TCAAGCGGTC AATTATCAAA

5301 GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA
     CGCGTTGCAA CAACGGTAAC GACGTCCGTA GCACCACAGT GCGAGCAGCA AACCATACCG AAGTAAGTCG AGGCCAAGGG TTGCTAGTTC CGCTCAATGT

5401 TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG
     ACTAGGGGGT ACAACACGTT TTTTCGCCAA TCGAGGAAGC CAGGAGGCTA GCAACAGTCT TCATTCAACC GGCGTCACAA TAGTGAGTAC CAATACCGTC

5501 CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC
     GTGACGTATT AAGAGAATGA CAGTACGGTA GGCATTCTAC GAAAAGACAC TGACCACTCA TGAGTTGGTT CAGTAAGACT CTTATCACAT ACGCCGCTGG
```

Fig. 8D

```
5601 GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC
     CTCAACGAGA ACGGGCCGCA GTTGTGCCCT ATTATGGCGC GGTGTATCGT CTTGAAATTT TCACGAGTAG TAACCTTTTG CAAGAAGCCC CGCTTTTGAG

5701 TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG
     AGTTCCTAGA ATGGCGACAA CTCTAGGTCA AGCTACATTG GGTGAGCACG TGGGTTGACT AGAAGTCGTA GAAAATGAAA GTGGTCGCAA AGACCCACTC

5801 CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA
     GTTTTTGTCC TTCCGTTTTA CGGCGTTTTT TCCCTTATTC CCGCTGTGCC TTTACAACTT ATGAGTATGA GAAGGAAAAA GTTATAATAA CTTCGTAAAT

5901 TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC
     AGTCCCAATA ACAGAGTACT CGCCTATGTA TAAACTTACA TAAATCTTTT TATTTGTTTA TCCCCAAGGC GCGTGTAAAG GGGCTTTTCA CGGTGGACTG

6001 GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTTC AA (SEQ ID NO: 99)
     CAGATTCTTT GGTAATAATA GTACTGTAAT TGGATATTTT TATCCGCATA GTGCTCCGGG AAAGCAGAAG TT
```

Fig. 8E

▨ = differences from F(ab)-12

```
                        10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
MB1.6    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
H2305.6  DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0101    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0192    DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
                                        CDR-L1
                    40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
MB1.6    KPGKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
H2305.6  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
Y0101    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0192    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
                    CDR-L2
                80                  90                  100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
MB1.6    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 101)
H2305.6  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:103)
Y0101    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:105)
Y0192    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 107)
                        CDR-L3
```

Fig. 9A

```
                        10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
MB1.6    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
H2305.6  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
Y0101    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0192    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVR
                                        CDR-H1
                    40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
MB1.6    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTISADTSSNIV
H2305.6  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSADTSSNIV
Y0101    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0192    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                    CDR-H2                          CDR-7
                80                  90                  100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
MB1.6    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:102)
H2305.6  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:104)
Y0101    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:106)
Y0192    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:108)
                                    CDR-H3
```

Fig. 9B

▩ = differences from F(ab)-12

```
                          10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0243-1  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0238-3  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0313-1  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0317    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
                                    CDR-L1
                  40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0243-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0238-3  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0313-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0317    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
             CDR-L2
          80          90                  100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
Y0243-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:109)
Y0238-3  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:111)
Y0313-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:113)
Y0317    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:115)
                        CDR-L3
```

Fig. 10A

```
                          10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0243-1  EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
Y0238-3  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVR
Y0313-1  EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
Y0317    EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
                                    CDR-H1
                  40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0243-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0238-3  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0313-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0317    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                CDR-H2                              CDR-7
          80          90          100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
Y0243-1  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:110)
Y0238-3  YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:112)
Y0313-1  YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:114)
Y0317    YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:116)
                                    CDR-H3
```

Fig. 10B

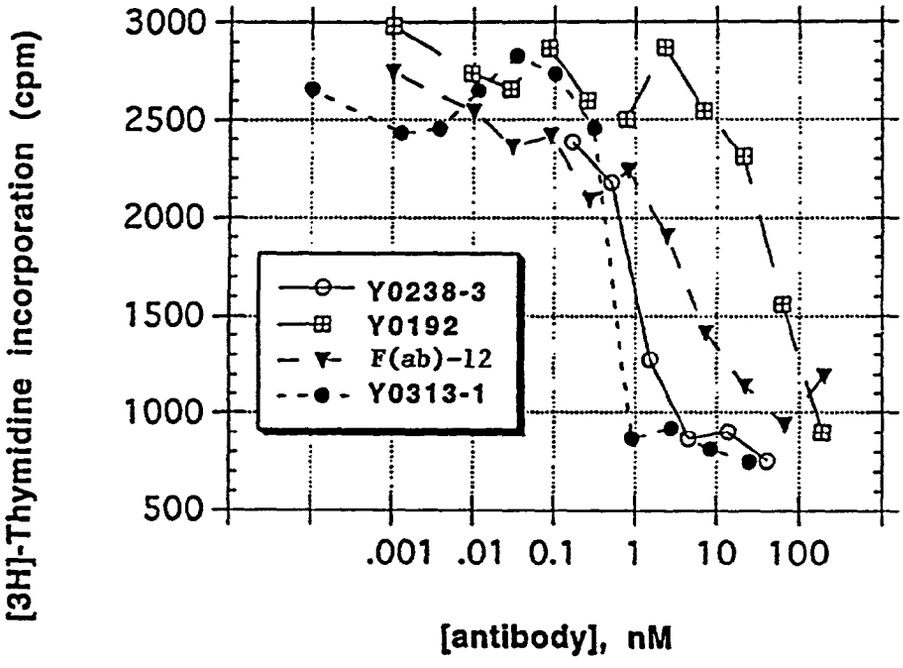
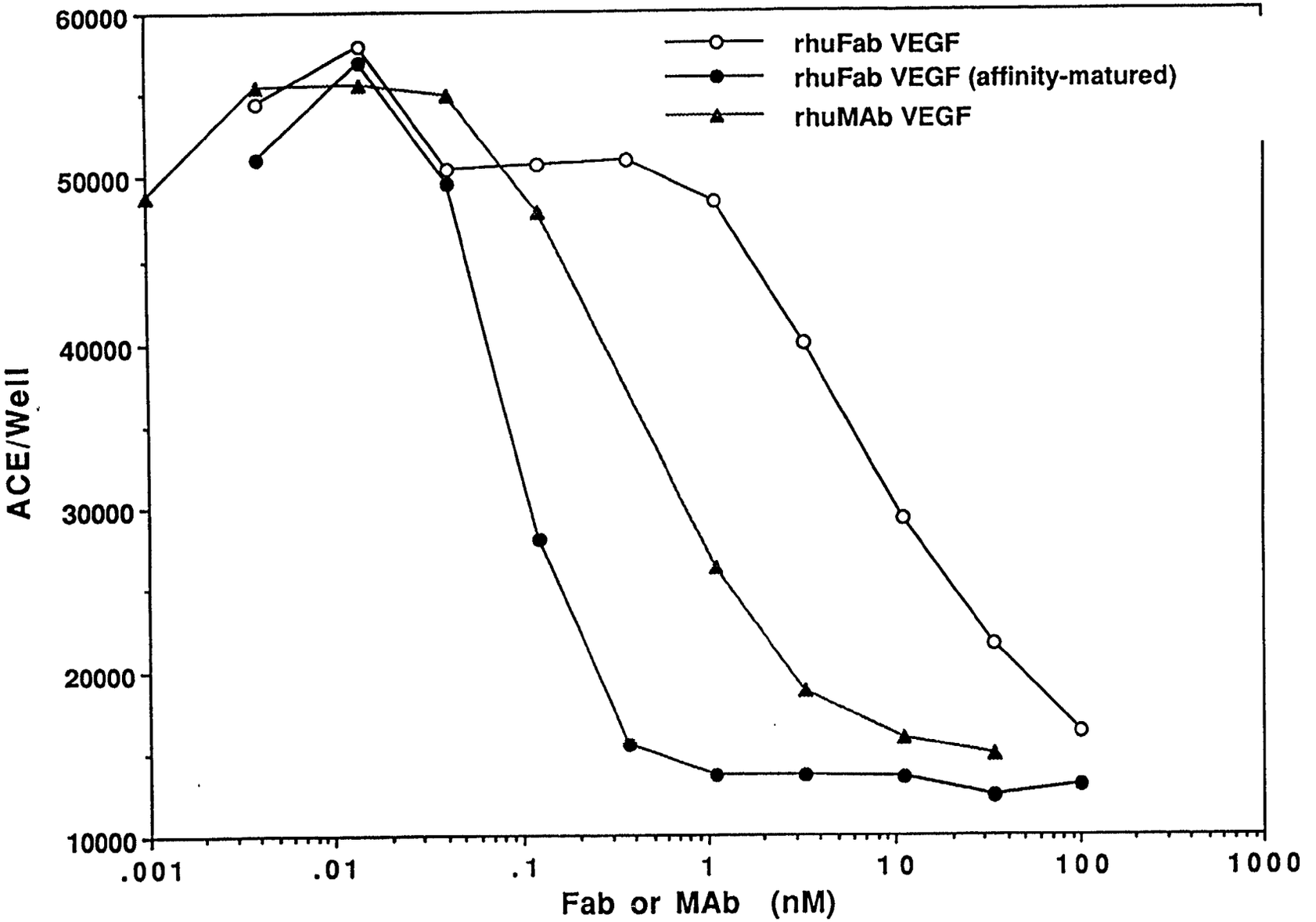

[3H]-Thymidine incorporation (cpm)
3000
2500
2000
1500
1000
500
.001
.01
0.1
1
10
100
[antibody], nM
Y0238-3
Y0192
F(ab)-12
Y0313-1

Fig. 11

Fig. 12



Express Mail No.: EL599584388US
Atty. Docket No.: P1093P1D1
Date Mailed: November 27, 2000

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY FOR CONTINUING APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTI-VEGF ANTIBODIES

the specification of which (check one) _ is attached hereto or X was filed on 06 August 1997 as Application Serial No. 08/908,469 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed Yes	No
Number	Country	Day/Month/Year Filed		

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Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/833,504	April 7, 1997	Pending
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: To Be Assigned Filed: Herewith For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on November 27, 2000 Eileen Ly

ASSOCIATE POWER OF ATTORNEY (37 CFR 1.34)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please recognize as Associate Attorney in this case:

Steven X. Cui - Reg. No. 44,637

Please direct all communications relative to said pending patent application to the following address:

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Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000

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Attorney Docket **P1093P1D1**
PATENT

CERTIFICATION UNDER 37 CFR 1.10

EL599584388US Express Mail Number **November 27, 2000 Date of Deposit**

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Eileen Ly

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Manuel Baca, Foster City, CA
James A. Wells, Burlingame, CA
Leonard G. Presta, San Francisco, CA
Henry B. Lowman, El Granada, CA
Yvonne Man-yee Chen, San Mateo, CA

Title: **ANTI-VEGF ANTIBODIES**

1. Type of Application

[ ] This application is for an original, non-provisional application.

[ ] This is a non-provisional application claiming priority to provisional application no. __ , filed ___, the entire disclosure of which is hereby incorporated by reference.

[X] This is a [ ] continuation-in-part [ ] continuation [X] divisional application claiming priority to application Serial Number 08/908,469 , filed August 6, 1997 , the entire disclosure of which is hereby incorporated by reference.

2. **Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)**

80 pages of specification
5 pages of claims
1 page(s) of abstract
16 sheet(s) of drawings
[] formal [X] informal

#83589

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)
___ An executed declaration of the inventor(s) [] is enclosed [] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)
__X__ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)
___ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)
___ An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [] will follow.

(for cont./div.)
__X__ The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

__X__ Cancel in this application original claims 1-38 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

_____ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

__X__ Amend the specification by inserting before the first line the sentence:

--This is a

_____ non-provisional application
_____ continuation
__X__ divisional
_____ continuation-in-part

of co-pending application(s)

__X__ Serial No. 08/908,469 filed on August 6, 1997, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --
_____ International Application _ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

#83589

_____ provisional application No. _ filed _, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--.

6. Payment of Fees

__X__ Applicants request deferral of payment of the filing fee until submission of the missing parts of application. **DO NOT CHARGE THE FILING FEE AT THIS TIME.**

7. Additional Papers Enclosed

[] Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
[] Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
[X] Associate Power of Attorney.
[] Other:

8. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

_____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
_____ A copy of the petition for extension of time in the ***prior*** application is attached.

9. Correspondence Address:

__X__ Address all future communications to:

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-8674

Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000

By: Steven X. Cui
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#83589

Express Mail No.: EL599584388US
Atty. Docket No.: P1093P1D1
Date Mailed: November 27, 2000

ANTI-VEGF ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of co-pending U.S. Application Serial No. 08/833,504, filed April 7, 1997, which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to anti-VEGF antibodies and, in particular, to humanized anti-VEGF antibodies and variant anti-VEGF antibodies.

Description of Related Art

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun

et al). The negative regulators so far identified include thrombospondin (Good *et al. Proc. Natl. Acad. Sci. USA.* 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039(1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

SUMMARY OF THE INVENTION

This application describes humanized anti-VEGF antibodies and anti-VEGF antibody variants with desirable properties from a therapeutic perspective, including strong binding affinity for VEGF; the ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and the ability

to inhibit VEGF-induced angiogenesis *in vivo*.

The preferred humanized anti-VEGF antibody or variant anti-VEGF antibody herein binds human VEGF with a K_d value of no more than about 1×10^{-8}M and preferably no more than about 5×10^{-9}M. In addition, the humanized or variant anti-VEGF antibody may have an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*. The humanized or variant anti-VEGF antibodies of particular interest herein are those which inhibit at least about 50% of tumor growth in an A673 *in vivo* tumor model, at an antibody dose of 5mg/kg.

In one embodiment, the anti-VEGF antibody has a heavy and light chain variable domain, wherein the heavy chain variable domain comprises hypervariable regions with the following amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129). For example, the heavy chain variable domain may comprise the amino acid sequences of CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR;SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV;SEQ ID NO:3). Preferably, the three heavy chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4.

The invention further provides an anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYX_1FTX_2YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSK STAYLQMNSLRAEDTAVYYCAKYPX_3YYGX_4SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T. One particularly useful heavy chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the heavy chain variable domain sequence of SEQ ID NO:7. Such preferred heavy chain variable domain sequences may be combined with the following preferred light chain variable domain sequences or with other light chain variable domain sequences, provided that the antibody so produced binds human VEGF.

The invention also provides preferred light chain variable domain sequences which may be combined with the above-identified heavy chain variable domain sequences or with other heavy

chain variable domain sequences, provided that the antibody so produced retains the ability to bind to human VEGF. For example, the light chain variable domain may comprise hypervariable regions with the following amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6). Preferably, the three light chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4.

In one embodiment, the invention provides a humanized anti-VEGF antibody light chain variable domain comprising the amino acid sequence:
DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L. One particularly useful light chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the light chain variable domain sequence of SEQ ID NO:8.

The invention also provides a variant of a parent anti-VEGF antibody (which parent antibody is preferably a humanized or human anti-VEGF antibody), wherein the variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of the heavy or light chain variable domain of the parent anti-VEGF antibody. The variant preferably has one or more substitution(s) in one or more hypervariable region(s) of the anti-VEGF antibody. Preferably, the substitution(s) are in the heavy chain variable domain of the parent antibody. For example, the amino acid subsition(s) may be in the CDRH1 and/or CDRH3 of the heavy chain variable domain. Preferably, there are substitutions in both these hypervariable regions. Such "affinity matured" variants are demonstrated herein to bind human VEGF more strongly than the parent anti-VEGF antibody from which they are generated, *i.e.*, they have a K_d value which is significantly less than that of the parent anti-VEGF antibody. Preferably, the variant has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower, preferably at least about 20 fold lower, and most preferably at least about 50 fold lower, than that of the parent anti-VEGF antibody. One particularly prefered variant is the Y0317 variant of Example 3, which has a CDRH1 comprising the amino acid sequence:GYDFTHYGMN (SEQ ID NO:126) and a CDRH3 comprising the amino acid sequence:YPYYYGTSHWYFDV (SEQ ID

NO:127). These hypervariable regions and CDRH2 are generally provided in a human framework region, *e.g.*, resulting in a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:116. Such heavy chain variable domain sequences are optionally combined with a light chain variable domain comprising the amino acid sequence of SEQ ID NO:124, and preferably the light chain variable domain amino acid sequence of SEQ ID NO:115.

Various forms of the antibody are contemplated herein. For example, the anti-VEGF antibody may be a full length antibody (*e.g.* having an intact human Fc region) or an antibody fragment (*e.g.* a Fab, Fab' or $F(ab')_2$). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of VEGF protein comprising exposing a sample suspected of containing the VEGF protein to the anti-VEGF antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the VEGF protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.* from the host cell culture medium). The invention also provides a composition comprising the anti-VEGF antibody and a pharmaceutically acceptable carrier or diluent. The composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a tumor or retinal disorder, comprising administering a therapeutically effective amount of the anti-VEGF antibody to the mammal.

Brief Description of the Drawings

Figs. 1A and 1B depict the amino acid sequences of variable heavy domain (SEQ ID NO:9) and light domain (SEQ ID NO:10) of muMAbVEGF A.4.6.1, variable heavy domain (SEQ ID NO:7)

and light domain (SEQ ID NO:8) of humanized F(ab) (F(ab)-12) and human consensus frameworks (hum III for heavy subgroup III (SEQ ID NO:11); humκ1 for light κ subgroup I (SEQ ID NO:12)). Fig. 1A aligns variable heavy domain sequences and Fig. 1B aligns variable light domain sequences. Asterisks indicate differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. Complementarity Determining Regions (CDRs) are underlined.

Fig. 2 is a ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in brown with CDRs in tan. The sidechain of residue L46 is shown in yellow. VH domain is shown in purple with CDRs in pink. Sidechains of VH residues changed from human to murine are shown in yellow.

Fig. 3 depicts inhibition of VEGF-induced mitogenesis by humanized anti-VEGF F(ab)-12 from Example 1. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6 X 10^3 cells/well in six well plates, as described in Example 1. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1; F(ab)-12) was added at the indicated concentrations. After 2-3 hours, rhVEGF165 was added at the final concentration of 3 ng/ml. After five or six days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

Fig. 4 shows inhibition of tumor growth *in vivo* by humanized anti-VEGF F(ab)-12 from Example 1. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2 x 10^6 per mouse. Starting 24 hours after tumor cell inoculation, animals were injected with a control MAb, muMAb VEGF A4.6.1 or rhuVEGF MAb (IgG1; F(ab)-12) twice weekly, intra peritoneally. The dose of the control Mab was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated (n = 10). Four weeks after tumor cell injection, animals were euthanized and tumors were removed and weighed. *: significant difference when compared to the control group by ANOVA ($p < 0.05$).

Figs. 5A and 5B show the acid sequences of the light and heavy variable domains respectively of murine antibody A4.6.1 (SEQ ID NO:10 for the VL and SEQ ID NO:9 for the VH) and humanized A4.6.1 variants hu2.0 (SEQ ID NO:13 for the VL and SEQ ID NO:14 for the VH) and hu2.10 (SEQ ID NO:15 for the VL and SEQ ID NO:16 for the VH) from Example 2. Sequence numbering is according to Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed.

Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain κ subgroup I consensus framework (SEQ ID NO:12) and heavy chain subgroup III consensus framework (SEQ ID NO:11). hu2.10 was the consensus humanized clone obtained from phage sorting experiments described herein.

Fig. 6 depicts framework residues targeted for randomization in Example 2.

Fig. 7 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in *supE E. coli)*, then the C-terminal region of the gene III protein (residues 249-406). Transformation into F^+ *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequence encoded thereby (SEQ ID NO:100).

Figs. 9A and 9B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants in Example 3, compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. Residues are numbered sequentially in the VL and VH domains, as opposed to the Kabat numbering scheme. The template molecule, MB1.6 (SEQ ID NO's 101 and 102 for light and heavy variable domains, respectively) is shown, along with variants: H2305.6 (SEQ ID NO's 103 and 104 for light and heavy variable domains, respectively), Y0101 (SEQ ID NO's 105 and 106 for light and heavy variable domains, respectively), and Y0192 (SEQ ID NO's 107 and 108 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Figs. 10A and 10B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants from Example 3 compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains,

respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. The variants are designated Y0243-1 (SEQ ID NO's 109 and 110 for light and heavy variable domains, respectively), Y0238-3 (SEQ ID NO's 111 and 112 for light and heavy variable domains, respectively), Y0313-1 (SEQ ID NO's 113 and 114 for light and heavy variable domains, respectively), and Y0317 (SEQ ID NO's 115 and 116 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Fig. 11 depicts the results of the HuVEC activity assay in Example 3 for variants Y0238-3, Y0192 and Y0313-1 as well as full length F(ab)-12 from Example 1.

Fig. 12 depicts inhibition of VEGF-induced mitogenesis by full length F(ab)-12 from Example 1 (rhuMAb VEGF), a Fab fragment of F(ab)-12 from Example 1 (rhuFab VEGF), and a Fab fragment of affinity matured variant Y0317 from Example 3 (rhuFab VEGF (affinity matured)).

Detailed Description of the Preferred Embodiments

I. Definitions

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an

extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the

constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.,* IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof . Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci.*

USA 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences (*e.g.* as in SEQ ID NO:7 or 8), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for

the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Mol. Cell. Biol.* 5(12):3610-3616(1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.*, *Protein Engineering* 3(6):547-553(1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the

function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containingprodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed

partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.,* agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by

ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. Modes for Carrying out the Invention

The examples hereinbelow describe the production of humanized and variant anti-VEGF antibodies with desirable properties from a therapeutic perspective including: (a) strong binding affinity for the VEGF antigen; (b) an ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro;* and (c) the ability to inhibit VEGF-induced angiogenesis *in vivo*.

Antibody affinities may be determined as described in the examples hereinbelow. Preferred humanized or variant antibodies are those which bind human VEGF with a K_d value of no more than about 1×10^{-7}M; preferably no more than about 1×10^{-8}M; and most preferably no more than about 5×10^{-9}M.

Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select humanized or variant antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits endothelial cell growth in response to VEGF. In one embodiment, the antibody may be able to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). Preferably, the antibody has an effective dose 50 (ED50) value of no more than about 5nM, preferably no more than about 1nM, and most preferably no more than about 0.5nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", *i.e.*, at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by 50%. A preferred "endothelial cell growth assay" involves culturing bovine adrenal cortex-derived capillary endothelial cells in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described in Example 1 below. These endothelial cells are seeded at a density of 6×10^3 cells per well, in 6-well plates in growth medium. Either parent anti-VEGF antibody (control), humanized or variant anti-VEGF antibody is then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified VEGF was added to a final concentration of 3 ng/ml. For specificity control, each antibody may be added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells are dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Data may be analyzed by a four-parameter curve fitting program (KaleidaGraph).

The preferred humanized or variant anti-VEGF antibody may also be one which has *in vivo* tumor suppression activity. For example, the antibody may suppress the growth of human A673 rhabdomyosarcoma cells or breast carcinoma MDA-MB-435 cells in nude mice. For *in vivo* tumor studies, human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) or MDA-MB-435 cells (available from the ATCC) are cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics as described in Example 1 below. Female BALB/c nude mice, 6-10 weeks old, are injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals are then treated with the humanized or variant antibody and a control antibody with no activity in this assay. The humanized or variant anti-VEGF MAb is administered at a dose of 0.5 and/or 5 mg/kg. Each MAb is administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Tumor size is determined at weekly intervals. Four weeks after tumor cell inoculation, animals are euthanized and the tumors are removed and weighed. Statistical analysis may be performed by ANOVA. Preferably, the antibody in this "*in vivo* tumor assay" inhibits about 50-100%, preferably about 70-100% and most preferably about 80-100% human A673 tumor cell growth at a dose of 5mg/kg.

In the preferred embodiment, the humanized or variant antibody fails to elicit an immunogenic response upon administration of a therapeutically effective amount of the antibody to a human patient. If an immunogenic response is elicited, preferably the response will be such that the antibody still provides a therapeutic benefit to the patient treated therewith.

The humanized or variant antibody is also preferably one which is able to inhibit VEGF-induced angiogenesis in a human, *e.g.* to inhibit human tumor growth and/or inhibit intraocular angiogenesis in retinal disorders.

Preferred antibodies bind the "epitope A4.6.1" as herein defined. To screen for antibodies which bind to the epitope on human VEGF bound by an antibody of interest (*e.g.*, those which block binding of the A4.6.1 antibody to human VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

The antibodies of the preferred embodiment herein have a heavy chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein "FR1-4" represent the four framework regions and "CDRH1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. Many human antibody framework region sequences are compiled in Kabat *et al.*, *supra*, for example. In one preferred embodiment, the variable heavy FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human heavy chains subgroup III (*e.g.* as in SEQ ID NO:11).

The human variable heavy FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding nonhuman residue (by "corresponding nonhuman residue" is meant the nonhuman residue with the same Kabat positional numbering as the human residue of interest when the human and nonhuman sequences are aligned), but replacement with the nonhuman residue is not necessary. For example, a replacement FR residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable heavy FR residues which may be substituted include any one or more of FR residue numbers: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H, 94H (Kabat residue numbering employed here). Preferably at least two, or at least three, or at least four of these residues are substituted. A particularly preferred combination of FR substitutions is: 49H, 69H, 71H, 73H, 76H, 78H, and 94H.

With respect to the heavy chain hypervariable regions, these preferably have amino acid sequences as follows:

CDRH1

$GYX_1X_2X_3X_4YGX_5N$ (SEQ ID NO:117), wherein X_1 is D, T or E, but preferably is D or T; X_2 is F, W, or Y, but preferably is F; X_3 is T, Q, G or S, but preferably is T; X_4 is H or N; and X_5 is M or I, but preferably is M.

CDRH2

WINTX$_1$TGEPTYAADFKR (SEQ ID NO:118), wherein X$_1$ is Y or W, but preferably is Y.

CDRH3

YPX$_1$YX$_2$X$_3$X$_4$X$_5$HWYFDV (SEQ ID NO:119), wherein X$_1$ is H or Y; X$_2$ is Y, R, K, I, T, E, or W, but preferably is Y; X$_3$ is G, N, A, D, Q, E, T, K, or S, but preferably is G; X$_4$ is S, T, K, Q, N, R, A, E, or G, but preferably is S or T; and X$_5$ is S or G, but preferably is S.

The heavy chain variable domain optionally comprises what has been designated "CDR7" herein within (*i.e.* forming part of) FR3 (see Figs. 9B and 10B), wherein CDR7 may have the following amino acid sequence:

CDR7

X$_1$SX$_2$DX$_3$X$_4$X$_5$X$_6$TX$_7$ (SEQ ID NO:120), wherein X$_1$ is F, I , V, L, or A, but preferably is F; X$_2$ is A, L, V, or I, but preferably is L; X$_3$ is T, V or K, but preferably is T; X$_4$ is S or W, but preferably is S; X$_5$ is S, or K, but preferably is K; X$_6$ is N, or S, but preferably is S; and X$_7$ is V, A, L or I, but preferably is A.

The antibodies of the preferred embodiment herein have a light chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein "FR1-4" represent the four framework regions and "CDRL1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. In one preferred embodiment, the variable light FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human kappa light chains subgroup I (*e.g.* as in SEQ ID NO:12).

The human variable light FR sequence preferably has substitutions therein, *e.g.* wherein the

human FR residue is replaced by a corresponding mouse residue, but replacement with the nonhuman residue is not necessary. For example, a replacement residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable light FR residues which may be substituted include any one or more of FR residue numbers: 4L, 46L and 71L (Kabat residue numbering employed here). Preferably only 46L is substituted. In another embodiment, both 4L and 46L are substituted.

With respect to the CDRs, these preferably have amino acid sequences as follows:

CDRL1

$X_1AX_2X_3X_4X_5SNYLN$ (SEQ ID NO:121), wherein X_1 is R or S, but preferably is S; X_2 is S or N, but preferably is S; X_3 is Q or E, but preferably is Q; X_4 is Q or D, but preferably is D; and X_5 is I or L, but preferably is I.

CDRL2

FTSSLHS (SEQ ID NO:122).

CDRL3

$QQYSX_1X_2PWT$ (SEQ ID NO:123), wherein X_1 is T, A or N, but preferably is T; and X_2 is V or T, but preferably is V.

Preferred humanized anti-VEGF antibodies are those having the heavy and/or light variable domain sequences of F(ab)-12 in Example 1 and variants thereof such as affinity matured forms including variants Y0317, Y0313-1 and Y0238-3 in Example 3, with Y0317 being the preferred variant. Methods for generating humanized anti-VEGF antibodies of interest herein are elaborated in more detail below.

A. Antibody Preparation

Methods for humanizing nonhuman VEGF antibodies and generating variants of anti-VEGF antibodies are described in the examples below. In order to humanize an anti-VEGF antibody, the nonhuman antibody starting material is prepared. Where a variant is to be generated, the parent antibody is prepared. Exemplary techniques for generating such nonhuman antibody

starting material and parent antibodies will be described in the following sections.

(i) Antigen preparation

The VEGF antigen to be used for production of antibodies may be, *e.g.*, intact VEGF or a fragment of VEGF (*e.g.* a VEGF fragment comprising "epitope A4.6.1"). Other forms of VEGF useful for generating antibodies will be apparent to those skilled in the art. The VEGF antigen used to generate the antibody, is preferably human VEGF, *e.g.* as described in Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991).

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard

methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) Humanization and amino acid sequence variants

Examples 1-2 below describe procedures for humanization of an anti-VEGF antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of these humanized antibodies, particularly where these improve the binding affinity or other biological properties of the humanized antibody. Example 3 describes methodologies for generating amino acid sequence variants of an anti-VEGF antibody with enhanced affinity relative to the parent antibody.

Amino acid sequence variants of the anti-VEGF antibody are prepared by introducing appropriate nucleotide changes into the anti-VEGF antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-VEGF antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-VEGF antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-VEGF antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with VEGF antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-VEGF antibody variants are screened for the desired activity. Alanine scanning mutagenesis is described in Example 3.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-VEGF antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-VEGF antibody molecule include the fusion to the N- or C-terminus of the anti-VEGF antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-VEGF antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-VEGF antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display (see Example 3 herein). Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis (see Example 3) can be performed

to identified hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-VEGF antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-VEGF antibody.

(v) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and US Patents 5,591,669, 5,589,369 and 5,545,807. Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); and US Patents 5,565,332 and 5,573,905). As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275)

(vi) Antibody fragments

In certain embodiments, the humanized or variant anti-VEGF antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al., Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vii) Multispecific antibodies

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized or variant anti-VEGF antibodies having binding specificities for at least two different

epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the VEGF protein. Alternatively, an anti-VEGF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the VEGF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express VEGF. These antibodies possess an VEGF-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to

thionitrobenzoate(TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from *E. coli* can be chemically coupled *in vitro* to form bispecific antibodies. Shalaby *et al., J. Exp. Med.* 175:217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al., J. Immunol.* 147:60 (1991).

(viii) Other modifications

Other modifications of the humanized or variant anti-VEGF antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain

disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al., Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al., Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.,* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.,* a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-VEGF antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al., Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an

exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.,* avidin) which is conjugated to a cytotoxic agent (*e.g.,* a radionuclide).

The anti-VEGF antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al., J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al., J. National Cancer Inst.*81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.,* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases,

such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-VEGF antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.,* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.,* by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain

of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence: PKNSSMISNTP (SEQ ID NO:17), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:18), HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s): HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21) and the sequence: PKNSSMISNTP (SEQ ID NO:17).

Covalent modifications of the humanized or variant anti-VEGF antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Exemplary covalent modifications of polypeptides are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized or variant anti-VEGF antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. In another embodiment, the antibody may be produced by homologous recombination, *e.g.* as described in US Patent 5,204,244, specifically incorporated herein by reference. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components

generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, *e.g.*, as described in US Patent 5,534,615 issued July 9, 1996 and specifically incorporated herein by reference.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-VEGF antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated anti-VEGF antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains

for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-VEGF antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the anti-VEGF antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and

phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzeneallow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin

(J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*,from about 0-0.25M salt).

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other (see Section F below). Such molecules are suitably present in

combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

D. Non-therapeutic Uses for the Antibody

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing

the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish

peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-VEGF antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc.

1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.,* US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulinantibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

E. Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.,* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.,* a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided

as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

F. Therapeutic Uses for the Antibody

For therapeutic applications, the anti-VEGF antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies also are suitably administered by intra tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodys of the present invention are expected to be especially useful in reducing the severity of AMD.

Depending on the type and severity of the disease, about 1 µg/kg to about 50 mg/kg (*e.g.*, 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 µg/kg to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak *et al.*, PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics,

pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy. The antibody and one or more other anti-VEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of anti-VEGF antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF antibody. Treatment with anti-VEGF antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

G. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials,

syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

EXAMPLE 1

This example describes the production of humanized anti-VEGF antibodies with desirable properties from a therapeutic standpoint.

MATERIALS AND METHODS

Cloning of Murine A4.6.1 MAb and Construction of Mouse-Human Chimeric Fab: The murine anti-VEGF mAb A4.6.1 has been previously described by Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993). Total RNA was isolated from hybridoma cells producing the anti-VEGF Mab A.4.6.1 using RNAsol (TEL-TEST) and reverse-transcribedto cDNA using Oligo-dT primer and the SuperScript II system (GIBCO BRL, Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the N-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup kV and heavy chain subgroup II (Kabat *et al. Sequences of Proteins of Immunological Interest.* 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). After polymerase chain reaction (PCR) amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned respectively into the pEMX1 vector containing the human CL and CH1 domains (Werther *et al. J. Immunol.* 157:4986-4995 (1996)), thus generating a

mouse-human chimera. This chimeric F(ab) consisted of the entire murine A4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113 and the entire murine A4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to that of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

Computer Graphics Models of Murine and Humanized F(ab): Sequences of the VL and VH domains (Figs.1A and 1B) were used to construct a computer graphics model of the murine A4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (Carter *et al. Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992) and Eigenbrot *et al. J.Mol. Biol.* 229:969-995 (1993)).

Construction of Humanized F(ab)s: The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *E. coli* has been described previously (Werther *et al., supra*). Briefly, the plasmid contains a DNA fragment encoding a consensus human k subgroup I light chain (VLkI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (Carter *et al., supra*).

To construct the first F(ab) variant of humanized A4.6.1, F(ab)-1, site-directed mutagenesis (Kunkel *et al., Proc. Natl. Acad. Sci. USA* 82:488-492 (1985)) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs according to Kabat *et al., supra*, were changed to the murine A4.6.1 sequence . F(ab)-1 therefore consisted of a complete human framework (VL k subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml Luria broth-100 mg/ml carbenicillin for

5-8 h at 37° C. The 5 ml culture was added to 500 ml AP5-50 µg/ml carbenicillin and allcwed to grow for 20 h in a 4 L baffled shake flask at 30° C. AP5 media consists of: 1.5 g glucose, 11.0 g Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO4 (anhydrous), 1.07 g NH4Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 mm Sealkeen filter. Cells were harvested by centrifugation in a 1 L centrifuge bottle at 3000x*g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM Tris-1 mM EDTA-20% sucrose, pH 8.0. 250 ml of 0.1 M benzamidine (Sigma, St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000x*g* for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (0.5 ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

Construction and Expression of Chimeric and Humanized IgG: For generation of human IgG1 variants of chimeric (chIgG1) and humanized (huIgG1) A4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12, Table 2) domains were subcloned into separate, previously described, pRK vectors (Eaton *et al.*, *Biochemistry* 25:8343-8347 (1986)). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were co-transfected into human 293 cells (Graham *et al.*, *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media was changed to serum-free and harvested daily for up to five days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore, Bedford, MA) and stored at 4° C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (Lucas *et al.*, *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line developed by L. Chasin (Columbia University), via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover,DM, Hames, BD. *DNA Cloning 4. Mammalian systems.* Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening. One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after this step was ~99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was < 0.10 eu/mg.

***F(ab) and IgG Quantitation*:** For quantitating F(ab) molecules, ELISA plates were coated with 2 µg/ml goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% bovine serum albumin (blocking buffer) at room temperature for 1 h. Standards (0.78 - 50 ng/ml human F(ab)) were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% bovine serum albumin-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a

Vmax plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program. Data points which fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples. The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

VEGF Binding Assay: For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 µg/ml rabbit $F(ab')_2$ to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (Park *et al., J. Biol. Chem.* 269:25646-25645 (1994)) in blocking buffer were incubated on the plate for 1 h. Standards (6.9 - 440 ng/ml chimeric F(ab)) and two-fold serial of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma, St. Louis, MO) followed by 3,3',5,5'-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

BIAcore™ Biosensor Assay: VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore™ biosensor (Karlsson *et al. Methods: A Comparison to Methods in Enzymology* 6:97-108 (1994)). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) (35 µl of 2 µM F(ab) at a flow rate of 20 µl/min) and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0 - 4500 sec were used for off-rate kinetic analysis. The dissociation rate constant (k_{off}) was obtained from the slope of the plot of ln(R0/R) versus time, where R0 is the signal at t=0 and R is the signal at each time point.

On-rate kinetics were measured using two-fold serial dilutions of F(ab) (0.0625 - 2 mM). The slope, K_s, was obtained from the plot of ln(-dR/dt) versus time for each F(ab) concentration using the BIAcore™ kinetics evaluation software as described in the Pharmacia Biosensor manual. R is the signal at time t. Data between 80 and 168, 148, 128, 114, 102, and 92 sec were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant (k_{on}) was obtained from the slope of the plot of K_s versus F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5 µl of 50 mM HCl at a flow rate of 20 µl/min to regenerate the chip.

Endothelial Cell Growth Assay: Bovine adrenal cortex-derived capillary endothelial cells were cultured in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as previously described (Leung *et al. Science* 246:1306-1309 (1989)). For mitogenic assays, endothelial cells were seeded at a density of 6 x 10^3 cells per well, in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF was then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified *E.coli*-expressed rhVEGF165 was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells were dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

In Vivo Tumor Studies: Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female BALB/c nude mice, 6-10 weeks old, were injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF or a control MAb directed against the gp120 protein (Kim *et al. Nature* 362:841-844 (1993)). Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks

after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

RESULTS

Humanization: The consensus sequence for the human heavy chain subgroup III and the light chain subgroup k I were used as the framework for the humanization (Kabat *et al.*, *supra*) (Figs. 1A and 1B). This framework has been successfully used in the humanization of other murine antibodies (Werther *et al., supra*; Carter *et al., supra*; Presta *et al. J. Immunol.* 151:2623-2632 (1993); and Eigenbrot *et al. Proteins* 18:49-62 (1994)). CDR-H1 included residues H26-H35. The other CDRs were according to Kabat *et al., supra*. All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli*. Typical yields from 500 ml shake flasks were 0.1-0.4 mg F(ab).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in human) was changed to the murine Gly. In addition, F(ab)s which consisted of the chimeric heavy chain/F(ab)-1 light chain (F(ab)-2) and F(ab)-1 heavy chain/chimeric light chain (F(ab)-3) were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 2). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered in order to increase binding.

Table 2: Binding of Humanized Anti-VEGF F(ab) Variants to VEGF[a]

Variant	Template	Changes[b]	Purpose	EC50 F(ab)-X		
				EC50 chimeric F(ab)[c]		
				Mean	S.D.	N
chim-F(ab)	Chimeric F(ab)			1.0		
F(ab)-1	Human FR		Straight CDR swap AlaH49Gly	>1350		2
F(ab)-2			Chimera Light Chain F(ab)-1 Heavy Chain	>145		3
F(ab)-3			F(ab)-1 Light Chain Chimera Heavy Chain	2.6	0.1	2
F(ab)-4	F(ab)-1	ArgH71Leu AsnH73Thr	CDR-H2 conformation Framework	>295		3
F(ab)-5	F(ab)-4	LeuL46Val	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78Ala	CDR-H1 conformation	36.4	4.2	2
F(ab)-7	F(ab)-5	IleH69Phe	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69Phe LeuH78Ala	CDR-H2 conformation CDR-H1 conformation	9.6	0.9	4
F(ab)-9	F(ab)-8	GlyH49Ala	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76Ser	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75Ala	Framework	3.3	0.4	2
F(ab)-12	F(ab)-10	ArgH94Lys	CDR-H3 conformation	1.6	0.6	4

[a]Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, *supra*).

[b]Murine residues are underlined; residue numbers are according to Kabat *et al.*, *supra*.

[c]Mean and standard deviation are the average of the ratios calculated for each of the independent assays; the EC50 for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

Changing human residues H71 and H73 to their murine counterparts in F(ab)-4 improved binding by 4-fold (Table 2). Inspection of the models of the murine and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 (F(ab)-5), the binding affinity increased by almost 4-fold (Table 2). Three other buried framework residues were evaluated based on the molecular models: H49, H69 and H78. Position H69 may affect the conformation of CDR-H2 while position H78 may affect the conformation of CDR-H1 (Figure 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case (F(ab)-6 and F(ab)-7, Table 2). When both were simultaneously changed, the improvement in binding was 8-fold (F(ab)-8, Table 2). Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala the binding was reduced by 15-fold (F(ab)-9, Table 2).

In F(ab)-10 and F(ab)-11 two residues in framework loop 3, FR-3, were changed to their murine counterparts: AsnH76 to murine Ser (F(ab)-10) and LysH75 to murine Ala (F(ab)-11). Both effected a relatively small improvement in binding (Table 2). Finally, at position H94 human and murine sequences most often have an Arg (Kabat *et al., supra*). In F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig.1A) and this resulted in binding which was less than 2-fold from the chimeric F(ab) (Table 2). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore™ system (Pharmacia). Using this technique the K_d of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower k_{on} and faster k_{off} (Table 3).

Table 3: Binding of Anti-VEGF F(ab) Variants to VEGF Using the BIAcore™ System[a]

Variant	Amount of (Fab) bound (RU)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
chim-F(ab)[b]	4250	$5.9x10^{-5}$	$6.5x10^{4}$	0.91
F(ab)-12	3740	$6.3x10^{-5}$	$3.5x10^{4}$	1.8

[a] The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore™ system when 2 µg F(ab) was injected onto a chip containing 2480 RU immobilized VEGF. Off-rate kinetics (k_{off}) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics (k_{on}) were measured using two-fold serial dilutions of F(ab). K_d, the equilibrium dissociation constant, was calculated as k_{off}/k_{on}.
b chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

Full length mAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human k light chain and human IgG1 heavy chain. The full length 12-IgG1 (F(ab)-12 fused to human IgG1) exhibited binding which was 1.7-fold weaker than the chimeric IgG1 (Table 4). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original murine mAb A4.6.1 (Table 4).

Table 4: Binding of Anti-VEGF IgG Variants to VEGF[a]

IgG1/chIgG1[b]			
Variant	**Mean**	**S.D.**	**N**
chIgG1	1.0		2
murIgG1[c]	0.759	0.001	2
12-IgG1[d]	1.71	0.03	2

[a] Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, (1994), *supra*).
[b] chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC50 for chIgG1 was 0.113 ± 0.013 µg/ml (0.75 nM).
[c] murIgG1 is muMAbVEGF A461 purified from ascites.
[d] 12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

Biological Studies: rhuMAb VEGF and muMAb VEGF A.4.6.1. were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). As illustrated in Figure 3, the two MAbs were essentially equivalent, both in potency and efficacy. The ED50 values were respectively 50 ± 5 ng/ml and 48 ± 8 ng/ml (~0.3 nM). In both cases 90% inhibition was achieved at the concentration of 500 ng/ml (~3 nM). Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any

effect on basal or bFGF-stimulated proliferation of capillary endothelial cells, confirming that the inhibition is specific for VEGF.

To determine whether such equivalency applies also to an *in vivo* system, the two antibodies were compared for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res* 56:4032-4039 (1996)). As shown in Figure 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements four weeks after cell inoculation. The decreases in tumor weight compared to the control group were respectively 85% and 93% at each dose in the animals treated with muMAb VEGF A.4.6.1. versus 90% and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435.

EXAMPLE 2

In this example, the murine anti-VEGF antibody A4.6.1 discussed above was humanized by randomizing a small set of framework residues and by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage in order to identify high affinity framework sequences via affinity-based selection.

MATERIALS AND METHODS

Construction of Anti-VEGF Phagemid Vector, pMB4-19: The murine anti-VEGF mAb A4.6.1 is discussed above in Example 1. The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 (Carter *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:4285-4289 (1992)) which codes for a human V_LkI-Cκ_1 light chain and human V_HIII-$C_H1\gamma_1$ heavy chain Fd fragment The transplanted A4.6.1 CDR sequences were chosen according to the sequence definition of Kabat *et al.*, *supra*, except for CDR-H1 which included residues 26-35. The Fab encoding sequence was subcloned into the phagemid vector phGHamg3 (Bass *et al. Proteins* 8:309-314 (1990) and Lowman *et al. Biochemistry* 30:10832-10838 (1991)). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion

of the M13 gene III coat protein. pMB4-19 is similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments (Garrard *et al. Biotechnology* 9:1373-1377 (1991)). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

Expression and Purification of Humanized A4.6.1 Fab Fragment: *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB4-19, or variants thereof. Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium (Chang *et al. Gene* 55:189-196 (1987)) containing 20 µg/mL carbenicillin and incubated for 26 hr at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 h. Cell pellets were then resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 min and centrifuged at 10,000 x g for 15 min. The supernatant was applied to a 1 mL streptococcal protein G-sepharose column (Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M Tris-HCl, pH 8.0. Fab preparations were buffer-exchanged into PBS and concentrated using Centricon-30 concentrators (Amicon). Typical yields of Fab were ~1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

Construction of the Anti-VEGF Fab Phagemid Library: The humanized A4.6.1 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel *et al. Methods Enzymol.* 204:125-139 (1991)). A derivative of pMB4-19 containing TAA stop triplets at V_H codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat *et al.*, *supra*). This modification was to prevent subsequent background contamination by wild type sequences. The codons targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

In order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60

and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol of 5' phosphorylated oligonucleotide 503-1 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:22)) or 503-2 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:23)) were combined with 1.5 nmol of 503-3 (5'-AGC CTG CGC GCT GAG GAC ACT GCC GTC TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG-3' (SEQ ID NO:24)) (randomized codons underlined; N=A/G/T/C; W=A/T; B=G/T/C; D=G/A/T; R=A/G; Y=C/T). Then, 1.5 nmol of template oligonucleotide (5'-CTC AGC GCG CAG GCT GTT CAT CTG CAG GTA-3' (SEQ ID NO:25)), with complementary sequence to the 5' ends of 503-1/2 and the 3' end of 503-3, was added to hybridize to each end of the ligation junction. *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95° C 1.25 min; 50° C for 5 min) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10mM Tris-HCl, 1mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons (V_L 4, 71; V_H 24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was effected in two steps. Firstly, V_L randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides 5'-GCT GAT ATC CAG TTG ACC CAG TCC CCG-3' (SEQ ID NO:26) 5'-and TCT GGG ACG GAT TAC ACT CTG ACC ATC-3' (SEQ ID NO:27). Deoxyuridine-containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (Table 5).

Oligonucleotides 504-1, a mixture of two 126-mer oligonucleotides (see above), and 5'-CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG-3' (SEQ ID NO:28) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 Blue cells (Stratagene) and combined. The total number of

independent transformants was estimated at >1.2 x 10^8, approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter *et al.*, *Ann. Rev. Immunol.* 12,433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard *et al.*, *Biotechn*, 9,1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 7). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8:309 (1990) and Lowman *et al.*, *Biochemistry* 30:10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37° C in 25 mL 2YT medium containing 50µg/mL carbenicillin and approximately 10^{10} M13KO7 helper phage (Vieira & Messing *Methods Enzymol.* 153:3-11 (1987)). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 µL PBS (~10^{14} phagemid/mL)

Selection of Humanized A4.6.1 Fab Variants: Purified $VEGF_{121}$ (100 µL at 10µg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well, in addition to an uncoated well, were blocked with 6% skim milk for 1 h and washed with PBS containing 0.05% TWEEN 20™ (detergent). Then, 10 µL of phagemid stock, diluted to 100 µL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05%TWEEN 20™, was added to each well. After 2 hours the wells were washed and the bound phage eluted with 100 µL of 0.1 M glycine (pH 2.0), and neutralized with 25 µL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger *et al. Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467 (1977)).

Determination of VEGF Binding Affinities: Association (k_{on}) and dissociation (k_{off}) rate constants for binding of humanized A4.6.1 Fab variants to $VEGF_{121}$ were measured by surface plasmon resonance (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)) on a Pharmacia BIAcore instrument. $VEGF_{121}$ was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN 20™ over the chip at a flow rate of 20 µL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 µL of 50 mM aqueous HCl at 3 µL/min. Binding profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

RESULTS

Construction of Humanized A4.6.1: An initial humanized A4.6.1 Fab fragment was constructed (hu2.0, Figs. 5A and 5B), in which the CDRs from A4.6.1 were grafted onto a human V_LKI-V_HIII framework. All other residues in hu2.0 were maintained as the human sequence. Binding of this variant to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants, the K_D for binding of hu2.0 was estimated at >7 µM. This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact V_L and V_H domains from murine A4.6.1 and human constant domains. Thus binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

Design of Antibody Library: The group of framework changes to the human framework sequence herein is shown in Table 5 and Fig. 6.

Table 5: Key Framework Residues Important for Antigen Binding and Targeted for Randomization

Framework residue		Human $V\kappa_L I$, $V_H III$ consensus residue	Murine A4.6.1 residue	Randomization[a]
V_L:	4	Met	Met	Met, Leu
	71	Phe	Tyr	Phe, Tyr
V_H:	24	Ala	Ala	Ala, Val, Thr
	37	Val	Val	Val, Ile

	67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
	69	Ile	Phe	Ile, Phe
	71	Arg	Leu	Arg[b], Leu[b]
	73	Asp	Thr	Asp[b], Thr[b]
	75	Lys	Ala	Lys[b], Ala[b]
	76	Asn	Ser	Asn[b], Ser[b]
	78	Leu	Ala	Leu, Ala, Val, Phe
	93	Ala	Ala	Ala, Val, Leu, Ser, Thr
	94	Arg	Lys	Arg, Lys

[a]Amino acid diversity in phagemid library
[b]V_H71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) V_HIII tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V_L and V_H sequences. Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V_L framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126-base oligonucleotides were preassembled from smaller synthetic fragments. This made possible randomization of V_H codons 67, 69, 71, 73, 75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

Selection of Tight Binding Humanized A4.6.1 Fab's: Variants from the humanized A4.6.1 Fab phagemid library were selected based on binding to VEGF. Enrichment of functional

phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results, summarized in Table 6, revealed strong consensus amongst the clones selected. Ten out of the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 (V_L 71; V_H 37, 71, 73, 75, 76, 78 and 94). Interestingly, residues V_H 37 (Ile) and 78 (Val) were selected neither as the human V $_H$III or murine A4.6.1 sequence. This result suggests that some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

Table 6: Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library

Variant	**Residue substitutions**												
	V_L		V_H										
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	F	A	V	F	I	R	N	K	N	L	A	R
Phage-selected clones:													
hu2.1(2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.2(2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.6(1)	L	-	-	I	T	-	L	T	A	S	V	-	K
hu2.7(1)	L	-	-	I	-	-	-	-	-	-	V	-	K
hu2.10(10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human V_LKI-V_HIII framework sequence (*i.e.* as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human V_HIII consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA assay (Cunningham *et al. EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

Expression and Binding Affinity of Humanized A4.6.1 Variants: Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes (K_D >7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

Table 7: VEGF Binding Affinity of Humanized A4.6.1 Fab Variants

Variant	k_{on} $M^{-1}s^{-1}/10^4$	k_{off} 10^4s^{-1}	K_D nM	$\frac{K_D(A4.6.1)}{K_D(mut)}$
A4.6.1 chimera	5.4	0.85	1.6	›4000
hu2.0	ND	ND	›7000**	
Phage selected clones:				
hu2.1	0.70	18	260	170

hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

*hu2.10V = hu2.10 with mutation V_L Leu->Val
Estimated errors in the Biacore binding measurements are +/-25%.
**Too weak to measure; estimate of lower bound

Additional Improvement of Humanized Variant hu2.1: Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human V_LκI-V_HIII framework (Figs. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V_L 46 sits at the V_L-V_H interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always leucine in most V_Lκ frameworks (Kabat *et al.*, *supra*), but is valine in A4.6.1. Accordingly, a Leu -> Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding, demonstrating the importance of valine at position V_L 46 in antibody A4.6.1. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

EXAMPLE 3

In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

Construction of Humanized Antibody pY0101: Phage-displayed antibody vector phMB4-19-1.6 (see Figs. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity for VEGF with oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

Table 8: Oligos for Directed Mutations

Oligo Number	Region	Substitution/ Comments	Sequence
HL-242	VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
HL-243	VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
HL-245	VH	CDR-7	5'-CGTCGTTTCACTTTTTCTGCAGACACCT CCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
HL-246	VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
HL-254	VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
HL-256	VH	I37V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
HL-257	VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTTCTTTAGACACCT CCAAAAGCACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

The resulting variant was termed Y0101 (Figs. 9A and 9B).

Construction of the First Generation of Antibody-Phage Libraries: To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis

with oligonucleotides using the degenerate NNS codon (where N is an equal mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement (Figs. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.

Table 9: Template Oligos for Mutagenesis

Oligo Number	Region Comments	Sequence
HL-248	VL1	5'-GGGTCACCATCACCTGCTAAGCATAATAATAATAAAGCAACT ATTTAAACTGG-3' (SEQ ID NO:36)
HL-249	VL1	5'-GCGCAAGTCAGGATATTTAATAATAATAATAATGGTATCAAC AGAAACCAGG-3' (SEQ ID NO:37)
HL-250	VH3	5'-GTCTATTACTGTGCAAAGTAATAACACTAATAAGGGAGCAG CCACTGG-3' (SEQ ID NO:38)
HL-251	VH3	5'-GGTACCCCCACTATTATTAATAATAATAATGGTATTTCGACG TCTGGGG-3' (SEQ ID NO:39)
HL-252	VH3	5'-CACTATTATGGGAGCAGCCACTAATAATAATAAGTCTGGGT CAAGGAACCCTG-3' (SEQ ID NO:40)
HL-263	VH1	5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATAATAAGGTATG AACTGGGTCCG-3' (SEQ ID NO:41)
HL-264	VH2	5'-GAATGGGTTGGATGGATTAACTAATAATAAGGTTAACCGAC CTATGCTGCGG-3' (SEQ ID NO:42)
YC-80	VH3	5'-CTGTGCAAAGTACCCGTAATATTAATAATAATAACACTGGTA TTTCGAC-3' (SEQ ID NO:43)
YC-100	CDR7	5'-CGTTTCACTTTTTCTTAAGACTAATCCAAATAAACAGCATAC CTGCAG-3' (SEQ ID NO:44)
YC-102	VH2	5'-GAATGGGTTGGATGGATTTAATAATAATAAGGTGAACCGAC CTATG-3' (SEQ ID NO:45)

Table 10: Random Oligos for Library Construction

Oligo Number	Region Comment	Sequence
HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNSNNSNNSNNSAGC AACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNSNNSNNSNNSTGGTATCAACA GAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSGGGAGCAGC CACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-GGTACCCCCACTATTATNNSNNSNNSNNSTGGTATTTCGACGT CTGGGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNSNNSNNSGTCTGGGGT CAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNNSNNSTTCNNSNNSNNSGGTATGA ACTGGGTCCG-3' (SEQ ID NO:51)
HL-266	VH2	5'-GAATGGGTTGGATGGATTAACNNSNNSNNSGGTNNSCCGACC TATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNSNNSNNSCACTGGTAT TTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTTCTNNSGACNNSTCCAAANNSACAGCATACCT GCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNSNNSNNSGGTGAACCGACC TATG-3' (SEQ ID NO:55)

The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from 2 x 10^7 to 1.5 x 10^8, was estimated based upon plating of the initial transformation onto carbenicillin plates.

Initial Affinity Selections: For each round of selection, approximately 10^9-10^{10} phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 µg/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEEN™ (0.05% TWEEN 20™

in PBS buffer). Typically, to select for enhanced affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEEN™ buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEEN™ buffer was removed, the remained phages were eluted with 0.1 M HCl and immediately neutralized with 1/3 volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

Phage-ELISA Assay of Binding Affinities: From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 µg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, *o*-phenylenediamine (Sigma). The reaction was stopped with addition of ½ volume of 2.5 M H_2SO_4. Optical density at 492nm was measured on a spectrophotometric plate reader.

Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R, S26N, Q27E, D28Q, and I29L in the VL region (Fig. 9A). In addition, this variant was found to have a spurious

mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

Ala-Scanning the CDRs of Anti-VEGF: To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to > 150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

Table 11: Relative VEGF Affinities of Ala-Scan Fab Variants

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
R24A	1	G26A	2
A25S	1	Y27A	34
N26A	1	T28A	1
E27A	1	F29A	16
Q28A	1	T30A	1
L29A	1	N31A	>150
S30A	2	Y32A	>150
N31A	2	G33A	6
Y32A	2	I34A	6
L33A	2	N35A	66
N34A	4		
		W50A	>150
F50A	1	I51A	4
T51A	1	N52A	>150

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
S52A	1	T53A	9
S53A	1	Y54A	9
L54A	1	T55A	4
H55A	1	G56A	1
S56A	1	E57A	2
		P58A	1
Q89A	4	T59A	3
Q90A	3	Y60A	2
Y91A	14	A61S	1
S92A	1	A62S	1
T93A	1	D63A	1
V94A	2	F64A	1
P95A	3	K65A	1
W96A	>150	R66A	1
T97A	1		
		Y99A	>150
		P100A	38
		H101A	4
		Y102A	4
		Y103A	5
		G104A	2
		S105A	1
		S106A	>150
		H107A	2
		W108A	>150
		Y109A	19
		F110A	25
		D111A	2

All variants are in the background of pY0192 ("wt"; see Figs. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction) and F110A (25-fold reduction). In contrast, only one VL substitution had a large impact on binding affinity, W96A

(>150-fold reduction). These results point to the three VH CDRs as the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

Design of Second-Generation CDR Mutation Libraries: Two additional libraries which randomized existing residues in anti-VEGF version Y0192 were designed based upon inspection of the crystal structure. In VH2, residues 52-55 were randomized because they lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see Fig. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

Second-Generation Selections of Anti-VEGF Libraries: Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from 6×10^7 to 5×10^8 which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

Table 12: Conditions for Secondary Selections of Fab Variants

Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (°C)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 µM VEGF/ELISA	room temp.

4	18	1 µM VEGF/ELISA	room temp.
5	37	1 µM VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37°C	1 µM VEGF/ELISA	room temp./37°C
7	63	1 µM VEGF/ELISA	37°C
8	121	1 µM VEGF/ELISA	37°C

ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

Phage-ELISA Assays of Second Generation Clones: After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

Table 13: Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries

Variants from library YC-81	
Name	**VH3 sequence (residues 99-111)**
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO:56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO:57)
Y0238-3	YPYYYGTSHWYFD (SEQ ID NO:58)
Y0238-4	YPYYYNQSHWYFD (SEQ ID NO:59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO:60)
Y0238-6	YPYYRDNSHWYFD (SEQ ID NO:61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO:62)

Y0238-8	YPYYRQNSHWYFD (SEQ ID NO:63)
Y0238-9	YPYYRQSSHWYFD (SEQ ID NO:64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO:65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO:66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO:67)
Y0228-21	YPYYRNASHWYFD (SEQ ID NO:68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO:69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO:70)
Y0228-24	YPYYRQRGHWYFD (SEQ ID NO:71)
Y0228-26	YPYYTGRSHWYFD (SEQ ID NO:72)
Y0228-27	YPYYTNTSHWYFD (SEQ ID NO:73)
Y0228-28	YPYYRKGSHWYFD (SEQ ID NO:74)
Y0228-29	YPYYTGSSHWYFD (SEQ ID NO:75)
Y0228-30	YPYYRSGSHWYFD (SEQ ID NO:76)
Y0229-20	YPYYTNRSHWYFD (SEQ ID NO:77)
Y0229-21	YPYYRNSSHWYFD (SEQ ID NO:78)
Y0229-22	YPYYKESSHWYFD (SEQ ID NO:79)
Y0229-23	YPYYRDASHWYFD (SEQ ID NO:80)
Y0229-24	YPYYRQKGHWYFD (SEQ ID NO:81)
Y0229-25	YPYYKGGSHWYFD (SEQ ID NO:82)
Y0229-26	YPYYYGASHWYFD (SEQ ID NO:83)
Y0229-27	YPYYRGESHWYFD (SEQ ID NO:84)
Y0229-28	YPYYRSTSHWYFD (SEQ ID NO:85)
Variants from library HL-265	
Name	**VH1 sequence (residue 26-35)**
Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO:86)
Y0243-2	GYEFQHYGMN (SEQ ID NO:87)

Y0243-3	GYEFTHYGMN (SEQ ID NO:88)
Y0243-4	GYDFGHYGMN (SEQ ID NO:89)
Y0243-5	GYDFSHYGMN (SEQ ID NO:90)
Y0243-6	GYEFSHYGMN (SEQ ID NO:91)
Variants from library YC-101	
Name	**VH "CDR7" sequence (residues 70-79)**
Y0244-1	FSVDVSKSTA (SEQ ID NO:92)
Y0244-2	FSLDKSKSTA (SEQ ID NO:93)
Y0244-3	FSLDVWKSTA (SEQ ID NO:94)
Y0244-4	FSIDKSKSTA (:95)

The sequence of the randomized region only is shown as deduced from DNA sequencing.

When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could be explained by the time-scale on which the assay was performed (< 3 hours).

In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants' DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

CDR Combination Variants: To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

Table 14: Combination CDR Anti-VEGF Variants

Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTACTATTATGGGAC GAGCCACTGGTATTTC-3' (SEQ ID NO:96)
Y0317	Y0313-1	YC-108 (revert VL1 back to wild type)	5'-GTCACCATCACCTGCAGCGCAAGTCA GGATATTAGCAACTATTTAAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCAGCCACTG GTATTTC-3' (SEQ ID NO:98)

Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

BIAcore Analysis: The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25° C at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, Kd's from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements				
Variant	**Kon (10^4/M/s)**	**koff (10^{-4}/s)**	**Kd (nM)**	**Kd (wt) / Kd (mut)**
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*
Y0313-3	1.2	0.081	0.65	4.5

* The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T28D and N31H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.15nM to 0.65 nM (see Table 15). The greater affinity enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

Cell-Based Assay of VEGF Inhibition: Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% diafiltered fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration),

and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

The results (Fig. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC50 for the mutant. For example, if the binding affinity (Kd) of the mutant is in fact < 0.2 nM, the IC50 in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*. Since the variant Y0317 differs from Y0313-1 only in the reversion of the VL1 sequence to wild-type (Fig. 10A), it is predicted that Y0317 will have similar activity to Y0313-1.

Variant Y0317 (Fab) and humanized variant F(ab)-12 from Example 1 (full length and Fab) were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF using the assay described in Example 1. As illustrated in Figure 12, Y0317 was markedly more effective at inhibiting bovine capillary endothelial cell proliferation than the full length and Fab forms of F(ab)-12 in this assay. The Y0317 affinity matured Fab demonstrated an ED50 value in this assay which was at least about 20 fold lower than F(ab)-12 Fab.

WHAT IS CLAIMED IS:

1. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M.

2. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 5 x 10^{-9}M.

3. A humanized anti-VEGF antibody which has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*.

4. A humanized anti-VEGF antibody which inhibits VEGF-induced angiogenesis *in vivo*.

5. The humanized anti-VEGF antibody of claim 4 wherein 5mg/kg of the antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

6. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129).

7. The humanized anti-VEGF antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:7.

8. The humanized anti-VEGF antibody of claim 6 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

10. The humanized anti-VEGF antibody of claim 9 comprising the amino acid sequence of SEQ ID NO:8.

11. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.

12. An anti-VEGF antibody light chain variable domain comprising the amino acid sequence: DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ KPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L.

13. An anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGYX $_1$FTX$_2$YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX $_3$YYGX$_4$SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T.

14. A variant of a parent anti-VEGF antibody, wherein said variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of a heavy chain variable domain of said parent antibody.

15. The variant of claim 14 wherein said parent antibody is a human or humanized antibody.

16. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 1×10^{-8}M.

17. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 5×10^{-9}M.

18. The variant of claim 14 wherein the substitution is in CDRH1 of the heavy chain variable domain.

19. The variant of claim 14 wherein the substitution is in CDRH3 of the heavy chain variable domain.

20. The variant of claim 14 which has amino acid substitutions in both CDRH1 and CDRH3.

21. The variant of claim 14 which binds human VEGF with a K_d value less than that of said parent antibody.

22. The variant of claim 14 which has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower than that of said parent antibody.

23. The variant of claim 18 wherein the CDRH1 comprises the amino acid sequence: GYDFTHYGMN (SEQ ID NO:126)

24. The variant of claim 19 wherein the CDRH3 comprises the amino acid sequence: YPYYYGTSHWYFDV (SEQ ID NO:127).

25. The variant of claim 14 wherein the heavy chain variable domain comprises the amino acid sequence of SEQ ID NO:116.

26. The variant of claim 25 further comprising the light chain variable domain amino acid sequence of SEQ ID NO:124.

27. The variant of claim 26 comprising the light chain variable domain amino acid sequence of SEQ ID NO:115.

28. The humanized anti-VEGF antibody of claim 1 which is a full length antibody.

29. The humanized anti-VEGF antibody of claim 28 which is a human IgG.

30. The humanized anti-VEGF antibody of claim 1 which is an antibody fragment.

31. The antibody fragment of claim 30 which is a Fab.

32. A composition comprising the humanized anti-VEGF antibody of claim 1 and a pharmaceutically acceptable carrier.

33. A composition comprising the variant anti-VEGF antibody of claim 14 and a pharmaceutically acceptable carrier.

34. Isolated nucleic acid encoding the antibody of claim 1.

35. A vector comprising the nucleic acid of claim 34.

36. A host cell comprising the vector of claim 35.

37. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 36 so that the nucleic acid is expressed.

38. The process of claim 37 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

39. A method for inhibiting VEGF-induced angiogenesis in a mammal comprising administering a therapeutically effective amount of the humanized anti-VEGF antibody of claim 1 to the mammal.

40. The method of claim 39 wherein the mammal is a human.

41. The method of claim 39 wherein the mammal has a tumor.

42. The method of claim 39 wherein the mammal has a retinal disorder.

ANTI-VEGF ANTIBODIES

Abstract of the Disclosure

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and inhibit tumor growth *in vivo*.

Variable Heavy

```
A4.6.1     EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQAPGKGLKWMG
            *   *  ** *   *** *  *                 *       * *
F(ab)-12   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG
                                      *  ** * *              *
humIII     EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS
           1        10        20        30        40

A4.6.1     WINTYTGEPTYAADFKRRFTFSLETSASTAYLQISNLKNDDTATYFCAK
                                    *  *      *** ***   * *
F(ab)-12   WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAK
           * **** ***  *** *   * * *  * *                   *
humIII     VISGDGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
          50  a      60        70        80  abc      90

A4.6.1     YPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
                          *  *
F(ab)-12   YPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:7)
           *           *
humIII     G----------FDYWGQGTLVTVSS (SEQ ID NO:11)
                           110
```

Fig. 1A

Variable Light

```
A4.6.1     DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKPDGTVKVLIY
                 **      *    * *                   ****
F(ab)-12   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIY
                                   *   *     *          *
humKI      DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKPGKAPKLLIY
           1        10        20        30        40

A4.6.1     FTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSTVPWTF
                                 **    * *   *
F(ab)-12   FTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTF
           **   *                                      ***
humKI      AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPWTF
          50        60        70        80        90

A4.6.1     GGGTKLEIKR (SEQ ID NO:10)
            *   *
F(ab)-12   GQGTKVEIKR (SEQ ID NO:8)

humKI      GQGTKVEIKR (SEQ ID NO:12)
          100
```

Fig. 1B

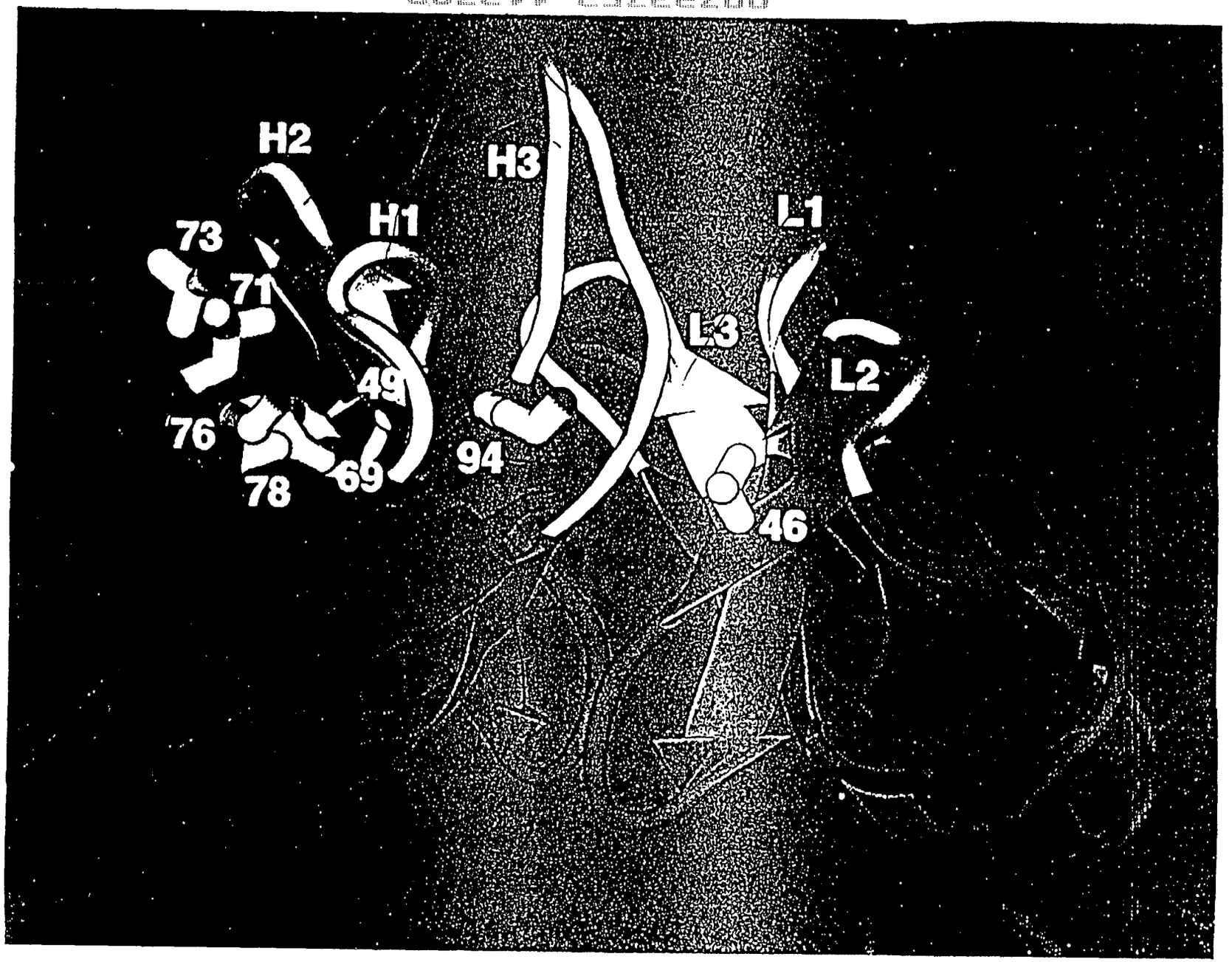

Fig. 2

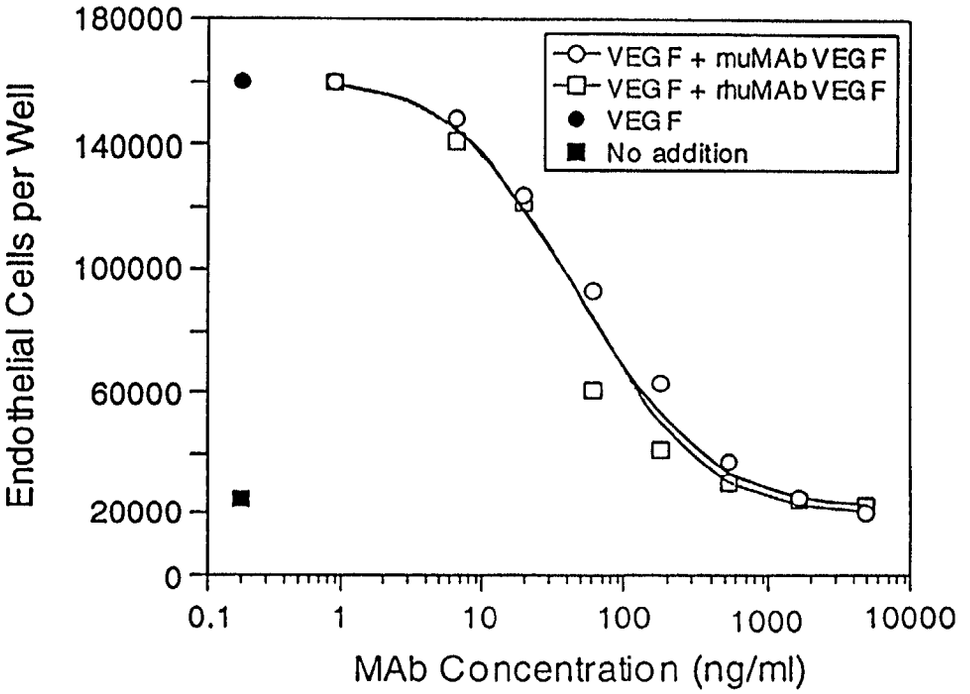

Fig. 3

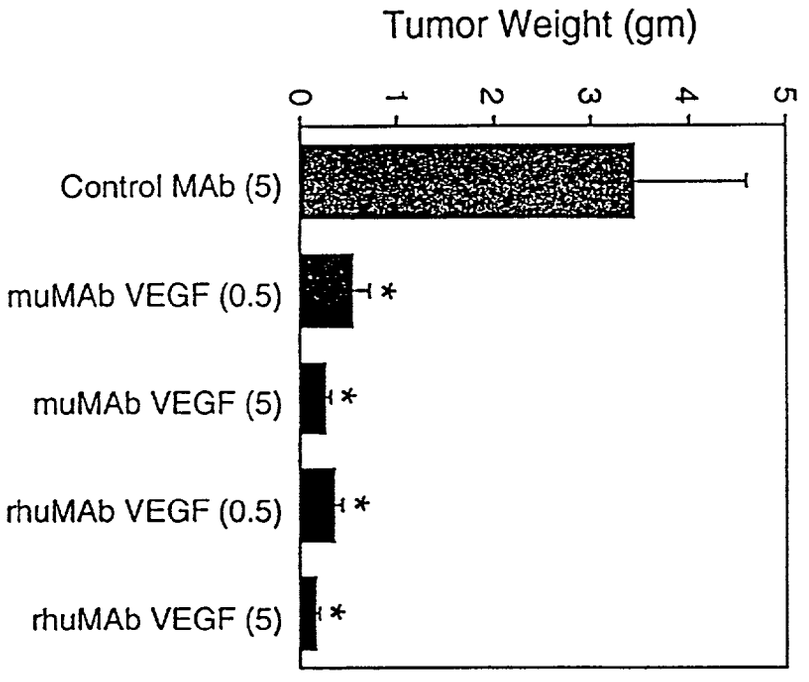

Fig. 4

V_L domain

```
                10        20        30        40
A4.6.1  DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
              **      *    * *
hu2.0   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP

hu2.10  DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP
```

Fig. 5A

```
                50        60        70        80
A4.6.1  DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
        **** *                          **    * *
hu2.0   GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
                                        .
hu2.10  GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTISSLQP


                90       100
A4.6.1  EDIATYYCQQYSTVPWTFGGGTKLEIK (SEQ ID NO:10)
          *                 *   *
hu2.0   EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:13)

hu2.10  EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:15)
```

- V_H domain

```
                10        20        30        40
A4.6.1  EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
          *   *  ** *   *** * .*               *
hu2.0   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                              .
hu2.10  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIRQA


                50 a      60        70        80
A4.6.1  PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
              * *                   * *** ** *
hu2.0   PGKGLEWVGWINTYTGEPTYAADFKRRFTISRDNSKNTLYL
                                      . . .. .
hu2.10  PGKGLEWVGWINTYTGEPTYAADFKRRFTISLDTSASTVYL


          abc      90       100abcdef       110
A4.6.1  QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
         *** ***   * *  *                   *  *
hu2.0   QMNSLRAEDTAVYYCARYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:14)
                    .
hu2.10  QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:16)
```

Fig. 5B

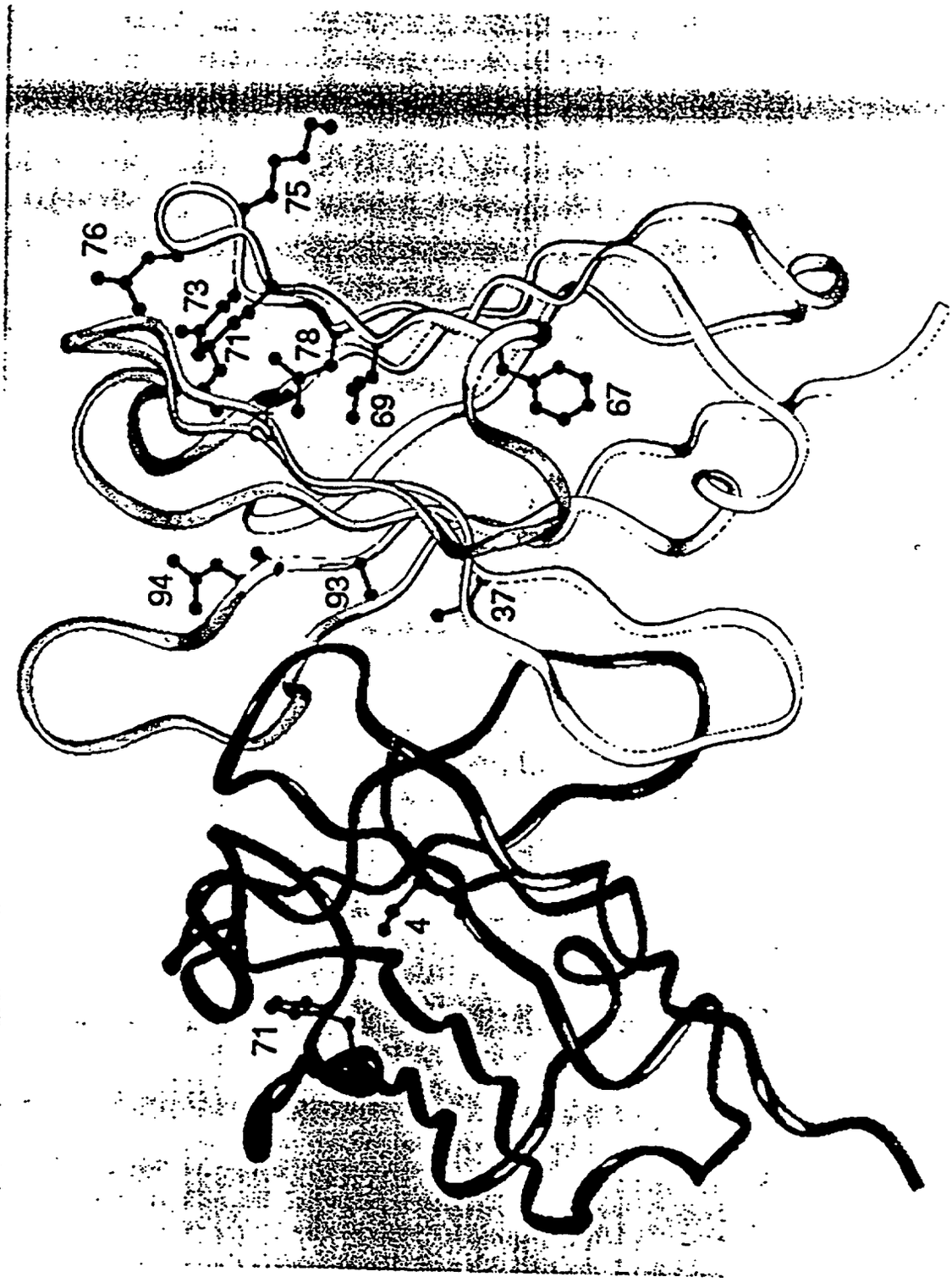

Fig. 6

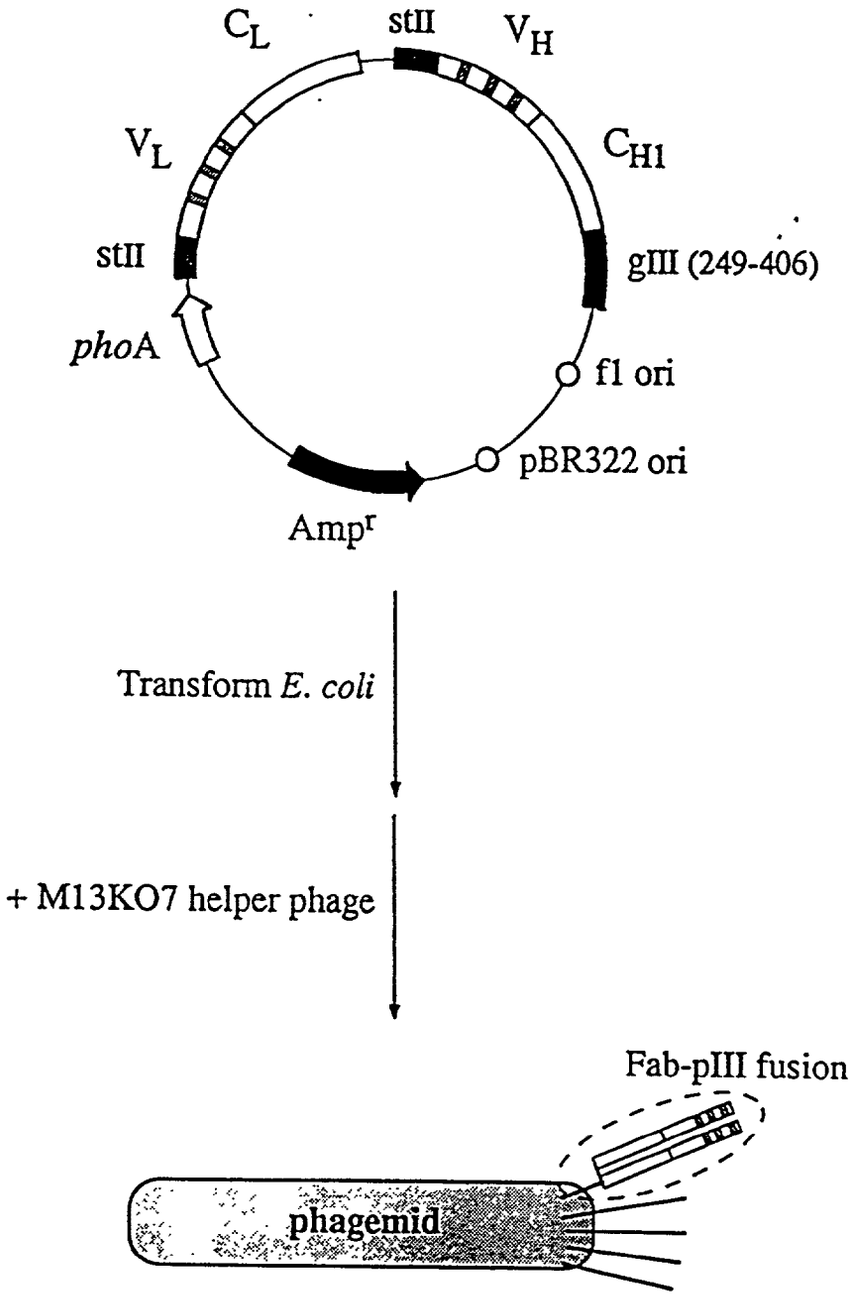

Fig. 7

```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC
     CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAAACCT CTAATAGCAG TGACGTTACG

 101 TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC
     AAGCGTTATA CCGCGTTTTA CTGGTTGTCG CCAACTAACT AGTCCATCTC CCCCGCGACA TGCTCCATTT CGGGCTACGG TCGTAAGGAC TGCTGCTATG

 201 GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT
     CCTCGACGAC GCGCTAATGC ATTTCTTCAA TAACTTCGTA GGAGCAGTCA TTTTTCAATT AGAAAAGTTG TCGACAGTAT TTCAACAGTG CCGGCTCTGA

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGGTACCC GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA
     ATATCAGCGA AACAAAAATA AAAAATTACA TAAACATTGA TCTTAAGCTC GAGCCATGGG CCCCTAGGAG ATCTCCAACT CCACTAAAAT ACTTTTTCTT
-23                                                                                                    M etLysLysAsn
                                                                                                Begin stII signal sequence
 401 TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG CGTACGCTGA TATCCAGTTG ACCCAGTCCC CGAGCTCCCT GTCCGCCTCT
     ATAGCGTAAA GAAGAACGTA GATACAAGCA AAAAAGATAA CGATGTTTGC GCATGCGACT ATAGGTCAAC TGGGTCAGGG GCTCGAGGGA CAGGCGGAGA
-19  IleAlaPhe LeuLeuAlaS erMetPheVa lPheSerIle AlaThrAsnA laTyrAlaAs pIleGlnLeu ThrGlnSerP roSerSerLe uSerAlaSer
                                                                Begin light chain
 501 GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG ATATTAGCAA CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAACTACTGA
     CACCCGCTAT CCCAGTGGTA GTGGACGTCG CGTTCAGTCC TATAATCGTT GATAAATTTG ACCATAGTTG TCTTTGGTCC TTTTCGAGGC TTTGATGACT
  15 ValGlyAspA rgValThrIl eThrCysSer AlaSerGlnA spIleSerAs nTyrLeuAsn TrpTyrGlnG lnLysProGl yLysAlaPro LysLeuLeuIle

 601 TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA TCCGGTTCTG GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA
     AAATGAAGTG GAGGAGAGAG GTGAGACCTC AGGGAAGAGC GAAGAGACCT AGGCCAAGAC CCTGCCTAAT GTGAGACTGG TAGTCGTCAG ACGTCGGTCT
  49   TyrPheTh rSerSerLeu HisSerGlyV alProSerAr gPheSerGly SerGlySerG lyThrAspTy rThrLeuThr IleSerSerL euGlnProGlu

 701 AGACTTCGCA ACTTATTACT GTCAACAGTA TAGCACCGTG CCGTGGACGT TTGGACAGGG TACCAAGGTG GAGATCAAAC GAACTGTGGC TGCACCATCT
     TCTGAAGCGT TGAATAATGA CAGTTGTCAT ATCGTGGCAC GGCACCTGCA AACCTGTCCC ATGGTTCCAC CTCTAGTTTG CTTGACACCG ACGTGGTAGA
  82  AspPheAla ThrTyrTyrC ysGlnGlnTy rSerThrVal ProTrpThrP heGlyGlnGl yThrLysVal GluIleLysA rgThrValAl aAlaProSer

 801 GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG GAACTGCTTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT
     CAGAAGTAGA AGGGCGGTAG ACTACTCGTC AACTTTAGAC CTTGACGAAG ACAACACACG GACGACTTAT TGAAGATAGG GTCTCTCCGG TTTCATGTCA
 115 ValPheIleP heProProSe rAspGluGln LeuLysSerG lyThrAlaSe rValValCys LeuLeuAsnA snPheTyrPr oArgGluAla LysValGlnTrp

 901 GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA GAGCAGGACA GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT
     CCTTCCACCT ATTGCGGGAG GTTAGCCCAT TGAGGGTCCT CTCACAGTGT CTCGTCCTGT CGTTCCTGTC GTGGATGTCG GAGTCGTCGT GGGACTGCGA
 149   LysValAs pAsnAlaLeu GlnSerGlyA snSerGlnGl uSerValThr GluGlnAspS erLysAspSe rThrTyrSer LeuSerSerT hrLeuThrLeu

1001 GAGCAAAGCA GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT GAGCTCGCCC GTCACAAAGA GCTTCAACAG GGGAGAGTGT
     CTCGTTTCGT CTGATGCTCT TTGTGTTTCA GATGCGGACG CTTCAGTGGG TAGTCCCGGA CTCGAGCGGG CAGTGTTTCT CGAAGTTGTC CCCTCTCACA
 182  SerLysAla AspTyrGluL ysHisLysVa lTyrAlaCys GluValThrH isGlnGlyLe uSerSerPro ValThrLysS erPheAsnAr gGlyGluCys
```

Fig. 8A

```
1101 TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA CGGAACTAGT GGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG
     ATTCGACTAG GAGATGCGGC CTGCGTAGCA CCGGGATCAT GCGTTGATCA GCATTTTTCC CATAGATCTC CAACTCCACT AAAATACTTT TTCTTATAGC
 215 OC*                                                                                                -23 MetLys LysAsnIleAla
                  Begin stII signal sequence
1201 CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC GCTGAGGTTC AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG
     GTAAAGAAGA ACGTAGATAC AAGCAAAAAA GATAACGATG TTTGCGCATG CGACTCCAAG TCGACCACCT CAGACCGCCA CCGGACCACG TCGGTCCCCC
  -17   PheLeuLe uAlaSerMet PheValPheS erIleAlaTh rAsnAlaTyr AlaGluValG lnLeuValGl uSerGlyGly GlyLeuValG lnProGlyGly
                                                                Begin heavy chain
1301 CTCACTCCGT TTGTCCTGTG CAGCTTCTGG CTATACCTTC ACCAACTATG GTATGAACTG GATCCGTCAG GCCCCGGGTA AGGGCCTGGA ATGGGTTGGA
     GAGTGAGGCA AACAGGACAC GTCGAAGACC GATATGGAAG TGGTTGATAC CATACTTGAC CTAGGCAGTC CGGGGCCCAT TCCCGGACCT TACCCAACCT
   17  SerLeuArg LeuSerCysA laAlaSerGl yTyrThrPhe ThrAsnTyrG lyMetAsnTr pIleArgGln AlaProGlyL ysGlyLeuGl uTrpValGly

1401 TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTTACT ATATCTGCAG ACACCTCCAG CAACACAGTT TACCTGCAGA
     ACCTAATTGT GGATATGGCC ACTTGGCTGG ATACGACGCC TAAAGTTTGC AGCAAAATGA TATAGACGTC TGTGGAGGTC GTTGTGTCAA ATGGACGTCT
   50 TrpIleAsnT hrTyrThrGl yGluProThr TyrAlaAlaA spPheLysAr gArgPheThr IleSerAlaA spThrSerSe rAsnThrVal TyrLeuGlnMet

1501 TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC CCGCACTATT ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG
     ACTTGTCGGA CGCGCGACTC CTGTGACGGC AGATAATGAC ACGTTTCATG GGCGTGATAA TACCCTCGTC GGTGACCATA AAGCTGCAGA CCCCAGTTCC
   84   AsnSerLe uArgAlaGlu AspThrAlaV alTyrTyrCy sAlaLysTyr ProHisTyrT yrGlySerSe rHisTrpTyr PheAspValT rpGlyGlnGly

1601 AACCCTGGTC ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC
     TTGGGACCAG TGGCAGAGGA GCCGGAGGTG GTTCCCGGGT AGCCAGAAGG GGGACCGTGG GAGGAGGTTC TCGTGGAGAC CCCCGTGTCG CCGGGACCCG
  117  ThrLeuVal ThrValSerS erAlaSerTh rLysGlyPro SerValPheP roLeuAlaPr oSerSerLys SerThrSerG lyGlyThrAl aAlaLeuGly

1701 TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT
     ACGGACCAGT TCCTGATGAA GGGGCTTGGC CACTGCCACA GCACCTTGAG TCCGCGGGAC TGGTCGCCGC ACGTGTGGAA GGGCCGACAG GATGTCAGGA
  150 CysLeuValL ysAspTyrPh eProGluPro ValThrValS erTrpAsnSe rGlyAlaLeu ThrSerGlyV alHisThrPh eProAlaVal LeuGlnSerSer

1801 CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA
     GTCCTGAGAT GAGGGAGTCG TCGCACCACT GGCACGGGAG GTCGTCGAAC CCGTGGGTCT GGATGTAGAC GTTGCACTTA GTGTTCGGGT CGTTGTGGTT
  184   GlyLeuTy rSerLeuSer SerValValT hrValProSe rSerSerLeu GlyThrGlnT hrTyrIleCy sAsnValAsn HisLysProS erAsnThrLys

1901 GGTCGACAAG AAAGTTGAGC CCAAATCTTG TGACAAAACT CACCTCTAGA GTGGCGGTGG CTCTGGTTCC GGTGATTTTG ATTATGAAAA GATGGCAAAC
     CCAGCTGTTC TTTCAACTCG GGTTTAGAAC ACTGTTTTGA GTGGAGATCT CACCGCCACC GAGACCAAGG CCACTAAAAC TAATACTTTT CTACCGTTTG
  217  ValAspLys LysValGluP roLysSerCy sAspLysThr HisLeuAM*S erGlyGlyGl ySerGlySer GlyAspPheA spTyrGluLy sMetAlaAsn
                                        end light chain   Begin g3p domain
2001 GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG CGCTACAGTC TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA
     CGATTATTCC CCCGATACTG GCTTTTACGG CTACTTTTGC GCGATGTCAG ACTGCGATTT CCGTTTGAAC TAAGACAGCG ATGACTAATG CCACGACGAT
  250 AlaAsnLysG lyAlaMetTh rGluAsnAla AspGluAsnA laLeuGlnSe rAspAlaLys GlyLysLeuA spSerValAl aThrAspTyr GlyAlaAlaIle

2101 TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG
     AGCTACCAAA GTAACCACTG CAAAGGCCGG AACGATTACC ATTACCACGA TGACCACTAA AACGACCGAG ATTAAGGGTT TACCGAGTTC AGCCACTGCC
  284   AspGlyPh eIleGlyAsp ValSerGlyL euAlaAsnGl yAsnGlyAla ThrGlyAspP heAlaGlySe rAsnSerGln MetAlaGlnV alGlyAspGly

2201 TGATAATTCA CCTTTAATGA ATAATTTCCG TCAATATTTA CCTTCCCTCC CTCAATCGGT TGAATGTCGC CCTTTTGTCT TTAGCGCTGG TAAACCATAT
     ACTATTAAGT GGAAATTACT TATTAAAGGC AGTTATAAAT GGAAGGGAGG GAGTTAGCCA ACTTACAGCG GGAAAACAGA AATCGCGACC ATTTGGTATA
  317  AspAsnSer ProLeuMetA snAsnPheAr gGlnTyrLeu ProSerLeuP roGlnServa lGluCysArg ProPheValP heSerAlaGl yLysProTyr
```

Fig. 8B

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2301 GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCGCGTG GTGTCTTTGC GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA
     CTTAAAAGAT AACTAACACT GTTTTATTTG AATAAGGCAC CACAGAAACG CAAAGAAAAT ATACAACGGT GGAAATACAT ACATAAAAGA TGCAAACGAT
 350 GluPheSerI leAspCysAs pLysIleAsn LeuPheArgG lyValPheAl aPheLeuLeu TyrValAlaT hrPheMetTy rValPheSer ThrPheAlaAsn

2401 ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC
     TGTATGACGC ATTATTCCTC AGAATTAGTA CGGTCAAGAA AACCGATCGC GGCGGGATAT GGAACAGACG GAGGGGCGCA ACGCAGCGCC ACGTACCTCG
 384   IleLeuAr gAsnLysGlu SerOC* (SEQ ID NO: 100)
                 end g3 protein
2501 CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC
     GCCCGGTGGA GCTGGACTTA CCTTCGGCCG CCGTGGAGCG ATTGCCTAAG TGGTGAGGTT CTTAACCTCG GTTAGTTAAG AACGCCTCTT GACACTTACG

2601 GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC
     CGTTTGGTTG GGAACCGTCT TGTATAGGTA GCGCAGGCGG TAGAGGTCGT CGGCGTGCGC CGCGTAGAGC CCGTCGCAAC CCAGGACCGG TGCCCACGCG

2701 ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT TACTGGTTAG CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT
     TACTAGCACG AGGACAGCAA CTCCTGGGCC GATCCGACCG CCCCAACGGA ATGACCAATC GTCTTACTTA GTGGCTATGC GCTCGCTTGC ACTTCGCTGA

2801 GCTGCTGCAA AACGTCTGCG ACCTGAGCAA CAACATGAAT GGTCTTCGGT TTCCGTGTTT CGTAAAGTCT GGAAACGCGG AAGTCAGCGC CCTGCACCAT
     CGACGACGTT TTGCAGACGC TGGACTCGTT GTTGTACTTA CCAGAAGCCA AAGGCACAAA GCATTTCAGA CCTTTGCGCC TTCAGTCGCG GGACGTGGTA

2901 TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC
     ATACAAGGCC TAGACGTAGC GTCCTACGAC GACCGATGGG ACACCTTGTG GATGTAGACA TAATTGCTTC GCGACCGTAA CTGGGACTCA CTAAAAAGAG

3001 TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG TAACCGGGCA TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC
     ACCAGGGCGG CGTAGGTATG GCGGTCAACA AATGGGAGTG TTGCAAGGTC ATTGGCCCGT ACAAGTAGTA GTCATTGGGC ATAGCACTCG TAGGAGAGAG

3101 GTTTCATCGG TATCATTACC CCCATGAACA GAAATTCCCC CTTACACGGA GGCATCAAGT GACCAAACAG GAAAAAACCG CCCTTAACAT GGCCCGCTTT
     CAAAGTAGCC ATAGTAATGG GGGTACTTGT CTTTAAGGGG GAATGTGCCT CCGTAGTTCA CTGGTTTGTC CTTTTTTGGC GGGAATTGTA CCGGGCGAAA

3201 ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG AGCTGGACGC GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC
     TAGTCTTCGG TCTGTAATTG CGAAGACCTC TTTGAGTTGC TCGACCTGCG CCTACTTGTC CGTCTGTAGA CACTTAGCGA AGTGCTGGTG CGACTACTCG

3301 TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC
     AAATGGCGTC CTAGGCCTTT AACATTTGCA ATTATAAAAC AATTTTAAGC GCAATTTAAA AACAATTTAG TCGAGTAAAA AATTGGTTAT CCGGCTTTAG

3401 GGCAAAATCC CTTATAAATC AAAAGAATAG ACCGAGATAG GGTTGAGTGT TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG
     CCGTTTTAGG GAATATTTAG TTTTCTTATC TGGCTCTATC CCAACTCACA ACAAGGTCAA ACCTTGTTCT CAGGTGATAA TTTCTTGCAC CTGAGGTTGC

3501 TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG
     AGTTTCCCGC TTTTTGGCAG ATAGTCCCGA TACCGGGTGA TGCACTTGGT AGTGGGATTA GTTCAAAAAA CCCCAGCTCC ACGGCATTTC GTGATTTAGC

3601 GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG
     CTTGGGATTT CCCTCGGGGG CTAAATCTCG AACTGCCCCT TTCGGCCGCT TGCACCGCTC TTTCCTTCCC TTCTTTCGCT TTCCTCGCCC GCGATCCCGC

3701 CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC CTGCCTCGCG CGTTTCGGTG
     GACCGTTCAC ATCGCCAGTG CGACGCGCAT TGGTGGTGTG GGCGGCGCGA ATTACGCGGC GATGTCCCGC GCAGGCCTAG GACGGAGCGC GCAAAGCCAC

3801 ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC
     TACTGCCACT TTTGGAGACT GTGTACGTCG AGGGCCTCTG CCAGTGTCGA ACAGACATTC GCCTACGGCC CTCGTCTGTT CGGGCAGTCC CGCGCAGTCG
```

Fig. 8C

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3901 GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA GCGGAGTGTA TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA
     CCCACAACCG CCCACAGCCC CGCGTCGGTA CTGGGTCAGT GCATCGCTAT CGCCTCACAT ATGACCGAAT TGATACGCCG TAGTCTCGTC TAACATGACT

4001 GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA GGCGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC
     CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT ATGGCGTAGT CCGCGAGAAG GCGAAGGAGC GAGTGACTGA GCGACGCGAG

4101 GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA
     CCAGCAAGCC GACGCCGCTC GCCATAGTCG AGTGAGTTTC CGCCATTATG CCAATAGGTG TCTTAGTCCC CTATTGCGTC CTTTCTTGTA CACTCGTTTT

4201 GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC
     CCGGTCGTTT TCCGGTCCTT GGCATTTTTC CGGCGCAACG ACCGCAAAAA GGTATCCGAG GCGGGGGGAC TGCTCGTAGT GTTTTTAGCT GCGAGTTCAG

4301 AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA
     TCTCCACCGC TTTGGGCTGT CCTGATATTT CTATGGTCCG CAAAGGGGGA CCTTCGAGGG AGCACGCGAG AGGACAAGGC TGGGACGGCG AATGGCCTAT

4401 CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT
     GGACAGGCGG AAAGAGGGAA GCCCTTCGCA CCGCGAAAGA GTATCGAGTG CGACATCCAT AGAGTCAAGC CACATCCAGC AAGCGAGGTT CGACCCGACA

4501 GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG
     CACGTGCTTG GGGGGCAAGT CGGGCTGGCG ACGCGGAATA GGCCATTGAT AGCAGAACTC AGGTTGGGCC ATTCTGTGCT GAATAGCGGT GACCGTCGTC

4601 CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG
     GGTGACCATT GTCCTAATCG TCTCGCTCCA TACATCCGCC ACGATGTCTC AAGAACTTCA CCACCGGATT GATGCCGATG TGATCTTCCT GTCATAAACC

4701 TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC
     ATAGACGCGA GACGACTTCG GTCAATGGAA GCCTTTTTCT CAACCATCGA GAACTAGGCC GTTTGTTTGG TGGCGACCAT CGCCACCAAA AAAACAAACG

4801 AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA
     TTCGTCGTCT AATGCGCGTC TTTTTTTCCT AGAGTTCTTC TAGGAAACTA GAAAAGATGC CCCAGACTGC GAGTCACCTT GCTTTTGAGT GCAATTCCCT

4901 TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC
     AAAACCAGTA CTCTAATAGT TTTTCCTAGA AGTGGATCTA GGAAAATTTA ATTTTTACTT CAAAATTTAG TTAGATTTCA TATATACTCA TTTGAACCAG

5001 TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG
     ACTGTCAATG GTTACGAATT AGTCACTCCG TGGATAGAGT CGCTAGACAG ATAAAGCAAG TAGGTATCAA CGGACTGAGG GGCAGCACAT CTATTGATGC

5101 ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA
     TATGCCCTCC CGAATGGTAG ACCGGGGTCA CGACGTTACT ATGGCGCTCT GGGTGCGAGT GGCCGAGGTC TAAATAGTCG TTATTTGGTC GGTCGGCCTT

5201 GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT
     CCCGGCTCGC GTCTTCACCA GGACGTTGAA ATAGGCGGAG GTAGGTCAGA TAATTAACAA CGGCCCTTCG ATCTCATTCA TCAAGCGGTC AATTATCAAA

5301 GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA
     CGCGTTGCAA CAACGGTAAC GACGTCCGTA GCACCACAGT GCGAGCAGCA AACCATACCG AAGTAAGTCG AGGCCAAGGG TTGCTAGTTC CGCTCAATGT

5401 TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG
     ACTAGGGGGT ACAACACGTT TTTTCGCCAA TCGAGGAAGC CAGGAGGCTA GCAACAGTCT TCATTCAACC GGCGTCACAA TAGTGAGTAC CAATACCGTC

5501 CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC
     GTGACGTATT AAGAGAATGA CAGTACGGTA GGCATTCTAC GAAAAGACAC TGACCACTCA TGAGTTGGTT CAGTAAGACT CTTATCACAT ACGCCGCTGG
```

Fig. 8D

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5601 GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC
     CTCAACGAGA ACGGGCCGCA GTTGTGCCCT ATTATGGCGC GGTGTATCGT CTTGAAATTT TCACGAGTAG TAACCTTTTG CAAGAAGCCC CGCTTTTGAG

5701 TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG
     AGTTCCTAGA ATGGCGACAA CTCTAGGTCA AGCTACATTG GGTGAGCACG TGGGTTGACT AGAAGTCGTA GAAAATGAAA GTGGTCGCAA AGACCCACTC

5801 CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA
     GTTTTTGTCC TTCCGTTTTA CGGCGTTTTT TCCCTTATTC CCGCTGTGCC TTTACAACTT ATGAGTATGA GAAGGAAAAA GTTATAATAA CTTCGTAAAT

5901 TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC
     AGTCCCAATA ACAGAGTACT CGCCTATGTA TAAACTTACA TAAATCTTTT TATTTGTTTA TCCCCAAGGC GCGTGTAAAG GGGCTTTTCA CGGTGGACTG

6001 GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTTC AA (SEQ ID NO: 99)
     CAGATTCTTT GGTAATAATA GTACTGTAAT TGGATATTTT TATCCGCATA GTGCTCCGGG AAAGCAGAAG TT
```

Fig. 8E

▨ = differences from F(ab)-12

```
                        10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
MB1.6    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
H2305.6  DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0101    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0192    DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
                                        CDR-L1
                    40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
MB1.6    KPGKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
H2305.6  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
Y0101    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0192    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
                    CDR-L2
                80                  90                  100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
MB1.6    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 101)
H2305.6  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:103)
Y0101    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:105)
Y0192    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 107)
                        CDR-L3
```

Fig. 9A

```
                        10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
MB1.6    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
H2305.6  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
Y0101    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0192    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVR
                                        CDR-H1
                    40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
MB1.6    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTISADTSSNIV
H2305.6  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSADTSSNIV
Y0101    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0192    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                    CDR-H2                          CDR-7
                80                  90                  100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
MB1.6    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 102)
H2305.6  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 104)
Y0101    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 106)
Y0192    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 108)
                                        CDR-H3
```

Fig. 9B

▩ = differences from F(ab)-12

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                          10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0243-1  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0238-3  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0313-1  DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
Y0317    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
                                        CDR-L1
                  40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0243-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0238-3  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0313-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0317    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
             CDR-L2
          80        90                  100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
Y0243-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:109)
Y0238-3  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:111)
Y0313-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:113)
Y0317    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:115)
                        CDR-L3
```

Fig. 10A

```
                          10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0243-1  EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
Y0238-3  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVR
Y0313-1  EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
Y0317    EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
                                        CDR-H1
                  40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0243-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0238-3  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0313-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0317    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                CDR-H2                          CDR-7
          80        90                  100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
Y0243-1  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:110)
Y0238-3  YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:112)
Y0313-1  YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:114)
Y0317    YLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTL (SEQ ID NO:116)
                                CDR-H3
```

Fig. 10B

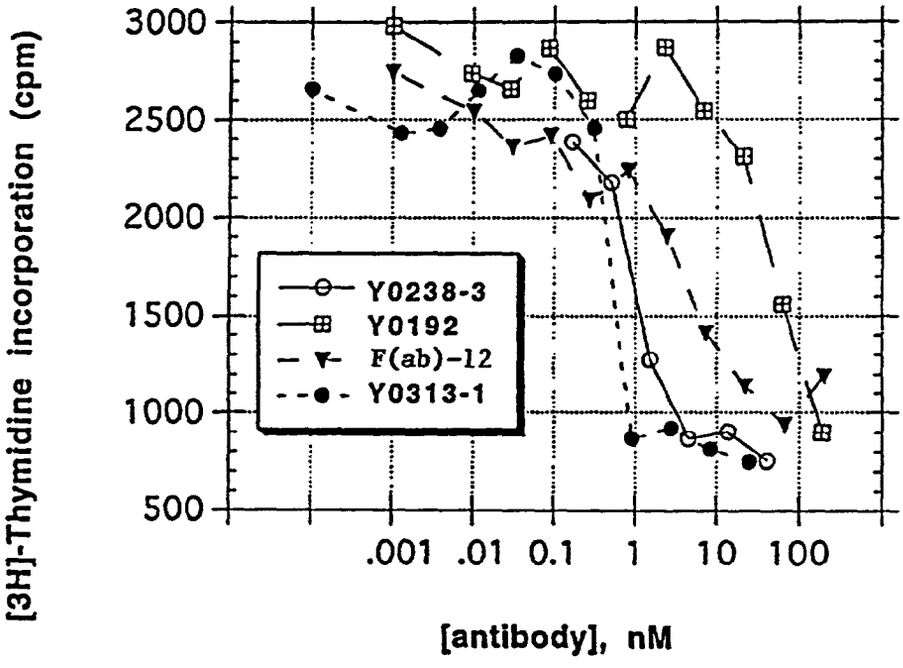
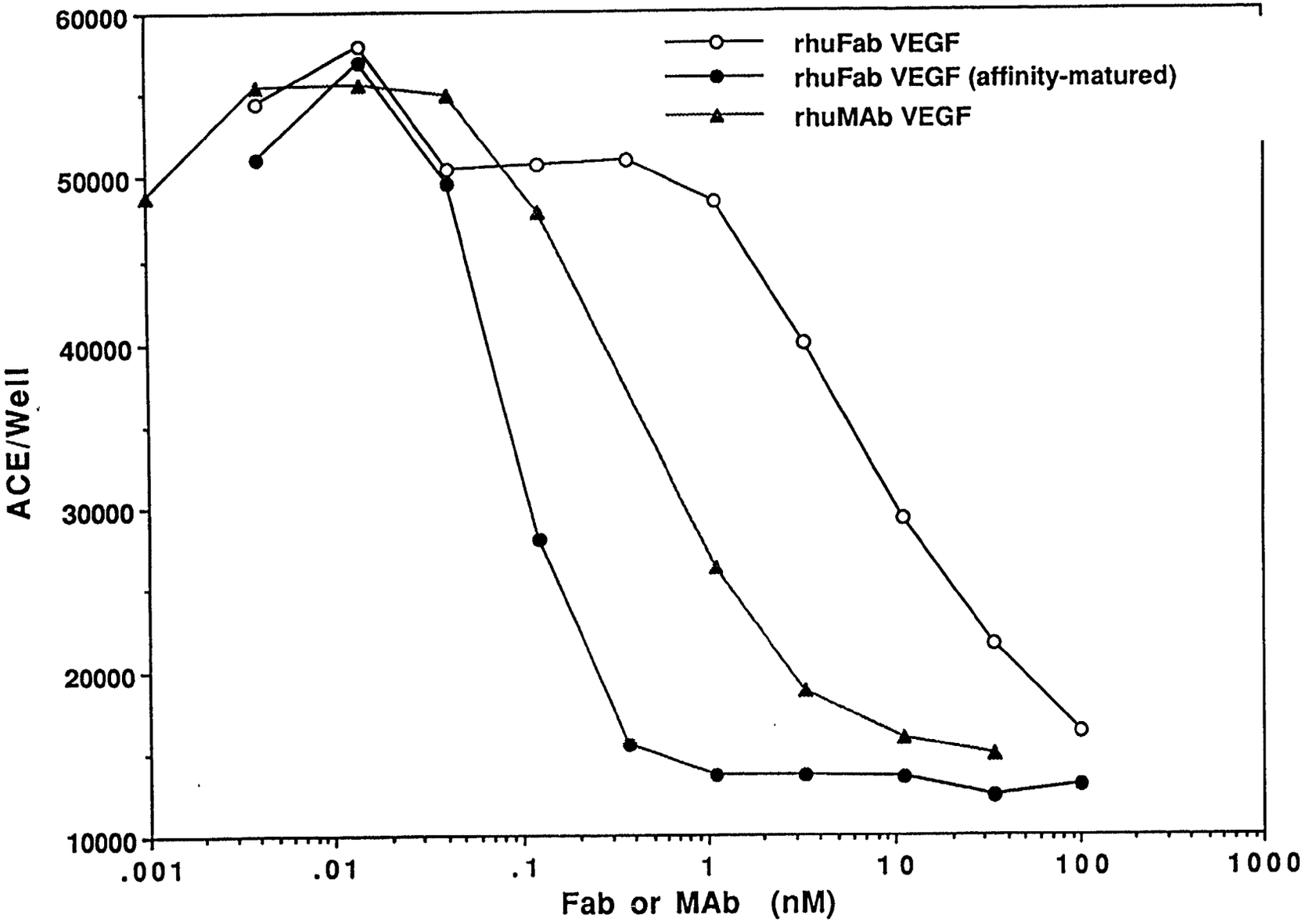

[3H]-Thymidine incorporation (cpm)
3000
2500
2000
1500
1000
500
.001
.01
0.1
1
10
100
[antibody], nM
Y0238-3
Y0192
F(ab)-12
Y0313-1

Fig. 11

Fig. 12



Express Mail No.: EL599584388US
Atty. Docket No.: P1093P1D1
Date Mailed: November 27, 2000

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY FOR CONTINUING APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTI-VEGF ANTIBODIES

the specification of which (check one) _ is attached hereto or X was filed on 06 August 1997 as Application Serial No. 08/908,469 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed Yes	No
Number	Country	Day/Month/Year Filed		

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/833,504	April 7, 1997	Pending
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Walter E. Buting - Reg. No. 23,092
Deirdre L. Conley - Reg. No. 36,487
Ginger R. Dreger - Reg. No. 33,055
Janet E. Hasak - Reg. No. 28,616
Sean A. Johnston - Reg. No. 35,910
Dennis G. Kleid - Reg. No. 32,037
Jeffrey S. Kubinec - Reg. No. 36,575
Wendy M. Lee - Reg. No. 40,378
Richard B. Love - Reg. No. 34,659
Diane L. Marschang - Reg. No. 35,600
Timothy R. Schwartz - Reg. No. 32,171
Craig G. Svoboda - Reg. No. 39,044
Timothy E. Torchia - Reg. No. 36,700
Daryl B. Winter - Reg. No. 32,637

Send correspondence to Genentech, Inc.

Attn: Wendy M. Lee
1 DNA Way
South San Francisco, CA 94080
Telephone: (650) 225-1994

I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Full name of sole or first inventor

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Inventor's signature Manuel Baca. Date 11/28/97

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Citizenship
Australia

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James A. Wells

Inventor's signature James A. Wells Date 10/29/97

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Citizenship
United States of America

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South San Francisco, CA 94080

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Leonard G. Presta

Inventor's signature Leonard G. Presta Date 10-31-97

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Inventor's signature Henry B. Lowman Date 10/29/97

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Citizenship
United States of America

Post Office Address
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Full name of fifth joint inventor, if any

Yvonne Man-yee Chen

Inventor's signature Yvonne Chen Date 10/29/97

Residence

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Citizenship
Canada

Post Office Address
1 DNA Way
South San Francisco, CA 94080

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: To Be Assigned Filed: Herewith For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on November 27, 2000 Eileen Ly

ASSOCIATE POWER OF ATTORNEY (37 CFR 1.34)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please recognize as Associate Attorney in this case:

Steven X. Cui - Reg. No. 44,637

Please direct all communications relative to said pending patent application to the following address:

Steven X. Cui
Genentech, Inc.
1 DNA Way
South San Francisco, CA 94080-4990
Telephone: (650) 225-8674

Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000

By: Janet E. Hasak
Janet E. Hasak
Reg. No. 28,616
Telephone No. 650-225-8674

09157
PATENT TRADEMARK OFFICE

#83625

Jc932 U.S. PTO 09/723752 11/21/00

ISSUE CLASSIFICATION	Subclass	
	Class	

PATENT NUMBER

U.S. **UTILITY** Patent Application

I.P.E.	PATENT DATE
TR. SCANNED BK ② Q.A. LH	

APPLICATION NO.	CONT/PRIOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
09/723752	D	~~514~~		~~1614~~	

APPLICANTS
Manuel Baca
James Wells
Leonard Presta
Henry Lowman

TITLE
Anti-VEGF antibodies

PTO-2040
12/99

ISSUING CLASSIFICATION

ORIGINAL		CROSS REFERENCE(S)	
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)
INTERNATIONAL CLASSIFICATION			

☐ Continued on Issue Slip Inside File Jacket

☐ TERMINAL DISCLAIMER	DRAWINGS			CLAIMS ALLOWED	
	Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
☐ The term of this patent subsequent to _____ (date) has been disclaimed.				NOTICE OF ALLOWANCE MAILED	
☐ The term of this patent shall not extend beyond the expiration date of U.S Patent. No. _____				ISSUE FEE	
				Amount Due	Date Paid
☐ The terminal ____months of this patent have been disclaimed.				ISSUE BATCH NUMBER	

(Assistant Examiner) (Date)

(Primary Examiner) (Date)

(Legal Instruments Examiner) (Date)

WARNING:
The information disclosed herein may be restricted. Unauthorized disclosure may be prohibited by the United States Code Title 35, Sections 122, 181 and 368. Possession outside the U.S. Patent & Trademark Office is restricted to authorized employees and contractors only.

Form PTO-436A
(Rev. 6/99)

FILED WITH: ☐ DISK (CRF) ☐ FICHE ☐ CD-ROM
(Attached in pocket on right inside flap)

(FACE)

SEARCHED

Class	Sub.	Date	Exmr.
424	130.1	11/3/02	LM
424	132.1	"	
424	135.1		
424	141.1		
424	155.1		
424	156.1		
530	387.1		
530	387.7		
530	388.1		
530	388.24		
530	388.8		
530	388.85		
UPDATED		8/21/03	LM

SEARCH NOTES
(INCLUDING SEARCH STRATEGY)

	Date	Exmr.
CAPLUS, BIOSIS, MEDLINE, WEST (US PGPUBS Dec 2002)	11/3/02	LM
INVENTORS NAMES		
SER ID # 1-8, 115-116		
08/908,716		
60/124,446		
VEGF		
ANTI-VEGF		
HUMANIZED WITH ANTI-VEGF		
LM APOPTOSIS		
A4.6.1		
TUMOR		
CANCER		
MACULAR DEGENERATION		
FABS		
IgG ANTIBODY		
BILL DIXON ABOUT DEC/OATH IS OK NO NEED FOR NEW ONE FOR PROVISIONAL	11/3/02	
UPDATED	8/21/03	LM

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.

(RIGHT OUTSIDE)

ISSUE SLIP STAPLE AREA (for additional cross references)

POSITION	INITIALS	ID NO.	DATE
FEE DETERMINATION			
O.I.P.E. CLASSIFIER	RSB		12/22/00
FORMALITY REVIEW	TL	912	04/15/01
RESPONSE FORMALITY REVIEW	MN	778	12/21/01

INDEX OF CLAIMS

✔ ..... Rejected
= ..... Allowed
— (Through numeral)... Canceled
÷ ..... Restricted
N ..... Non-elected
I ..... Interference
A ..... Appeal
O ..... Objected

Claim	Date																			
Final	Original	8/21/03																		
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If more than 150 claims or 10 actions staple additional sheet here

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Attorney Docket **P1093P1D1**
PATENT

CERTIFICATION UNDER 37 CFR 1.10

EL599584388US: Express Mail Number **November 27, 2000: Date of Deposit**

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Eileen Ly

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Manuel Baca, Foster City, CA
James A. Wells, Burlingame, CA
Leonard G. Presta, San Francisco, CA
Henry B. Lowman, El Granada, CA
Yvonne Man-yee Chen, San Mateo, CA

Title: **ANTI-VEGF ANTIBODIES**

1. Type of Application

[ ] This application is for an original, non-provisional application.

[ ] This is a non-provisional application claiming priority to provisional application no. __ , filed ___, the entire disclosure of which is hereby incorporated by reference.

[X] This is a [ ] continuation-in-part [ ] continuation [X] divisional application claiming priority to application Serial Number 08/908,469 , filed August 6, 1997 , the entire disclosure of which is hereby incorporated by reference.

2. **Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)**

80 pages of specification
5 pages of claims
1 page(s) of abstract
16 sheet(s) of drawings
[] formal [X] informal

#83589

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)
___ An executed declaration of the inventor(s) [] is enclosed [] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)
__X__ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)
___ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)
___ An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [] will follow.

(for cont./div.)
__X__ The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

__X__ Cancel in this application original claims 1-38 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

_____ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

__X__ Amend the specification by inserting before the first line the sentence:

--This is a

_____ non-provisional application
_____ continuation
__X__ divisional
_____ continuation-in-part

of co-pending application(s)

__X__ Serial No. 08/908,469 filed on August 6, 1997, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --
_____ International Application _ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

#83589

_____ provisional application No. _ filed _, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.–.

6. Payment of Fees

__X__ Applicants request deferral of payment of the filing fee until submission of the missing parts of application. **DO NOT CHARGE THE FILING FEE AT THIS TIME.**

7. Additional Papers Enclosed

[] Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
[] Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
[X] Associate Power of Attorney.
[] Other:

8. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

_____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
_____ A copy of the petition for extension of time in the ***prior*** application is attached.

9. Correspondence Address:

__X__ Address all future communications to:

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-8674

Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000 By: Steven X. Cui
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#83589

Variable Heavy

```
A4.6.1      EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQAPGKGLKWMG
             *   *  **  *   ***  *  *          *       * *
F(ab)-12    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG
                                      *  **  *  *           *
humIII      EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS
            1        10        20        30        40

A4.6.1      WINTYTGEPTYAADFKRRFTFSLETSASTAYLQISNLKNDDTATYFCAK
                                    *  *      *** ***   * *
F(ab)-12    WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAK
            * **** ***  *** *   * * *  * *                  *
humIII      VISGDGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
          50  a      60        70        80  abc       90

A4.6.1      YPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
                          *  *
F(ab)-12    YPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:7)
            *           *
humIII      G----------FDYWGQGTLVTVSS (SEQ ID NO:11)
                             110
```

Fig. 1A

Variable Light

```
A4.6.1      DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKPDGTVKVLIY
                 **      *    * *                  ****
F(ab)-12    DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIY
                                  *   *     *          *
humKI       DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKPGKAPKLLIY
            1        10        20        30        40

A4.6.1      FTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSTVPWTF
                                 **    * *   *
F(ab)-12    FTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTF
            **   *                                    ***
humKI       AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPWTF
          50        60        70        80        90

A4.6.1      GGGTKLEIKR (SEQ ID NO:10)
             *   *
F(ab)-12    GQGTKVEIKR (SEQ ID NO:8)
humKI       GQGTKVEIKR (SEQ ID NO:12)
          100
```

Fig. 1B

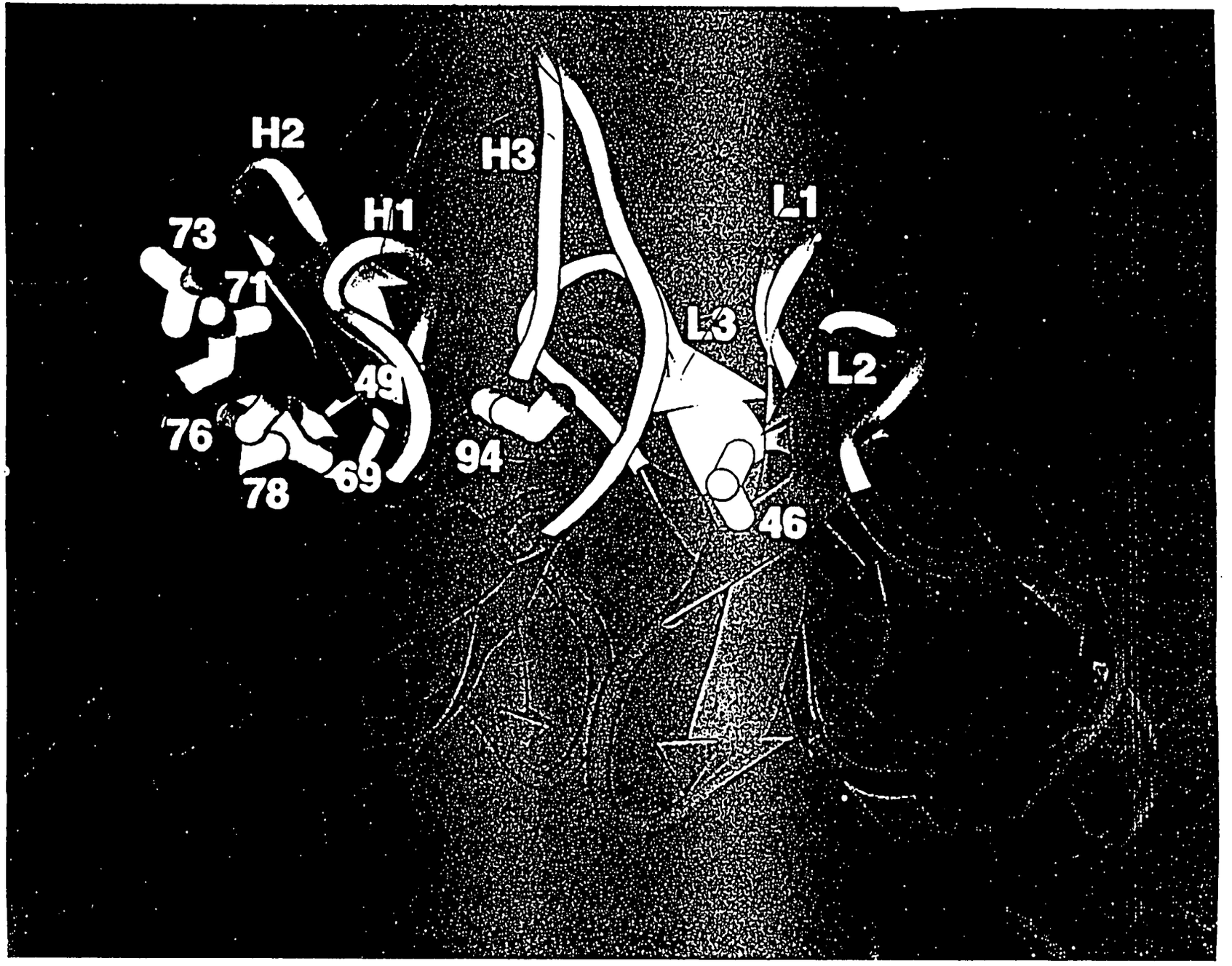

Fig. 2

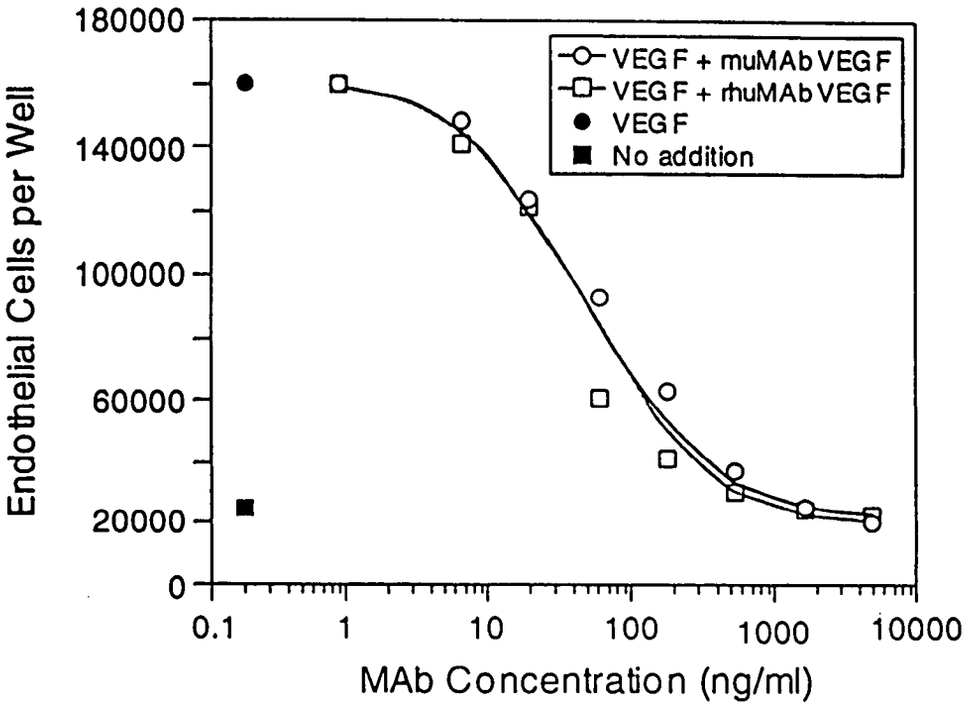

Fig. 3

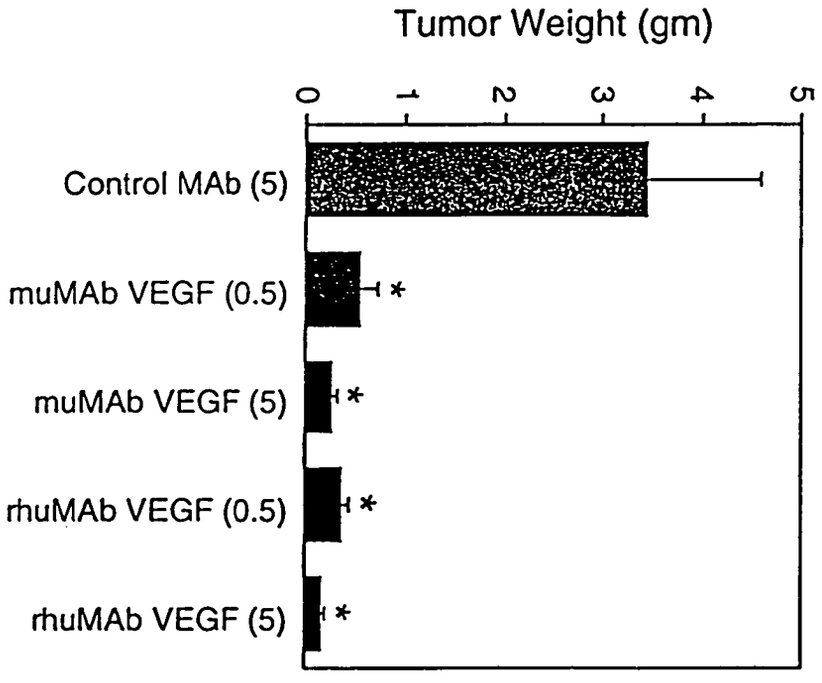

Fig. 4

V_L domain

```
                10        20        30        40
A4.6.1  DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
          **      *   * *
hu2.0   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP

hu2.10  DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP
```

Fig. 5A

```
                50        60        70        80
A4.6.1  DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
        **** *                     **    * *
hu2.0   GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
                                     .
hu2.10  GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTISSLQP

                90       100
A4.6.1  EDIATYYCQQYSTVPWTFGGGTKLEIK (SEQ ID NO:10)
          *                *   *
hu2.0   EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:13)

hu2.10  EDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO:15)
```

- V_H domain

```
                10        20        30        40
A4.6.1  EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
          *   *  ** *   *** * *                *
hu2.0   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                           .
hu2.10  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIRQA

                50 a      60        70        80
A4.6.1  PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
          * *                    * *** ** *
hu2.0   PGKGLEWVGWINTYTGEPTYAADFKRRFTISRDNSKNTLYL
                                   . . .. .
hu2.10  PGKGLEWVGWINTYTGEPTYAADFKRRFTISLDTSASTVYL

          abc      90       100abcdef       110
A4.6.1  QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS (SEQ ID NO:9)
        *** ***   * *  *                  *  *
hu2.0   QMNSLRAEDTAVYYCARYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:14)
                  .
hu2.10  QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO:16)
```

Fig. 5B

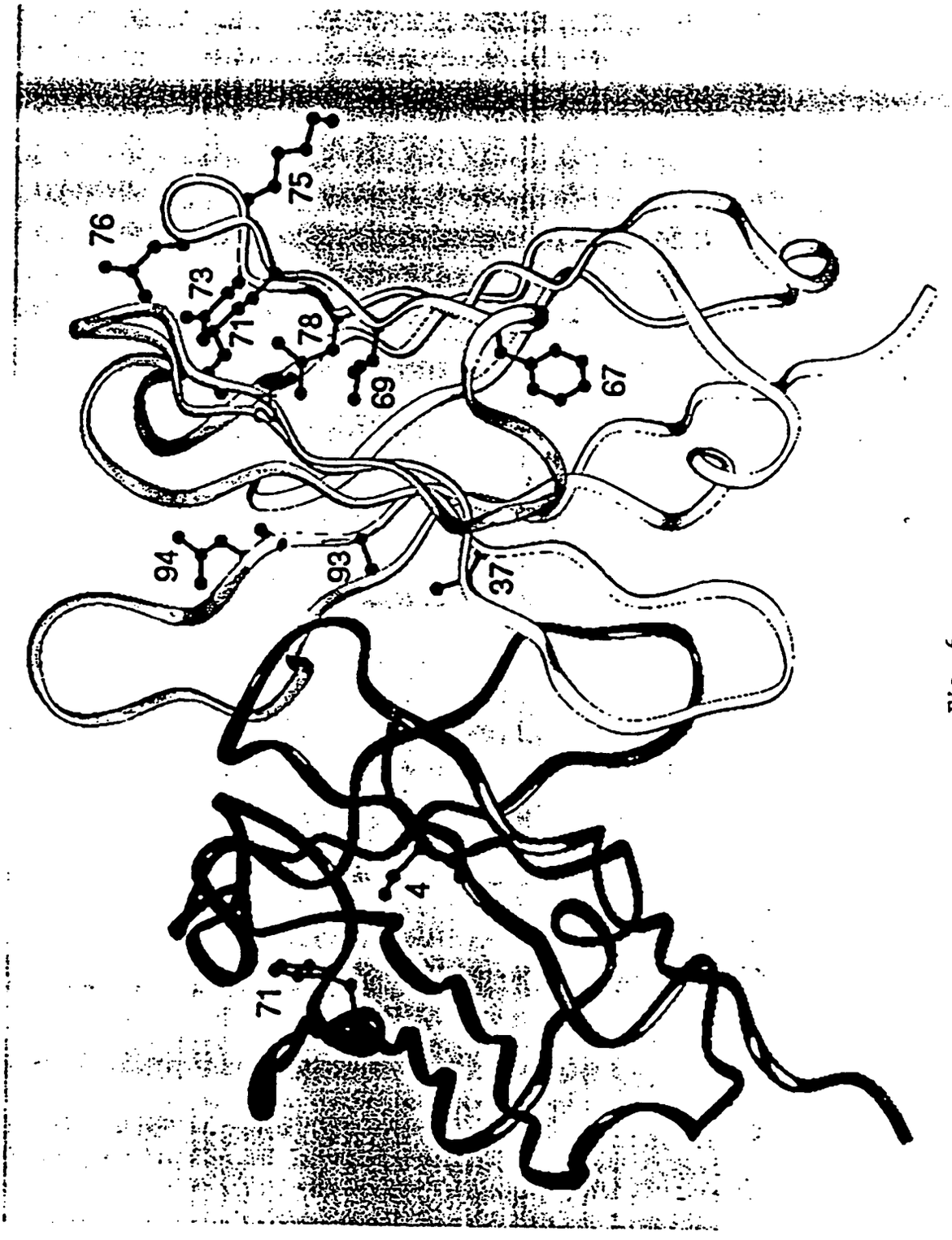

Fig. 6

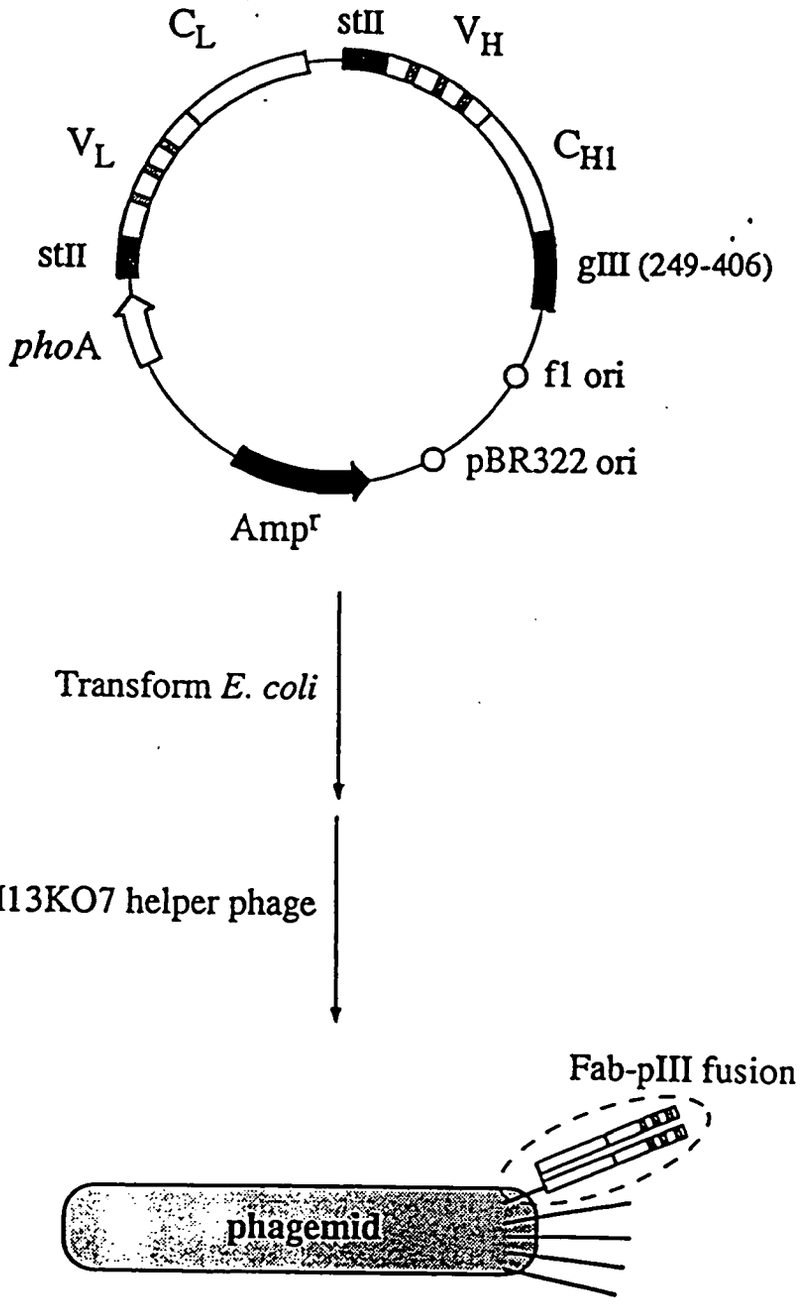

Fig. 7

```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC
     CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAAACCT CTAATAGCAG TGACGTTACG

 101 TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC
     AAGCGTTATA CCGCGTTTTA CTGGTTGTCG CCAACTAACT AGTCCATCTC CCCCGCGACA TGCTCCATTT CGGGCTACGG TCGTAAGGAC TGCTGCTATG

 201 GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT
     CCTCGACGAC GCGCTAATGC ATTTCTTCAA TAACTTCGTA GGAGCAGTCA TTTTTCAATT AGAAAAGTTG TCGACAGTAT TTCAACAGTG CCGGCTCTGA

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGGTACCC GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA
     ATATCAGCGA AACAAAAATA AAAAATTACA TAAACATTGA TCTTAAGCTC GAGCCATGGG CCCCTAGGAG ATCTCCAACT CCACTAAAAT ACTTTTTCTT
-23                                                                                                    M etLysLysAsn
                                                                                                Begin stII signal sequence
 401 TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG CGTACGCTGA TATCCAGTTG ACCCAGTCCC CGAGCTCCCT GTCCGCCTCT
     ATAGCGTAAA GAAGAACGTA GATACAAGCA AAAAAGATAA CGATGTTTGC GCATGCGACT ATAGGTCAAC TGGGTCAGGG GCTCGAGGGA CAGGCGGAGA
-19  IleAlaPhe LeuLeuAlaS erMetPheVa lPheSerIle AlaThrAsnA laTyrAlaAs pIleGlnLeu ThrGlnSerP roSerSerLe uSerAlaSer
                                                                Begin light chain
 501 GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG ATATTAGCAA CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAACTACTGA
     CACCCGCTAT CCCAGTGGTA GTGGACGTCG CGTTCAGTCC TATAATCGTT GATAAATTTG ACCATAGTTG TCTTTGGTCC TTTTCGAGGC TTTGATGACT
  15 ValGlyAspA rgValThrIl eThrCysSer AlaSerGlnA spIleSerAs nTyrLeuAsn TrpTyrGlnG lnLysProGl yLysAlaPro LysLeuLeuIle

 601 TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA TCCGGTTCTG GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA
     AAATGAAGTG GAGGAGAGAG GTGAGACCTC AGGGAAGAGC GAAGAGACCT AGGCCAAGAC CCTGCCTAAT GTGAGACTGG TAGTCGTCAG ACGTCGGTCT
  49    TyrPheTh rSerSerLeu HisSerGlyV alProSerAr gPheSerGly SerGlySerG lyThrAspTy rThrLeuThr IleSerSerL euGlnProGlu

 701 AGACTTCGCA ACTTATTACT GTCAACAGTA TAGCACCGTG CCGTGGACGT TTGGACAGGG TACCAAGGTG GAGATCAAAC GAACTGTGGC TGCACCATCT
     TCTGAAGCGT TGAATAATGA CAGTTGTCAT ATCGTGGCAC GGCACCTGCA AACCTGTCCC ATGGTTCCAC CTCTAGTTTG CTTGACACCG ACGTGGTAGA
  82  AspPheAla ThrTyrTyrC ysGlnGlnTy rSerThrVal ProTrpThrP heGlyGlnGl yThrLysVal GluIleLysA rgThrValAl aAlaProSer

 801 GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG GAACTGCTTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT
     CAGAAGTAGA AGGGCGGTAG ACTACTCGTC AACTTTAGAC CTTGACGAAG ACAACACACG GACGACTTAT TGAAGATAGG GTCTCTCCGG TTTCATGTCA
 115 ValPheIleP heProProSe rAspGluGln LeuLysSerG lyThrAlaSe rValValCys LeuLeuAsnA snPheTyrPr oArgGluAla LysValGlnTrp

 901 GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA GAGCAGGACA GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT
     CCTTCCACCT ATTGCGGGAG GTTAGCCCAT TGAGGGTCCT CTCACAGTGT CTCGTCCTGT CGTTCCTGTC GTGGATGTCG GAGTCGTCGT GGGACTGCGA
 149   LysValAs pAsnAlaLeu GlnSerGlyA snSerGlnGl uSerValThr GluGlnAspS erLysAspSe rThrTyrSer LeuSerSerT hrLeuThrLeu

1001 GAGCAAAGCA GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT GAGCTCGCCC GTCACAAAGA GCTTCAACAG GGGAGAGTGT
     CTCGTTTCGT CTGATGCTCT TTGTGTTTCA GATGCGGACG CTTCAGTGGG TAGTCCCGGA CTCGAGCGGG CAGTGTTTCT CGAAGTTGTC CCCTCTCACA
 182  SerLysAla AspTyrGluL ysHisLysVa lTyrAlaCys GluValThrH isGlnGlyLe uSerSerPro ValThrLysS erPheAsnAr gGlyGluCys
```

Fig. 8A

```
1101 TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA CGCAACTAGT CGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG
     ATTCGACTAG GAGATGCGGC CTGCGTAGCA CCGGGATCAT GCGTTGATCA GCATTTTTCC CATAGATCTC CAACTCCACT AAAATACTTT TTCTTATAGC
 215 OC*                                                                                                -23 MetLys LysAsnIleAla
                  Begin stII signal sequence
1201 CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC GCTGAGGTTC AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG
     GTAAAGAAGA ACGTAGATAC AAGCAAAAAA GATAACGATG TTTGCGCATG CGACTCCAAG TCGACCACCT CAGACCGCCA CCGGACCACG TCGGTCCCCC
 -17   PheLeuLe uAlaSerMet PheValPheS erIleAlaTh rAsnAlaTyr AlaGluValG lnLeuValGl uSerGlyGly GlyLeuValG lnProGlyGly
                                                                Begin heavy chain
1301 CTCACTCCGT TTGTCCTGTG CAGCTTCTGG CTATACCTTC ACCAACTATG GTATGAACTG GATCCGTCAG GCCCCGGGTA AGGGCCTGGA ATGGGTTGGA
     GAGTGAGGCA AACAGGACAC GTCGAAGACC GATATGGAAG TGGTTGATAC CATACTTGAC CTAGGCAGTC CGGGGCCCAT TCCCGGACCT TACCCAACCT
  17  SerLeuArg LeuSerCysA laAlaSerGl yTyrThrPhe ThrAsnTyrG lyMetAsnTr pIleArgGln AlaProGlyL ysGlyLeuGl uTrpValGly

1401 TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTTACT ATATCTGCAG ACACCTCCAG CAACACAGTT TACCTGCAGA
     ACCTAATTGT GGATATGGCC ACTTGGCTGG ATACGACGCC TAAAGTTTGC AGCAAAATGA TATAGACGTC TGTGGAGGTC GTTGTGTCAA ATGGACGTCT
  50 TrpIleAsnT hrTyrThrGl yGluProThr TyrAlaAlaA spPheLysAr gArgPheThr IleSerAlaA spThrSerSe rAsnThrVal TyrLeuGlnMet

1501 TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC CCGCACTATT ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG
     ACTTGTCGGA CGCGCGACTC CTGTGACGGC AGATAATGAC ACGTTTCATG GGCGTGATAA TACCCTCGTC GGTGACCATA AAGCTGCAGA CCCCAGTTCC
  84   AsnSerLe uArgAlaGlu AspThrAlaV alTyrTyrCy sAlaLysTyr ProHisTyrT yrGlySerSe rHisTrpTyr PheAspValT rpGlyGlnGly

1601 AACCCTGGTC ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC
     TTGGGACCAG TGGCAGAGGA GCCGGAGGTG GTTCCCGGGT AGCCAGAAGG GGGACCGTGG GAGGAGGTTC TCGTGGAGAC CCCCGTGTCG CCGGGACCCG
 117  ThrLeuVal ThrValSerS erAlaSerTh rLysGlyPro SerValPheP roLeuAlaPr oSerSerLys SerThrSerG lyGlyThrAl aAlaLeuGly

1701 TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT
     ACGGACCAGT TCCTGATGAA GGGGCTTGGC CACTGCCACA GCACCTTGAG TCCGCGGGAC TGGTCGCCGC ACGTGTGGAA GGGCCGACAG GATGTCAGGA
 150 CysLeuValL ysAspTyrPh eProGluPro ValThrValS erTrpAsnSe rGlyAlaLeu ThrSerGlyV alHisThrPh eProAlaVal LeuGlnSerSer

1801 CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA
     GTCCTGAGAT GAGGGAGTCG TCGCACCACT GGCACGGGAG GTCGTCGAAC CCGTGGGTCT GGATGTAGAC GTTGCACTTA GTGTTCGGGT CGTTGTGGTT
 184   GlyLeuTy rSerLeuSer SerValValT hrValProSe rSerSerLeu GlyThrGlnT hrTyrIleCy sAsnValAsn HisLysProS erAsnThrLys

1901 GGTCGACAAG AAAGTTGAGC CCAAATCTTG TGACAAAACT CACCTCTAGA GTGGCGGTGG CTCTGGTTCC GGTGATTTTG ATTATGAAAA GATGGCAAAC
     CCAGCTGTTC TTTCAACTCG GGTTTAGAAC ACTGTTTTGA GTGGAGATCT CACCGCCACC GAGACCAAGG CCACTAAAAC TAATACTTTT CTACCGTTTG
 217  ValAspLys LysValGluP roLysSerCy sAspLysThr HisLeuAM*S erGlyGlyGl ySerGlySer GlyAspPheA spTyrGluLy sMetAlaAsn
                                      end light chain   Begin g3p domain
2001 GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG CGCTACAGTC TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA
     CGATTATTCC CCCGATACTG GCTTTTACGG CTACTTTTGC GCGATGTCAG ACTGCGATTT CCGTTTGAAC TAAGACAGCG ATGACTAATG CCACGACGAT
 250 AlaAsnLysG lyAlaMetTh rGluAsnAla AspGluAsnA laLeuGlnSe rAspAlaLys GlyLysLeuA spSerValAl aThrAspTyr GlyAlaAlaIle

2101 TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG
     AGCTACCAAA GTAACCACTG CAAAGGCCGG AACGATTACC ATTACCACGA TGACCACTAA AACGACCGAG ATTAAGGGTT TACCGAGTTC AGCCACTGCC
 284   AspGlyPh eIleGlyAsp ValSerGlyL euAlaAsnGl yAsnGlyAla ThrGlyAspP heAlaGlySe rAsnSerGln MetAlaGlnV alGlyAspGly

2201 TGATAATTCA CCTTTAATGA ATAATTTCCG TCAATATTTA CCTTCCCTCC CTCAATCGGT TGAATGTCGC CCTTTTGTCT TTAGCGCTGG TAAACCATAT
     ACTATTAAGT GGAAATTACT TATTAAAGGC AGTTATAAAT GGAAGGGAGG GAGTTAGCCA ACTTACAGCG GGAAAACAGA AATCGCGACC ATTTGGTATA
 317  AspAsnSer ProLeuMetA snAsnPheAr gGlnTyrLeu ProSerLeuP roGlnServa lGluCysArg ProPheValP heSerAlaGl yLysProTyr
```

Fig. 8B

```
2301 GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCCGTG GTGTCTTTGC GTTTGTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA
     CTTAAAAGAT AACTAACACT GTTTTATTTG AATAAGGCAC CACAGAAACG CAAAGAAAAT ATACAACGGT GGAAATACAT ACATAAAAGA TGCAAACGAT
 350 GluPheSerI leAspCysAs pLysIleAsn LeuPheArgG lyValPheAl aPheLeuLeu TyrValAlaT hrPheMetTy rValPheSer ThrPheAlaAsn

2401 ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC
     TGTATGACGC ATTATTCCTC AGAATTAGTA CGGTCAAGAA AACCGATCGC GGCGGGATAT GGAACAGACG GAGGGGCGCA ACGCAGCGCC ACGTACCTCG
 384   IleLeuAr gAsnLysGlu SerOC* (SEQ ID NO: 100)
                end g3 protein
2501 CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC
     GCCCGGTGGA GCTGGACTTA CCTTCGGCCG CCGTGGAGCG ATTGCCTAAG TGGTGAGGTT CTTAACCTCG GTTAGTTAAG AACGCCTCTT GACACTTACG

2601 GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC
     CGTTTGGTTG GGAACCGTCT TGTATAGGTA GCGCAGGCGG TAGAGGTCGT CGGCGTGCGC CGCGTAGAGC CCGTCGCAAC CCAGGACCGG TGCCCACGCG

2701 ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT TACTGGTTAG CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT
     TACTAGCACG AGGACAGCAA CTCCTGGGCC GATCCGACCG CCCCAACGGA ATGACCAATC GTCTTACTTA GTGGCTATGC GCTCGCTTGC ACTTCGCTGA

2801 GCTGCTGCAA AACGTCTGCG ACCTGAGCAA CAACATGAAT GGTCTTCGGT TTCCGTGTTT CGTAAAGTCT GGAAACGCGG AAGTCAGCGC CCTGCACCAT
     CGACGACGTT TTGCAGACGC TGGACTCGTT GTTGTACTTA CCAGAAGCCA AAGGCACAAA GCATTTCAGA CCTTTGCGCC TTCAGTCGCG GGACGTGGTA

2901 TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC
     ATACAAGGCC TAGACGTAGC GTCCTACGAC GACCGATGGG ACACCTTGTG GATGTAGACA TAATTGCTTC GCGACCGTAA CTGGGACTCA CTAAAAAGAG

3001 TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG TAACCGGGCA TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC
     ACCAGGGCGG CGTAGGTATG GCGGTCAACA AATGGGAGTG TTGCAAGGTC ATTGGCCCGT ACAAGTAGTA GTCATTGGGC ATAGCACTCG TAGGAGAGAG

3101 GTTTCATCGG TATCATTACC CCCATGAACA GAAATTCCCC CTTACACGGA GGCATCAAGT GACCAAACAG GAAAAAACCG CCCTTAACAT GGCCCGCTTT
     CAAAGTAGCC ATAGTAATGG GGGTACTTGT CTTTAAGGGG GAATGTGCCT CCGTAGTTCA CTGGTTTGTC CTTTTTTGGC GGGAATTGTA CCGGGCGAAA

3201 ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG AGCTGGACGC GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC
     TAGTCTTCGG TCTGTAATTG CGAAGACCTC TTTGAGTTGC TCGACCTGCG CCTACTTGTC CGTCTGTAGA CACTTAGCGA AGTGCTGGTG CGACTACTCG

3301 TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC
     AAATGGCGTC CTAGGCCTTT AACATTTGCA ATTATAAAAC AATTTTAAGC GCAATTTAAA AACAATTTAG TCGAGTAAAA AATTGGTTAT CCGGCTTTAG

3401 GGCAAAATCC CTTATAAATC AAAAGAATAG ACCGAGATAG GGTTGAGTGT TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG
     CCGTTTTAGG GAATATTTAG TTTTCTTATC TGGCTCTATC CCAACTCACA ACAAGGTCAA ACCTTGTTCT CAGGTGATAA TTTCTTGCAC CTGAGGTTGC

3501 TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG
     AGTTTCCCGC TTTTTGGCAG ATAGTCCCGA TACCGGGTGA TGCACTTGGT AGTGGGATTA GTTCAAAAAA CCCCAGCTCC ACGGCATTTC GTGATTTAGC

3601 GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG
     CTTGGGATTT CCCTCGGGGG CTAAATCTCG AACTGCCCCT TTCGGCCGCT TGCACCGCTC TTTCCTTCCC TTCTTTCGCT TTCCTCGCCC GCGATCCCGC

3701 CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC CTGCCTCGCG CGTTTCGGTG
     GACCGTTCAC ATCGCCAGTG CGACGCGCAT TGGTGGTGTG GGCGGCGCGA ATTACGCGGC GATGTCCCGC GCAGGCCTAG GACGGAGCGC GCAAAGCCAC

3801 ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC
     TACTGCCACT TTTGGAGACT GTGTACGTCG AGGGCCTCTG CCAGTGTCGA ACAGACATTC GCCTACGGCC CTCGTCTGTT CGGGCAGTCC CGCGCAGTCG
```

Fig. 8C

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3901 GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA GCGGAGTGTA TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA
     CCCACAACCG CCCACAGCCC CGCGTCGGTA CTGGGTCAGT GCATCGCTAT CGCCTCACAT ATGACCGAAT TGATACGCCG TAGTCTCGTC TAACATGACT

4001 GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA GGCGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC
     CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT ATGGCGTAGT CCGCGAGAAG GCGAAGGAGC GAGTGACTGA GCGACGCGAG

4101 GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA
     CCAGCAAGCC GACGCCGCTC GCCATAGTCG AGTGAGTTTC CGCCATTATG CCAATAGGTG TCTTAGTCCC CTATTGCGTC CTTTCTTGTA CACTCGTTTT

4201 GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC
     CCGGTCGTTT TCCGGTCCTT GGCATTTTTC CGGCGCAACG ACCGCAAAAA GGTATCCGAG GCGGGGGGAC TGCTCGTAGT GTTTTTAGCT GCGAGTTCAG

4301 AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA
     TCTCCACCGC TTTGGGCTGT CCTGATATTT CTATGGTCCG CAAAGGGGGA CCTTCGAGGG AGCACGCGAG AGGACAAGGC TGGGACGGCG AATGGCCTAT

4401 CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT
     GGACAGGCGG AAAGAGGGAA GCCCTTCGCA CCGCGAAAGA GTATCGAGTG CGACATCCAT AGAGTCAAGC CACATCCAGC AAGCGAGGTT CGACCCGACA

4501 GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG
     CACGTGCTTG GGGGGCAAGT CGGGCTGGCG ACGCGGAATA GGCCATTGAT AGCAGAACTC AGGTTGGGCC ATTCTGTGCT GAATAGCGGT GACCGTCGTC

4601 CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG
     GGTGACCATT GTCCTAATCG TCTCGCTCCA TACATCCGCC ACGATGTCTC AAGAACTTCA CCACCGGATT GATGCCGATG TGATCTTCCT GTCATAAACC

4701 TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC
     ATAGACGCGA GACGACTTCG GTCAATGGAA GCCTTTTTCT CAACCATCGA GAACTAGGCC GTTTGTTTGG TGGCGACCAT CGCCACCAAA AAAACAAACG

4801 AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA
     TTCGTCGTCT AATGCGCGTC TTTTTTTCCT AGAGTTCTTC TAGGAAACTA GAAAAGATGC CCCAGACTGC GAGTCACCTT GCTTTTGAGT GCAATTCCCT

4901 TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC
     AAAACCAGTA CTCTAATAGT TTTTCCTAGA AGTGGATCTA GGAAAATTTA ATTTTTACTT CAAAATTTAG TTAGATTTCA TATATACTCA TTTGAACCAG

5001 TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG
     ACTGTCAATG GTTACGAATT AGTCACTCCG TGGATAGAGT CGCTAGACAG ATAAAGCAAG TAGGTATCAA CGGACTGAGG GGCAGCACAT CTATTGATGC

5101 ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA
     TATGCCCTCC CGAATGGTAG ACCGGGGTCA CGACGTTACT ATGGCGCTCT GGGTGCGAGT GGCCGAGGTC TAAATAGTCG TTATTTGGTC GGTCGGCCTT

5201 GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT
     CCCGGCTCGC GTCTTCACCA GGACGTTGAA ATAGGCGGAG GTAGGTCAGA TAATTAACAA CGGCCCTTCG ATCTCATTCA TCAAGCGGTC AATTATCAAA

5301 GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA
     CGCGTTGCAA CAACGGTAAC GACGTCCGTA GCACCACAGT GCGAGCAGCA AACCATACCG AAGTAAGTCG AGGCCAAGGG TTGCTAGTTC CGCTCAATGT

5401 TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG
     ACTAGGGGGT ACAACACGTT TTTTCGCCAA TCGAGGAAGC CAGGAGGCTA GCAACAGTCT TCATTCAACC GGCGTCACAA TAGTGAGTAC CAATACCGTC

5501 CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC
     GTGACGTATT AAGAGAATGA CAGTACGGTA GGCATTCTAC GAAAAGACAC TGACCACTCA TGAGTTGGTT CAGTAAGACT CTTATCACAT ACGCCGCTGG
```

Fig. 8D

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5601 GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC
     CTCAACGAGA ACGGGCCGCA GTTGTGCCCT ATTATGGCGC GGTGTATCGT CTTGAAATTT TCACGAGTAG TAACCTTTTG CAAGAAGCCC CGCTTTTGAG

5701 TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG
     AGTTCCTAGA ATGGCGACAA CTCTAGGTCA AGCTACATTG GGTGAGCACG TGGGTTGACT AGAAGTCGTA GAAAATGAAA GTGGTCGCAA AGACCCACTC

5801 CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA
     GTTTTTGTCC TTCCGTTTTA CGGCGTTTTT TCCCTTATTC CCGCTGTGCC TTTACAACTT ATGAGTATGA GAAGGAAAAA GTTATAATAA CTTCGTAAAT

5901 TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC
     AGTCCCAATA ACAGAGTACT CGCCTATGTA TAAACTTACA TAAATCTTTT TATTTGTTTA TCCCCAAGGC GCGTGTAAAG GGGCTTTTCA CGGTGGACTG

6001 GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTTC AA (SEQ ID NO: 99)
     CAGATTCTTT GGTAATAATA GTACTGTAAT TGGATATTTT TATCCGCATA GTGCTCCGGG AAAGCAGAAG TT
```

Fig. 8E

▨ = differences from F(ab)-12

```
                        10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
MB1.6    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
H2305.6  DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0101    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0192    DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQ
                                    CDR-L1
                    40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
MB1.6    KPGKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
H2305.6  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDYTLTIS
Y0101    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0192    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
                CDR-L2
                80                  90                  100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
MB1.6    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 101)
H2305.6  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:103)
Y0101    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:105)
Y0192    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO: 107)
                        CDR-L3
```

Fig. 9A

```
                        10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
MB1.6    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
H2305.6  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIR
Y0101    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0192    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVR
                                    CDR-H1
                    40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
MB1.6    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTISADTSSNIV
H2305.6  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSADTSSNIV
Y0101    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0192    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                        CDR-H2                          CDR-7
                80                  90                  100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
MB1.6    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 102)
H2305.6  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 104)
Y0101    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 106)
Y0192    YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO: 108)
                                    CDR-H3
```

Fig. 9B

▨ = differences from)-12

```
                          10                  20                  30
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
Y0243-1  DIQ▨TQSPSSLSASVGDRVTITC▨A▨E▨L▨SNYLNWYQQ
Y0238-3  DIQ▨TQSPSSLSASVGDRVTITC▨A▨E▨L▨SNYLNWYQQ
Y0313-1  DIQ▨TQSPSSLSASVGDRVTITC▨A▨E▨L▨SNYLNWYQQ
Y0317    DIQ▨TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
                                    CDR-L1
                 40                  50                  60                  70
F(ab)-12 KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0243-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0238-3  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0313-1  KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0317    KPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
              CDR-L2
            80                  90                 100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:8)
Y0243-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:109)
Y0238-3  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:111)
Y0313-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:113)
Y0317    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV (SEQ ID NO:115)
                        CDR-L3
```

Fig. 10A

```
                          10                  20                  30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0243-1  EVQLVESGGGLVQPGGSLRLSCAASGY▨F▨T▨HYGMNWVR
Y0238-3  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYG▨NWVR
Y0313-1  EVQLVESGGGLVQPGGSLRLSCAASGY▨F▨T▨HYGMNWVR
Y0317    EVQLVESGGGLVQPGGSLRLSCAASGY▨F▨T▨HYGMNWVR
                                    CDR-H1
                 40                  50                  60                  70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0243-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0238-3  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0313-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0317    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
                   CDR-H2                          CDR-7
            80                  90                 100                 110
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:7)
Y0243-1  YLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTL (SEQ ID NO:110)
Y0238-3  YLQMNSLRAEDTAVYYCAKYP▨YYG▨SHWYFDVWGQGTL (SEQ ID NO:112)
Y0313-1  YLQMNSLRAEDTAVYYCAKYP▨YYG▨SHWYFDVWGQGTL (SEQ ID NO:114)
Y0317    YLQMNSLRAEDTAVYYCAKYP▨YYG▨SHWYFDVWGQGTL (SEQ ID NO:116)
                                    CDR-H3
```

Fig. 10B

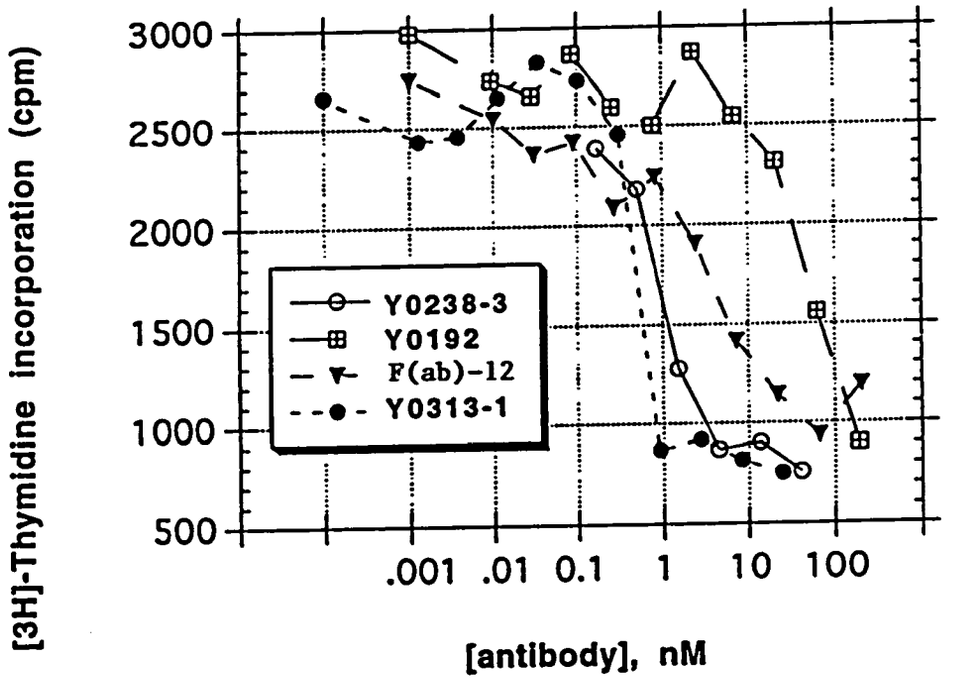
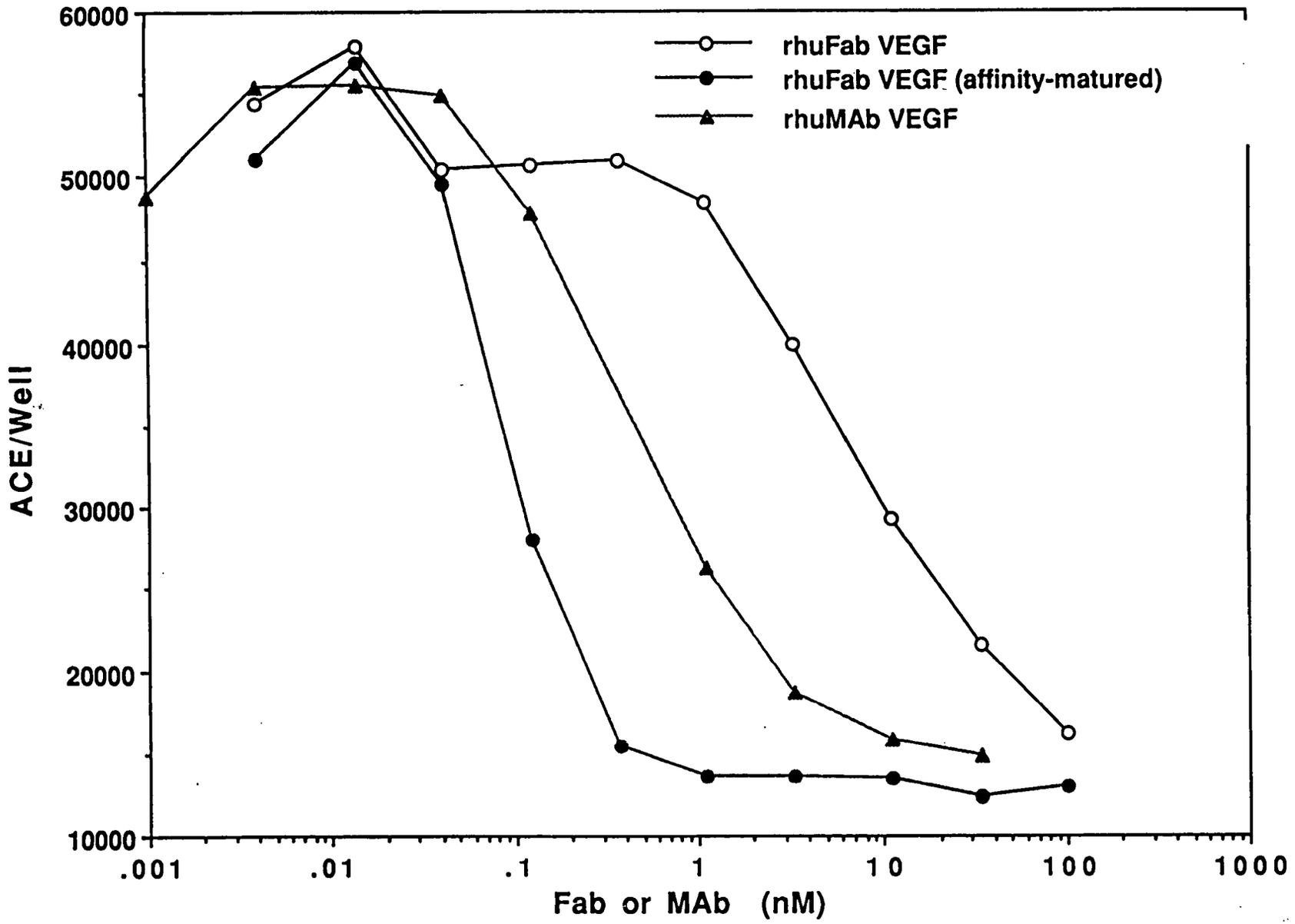


Fig. 11

Fig. 12



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ANTI-VEGF ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of co-pending U.S. Application Serial No. 08/833,504, filed April 7, 1997, which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to anti-VEGF antibodies and, in particular, to humanized anti-VEGF antibodies and variant anti-VEGF antibodies.

Description of Related Art

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun

et al). The negative regulators so far identified include thrombospondin (Good *et al. Proc. Natl. Acad. Sci. USA*. 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

SUMMARY OF THE INVENTION

This application describes humanized anti-VEGF antibodies and anti-VEGF antibody variants with desirable properties from a therapeutic perspective, including strong binding affinity for VEGF; the ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and the ability

to inhibit VEGF-induced angiogenesis *in vivo*.

The preferred humanized anti-VEGF antibody or variant anti-VEGF antibody herein binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M and preferably no more than about 5 x 10^{-9}M. In addition, the humanized or variant anti-VEGF antibody may have an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*. The humanized or variant anti-VEGF antibodies of particular interest herein are those which inhibit at least about 50% of tumor growth in an A673 *in vivo* tumor model, at an antibody dose of 5mg/kg.

In one embodiment, the anti-VEGF antibody has a heavy and light chain variable domain, wherein the heavy chain variable domain comprises hypervariable regions with the following amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129). For example, the heavy chain variable domain may comprise the amino acid sequences of CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR;SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV;SEQ ID NO:3). Preferably, the three heavy chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4.

The invention further provides an anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYX_1FTX_2YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSK STAYLQMNSLRAEDTAVYYCAKYPX_3YYGX_4SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T. One particularly useful heavy chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the heavy chain variable domain sequence of SEQ ID NO:7. Such preferred heavy chain variable domain sequences may be combined with the following preferred light chain variable domain sequences or with other light chain variable domain sequences, provided that the antibody so produced binds human VEGF.

The invention also provides preferred light chain variable domain sequences which may be combined with the above-identified heavy chain variable domain sequences or with other heavy

chain variable domain sequences, provided that the antibody so produced retains the ability to bind to human VEGF. For example, the light chain variable domain may comprise hypervariable regions with the following amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6). Preferably, the three light chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4.

In one embodiment, the invention provides a humanized anti-VEGF antibody light chain variable domain comprising the amino acid sequence:
DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L. One particularly useful light chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the light chain variable domain sequence of SEQ ID NO:8.

The invention also provides a variant of a parent anti-VEGF antibody (which parent antibody is preferably a humanized or human anti-VEGF antibody), wherein the variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of the heavy or light chain variable domain of the parent anti-VEGF antibody. The variant preferably has one or more substitution(s) in one or more hypervariable region(s) of the anti-VEGF antibody. Preferably, the substitution(s) are in the heavy chain variable domain of the parent antibody. For example, the amino acid subsition(s) may be in the CDRH1 and/or CDRH3 of the heavy chain variable domain. Preferably, there are substitutions in both these hypervariable regions. Such "affinity matured" variants are demonstrated herein to bind human VEGF more strongly than the parent anti-VEGF antibody from which they are generated, *i.e.*, they have a K_d value which is significantly less than that of the parent anti-VEGF antibody. Preferably, the variant has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower, preferably at least about 20 fold lower, and most preferably at least about 50 fold lower, than that of the parent anti-VEGF antibody. One particularly prefered variant is the Y0317 variant of Example 3, which has a CDRH1 comprising the amino acid sequence:GYDFTHYGMN (SEQ ID NO:126) and a CDRH3 comprising the amino acid sequence:YPYYYGTSHWYFDV (SEQ ID

NO:127). These hypervariable regions and CDRH2 are generally provided in a human framework region, *e.g.*, resulting in a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:116. Such heavy chain variable domain sequences are optionally combined with a light chain variable domain comprising the amino acid sequence of SEQ ID NO:124, and preferably the light chain variable domain amino acid sequence of SEQ ID NO:115.

Various forms of the antibody are contemplated herein. For example, the anti-VEGF antibody may be a full length antibody (*e.g.* having an intact human Fc region) or an antibody fragment (*e.g.* a Fab, Fab' or $F(ab')_2$). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of VEGF protein comprising exposing a sample suspected of containing the VEGF protein to the anti-VEGF antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the VEGF protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.* from the host cell culture medium). The invention also provides a composition comprising the anti-VEGF antibody and a pharmaceutically acceptable carrier or diluent. The composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a tumor or retinal disorder, comprising administering a therapeutically effective amount of the anti-VEGF antibody to the mammal.

Brief Description of the Drawings

Figs. 1A and 1B depict the amino acid sequences of variable heavy domain (SEQ ID NO:9) and light domain (SEQ ID NO:10) of muMAbVEGF A.4.6.1, variable heavy domain (SEQ ID NO:7)

and light domain (SEQ ID NO:8) of humanized F(ab) (F(ab)-12) and human consensus frameworks (hum III for heavy subgroup III (SEQ ID NO:11); humκ1 for light κ subgroup I (SEQ ID NO:12)). Fig. 1A aligns variable heavy domain sequences and Fig. 1B aligns variable light domain sequences. Asterisks indicate differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. Complementarity Determining Regions (CDRs) are underlined.

Fig. 2 is a ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in brown with CDRs in tan. The sidechain of residue L46 is shown in yellow. VH domain is shown in purple with CDRs in pink. Sidechains of VH residues changed from human to murine are shown in yellow.

Fig. 3 depicts inhibition of VEGF-induced mitogenesis by humanized anti-VEGF F(ab)-12 from Example 1. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6 X 10^3 cells/well in six well plates, as described in Example 1. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1; F(ab)-12) was added at the indicated concentrations. After 2-3 hours, rhVEGF165 was added at the final concentration of 3 ng/ml. After five or six days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

Fig. 4 shows inhibition of tumor growth *in vivo* by humanized anti-VEGF F(ab)-12 from Example 1. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2 x 10^6 per mouse. Starting 24 hours after tumor cell inoculation, animals were injected with a control MAb, muMAb VEGF A4.6.1 or rhuVEGF MAb (IgG1; F(ab)-12) twice weekly, intra peritoneally. The dose of the control Mab was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated (n = 10). Four weeks after tumor cell injection, animals were euthanized and tumors were removed and weighed. *: significant difference when compared to the control group by ANOVA ($p < 0.05$).

Figs. 5A and 5B show the acid sequences of the light and heavy variable domains respectively of murine antibody A4.6.1 (SEQ ID NO:10 for the VL and SEQ ID NO:9 for the VH) and humanized A4.6.1 variants hu2.0 (SEQ ID NO:13 for the VL and SEQ ID NO:14 for the VH) and hu2.10 (SEQ ID NO:15 for the VL and SEQ ID NO:16 for the VH) from Example 2. Sequence numbering is according to Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed.

Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain κ subgroup I consensus framework (SEQ ID NO:12) and heavy chain subgroup III consensus framework (SEQ ID NO:11). hu2.10 was the consensus humanized clone obtained from phage sorting experiments described herein.

Fig. 6 depicts framework residues targeted for randomization in Example 2.

Fig. 7 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in *supE E. coli)*, then the C-terminal region of the gene III protein (residues 249-406). Transformation into F^+ *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequence encoded thereby (SEQ ID NO:100).

Figs. 9A and 9B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants in Example 3, compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. Residues are numbered sequentially in the VL and VH domains, as opposed to the Kabat numbering scheme. The template molecule, MB1.6 (SEQ ID NO's 101 and 102 for light and heavy variable domains, respectively) is shown, along with variants: H2305.6 (SEQ ID NO's 103 and 104 for light and heavy variable domains, respectively), Y0101 (SEQ ID NO's 105 and 106 for light and heavy variable domains, respectively), and Y0192 (SEQ ID NO's 107 and 108 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Figs. 10A and 10B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants from Example 3 compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains,

respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. The variants are designated Y0243-1 (SEQ ID NO's 109 and 110 for light and heavy variable domains, respectively), Y0238-3 (SEQ ID NO's 111 and 112 for light and heavy variable domains, respectively), Y0313-1 (SEQ ID NO's 113 and 114 for light and heavy variable domains, respectively), and Y0317 (SEQ ID NO's 115 and 116 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Fig. 11 depicts the results of the HuVEC activity assay in Example 3 for variants Y0238-3, Y0192 and Y0313-1 as well as full length F(ab)-12 from Example 1.

Fig. 12 depicts inhibition of VEGF-induced mitogenesis by full length F(ab)-12 from Example 1 (rhuMAb VEGF), a Fab fragment of F(ab)-12 from Example 1 (rhuFab VEGF), and a Fab fragment of affinity matured variant Y0317 from Example 3 (rhuFab VEGF (affinity matured)).

Detailed Description of the Preferred Embodiments

I. Definitions

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an

extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the

constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.,* IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full l ngth monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof . Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci.*

USA 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences (*e.g.* as in SEQ ID NO:7 or 8), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for

the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Mol. Cell. Biol.* 5(12):3610-3616(1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering* 3(6):547-553(1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the

function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containingprodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed

partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by

ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. M des for Carrying out th Inv ntion

The examples hereinbelow describe the production of humanized and variant anti-VEGF antibodies with desirable properties from a therapeutic perspective including: (a) strong binding affinity for the VEGF antigen; (b) an ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro;* and (c) the ability to inhibit VEGF-induced angiogenesis *in vivo.*

Antibody affinities may be determined as described in the examples hereinbelow. Preferred humanized or variant antibodies are those which bind human VEGF with a K_d value of no more than about 1 x 10^{-7}M; preferably no more than about 1 x 10^{-8}M; and most preferably no more than about 5 x 10^{-9}M.

Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select humanized or variant antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits endothelial cell growth in response to VEGF. In one embodiment, the antibody may be able to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). Preferably, the antibody has an effective dose 50 (ED50) value of no more than about 5nM, preferably no more than about 1nM, and most preferably no more than about 0.5nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", *i.e.*, at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by 50%. A preferred "endothelial cell growth assay" involves culturing bovine adrenal cortex-derived capillary endothelial cells in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described in Example 1 below. These endothelial cells are seeded at a density of 6 x 10^3 cells per well, in 6-well plates in growth medium. Either parent anti-VEGF antibody (control), humanized or variant anti-VEGF antibody is then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified VEGF was added to a final concentration of 3 ng/ml. For specificity control, each antibody may be added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells are dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Data may be analyzed by a four-parameter curve fitting program (KaleidaGraph).

The preferred humanized or variant anti-VEGF antibody may also be one which has *in vivo* tumor suppression activity. For example, the antibody may suppress the growth of human A673 rhabdomyosarcoma cells or breast carcinoma MDA-MB-435 cells in nude mice. For *in vivo* tumor studies, human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) or MDA-MB-435 cells (available from the ATCC) are cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics as described in Example 1 below. Female BALB/c nude mice, 6-10 weeks old, are injected subcutaneously with 2 x 10^5 tumor cells in the dorsal area in a volume of 200 µl. Animals are then treated with the humanized or variant antibody and a control antibody with no activity in this assay. The humanized or variant anti-VEGF MAb is administered at a dose of 0.5 and/or 5 mg/kg. Each MAb is administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Tumor size is determined at weekly intervals. Four weeks after tumor cell inoculation, animals are euthanized and the tumors are removed and weighed. Statistical analysis may be performed by ANOVA. Preferably, the antibody in this "*in vivo* tumor assay" inhibits about 50-100%, preferably about 70-100% and most preferably about 80-100% human A673 tumor cell growth at a dose of 5mg/kg.

In the preferred embodiment, the humanized or variant antibody fails to elicit an immunogenic response upon administration of a therapeutically effective amount of the antibody to a human patient. If an immunogenic response is elicited, preferably the response will be such that the antibody still provides a therapeutic benefit to the patient treated therewith.

The humanized or variant antibody is also preferably one which is able to inhibit VEGF-induced angiogenesis in a human, *e.g.* to inhibit human tumor growth and/or inhibit intraocular angiogenesis in retinal disorders.

Preferred antibodies bind the "epitope A4.6.1" as herein defined. To screen for antibodies which bind to the epitope on human VEGF bound by an antibody of interest (*e.g.,* those which block binding of the A4.6.1 antibody to human VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

The antibodies of the preferred embodiment herein have a heavy chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein "FR1-4" represent the four framework regions and "CDRH1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. Many human antibody framework region sequences are compiled in Kabat *et al.*, *supra*, for example. In one preferred embodiment, the variable heavy FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human heavy chains subgroup III (*e.g.* as in SEQ ID NO:11).

The human variable heavy FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding nonhuman residue (by "corresponding nonhuman residue" is meant the nonhuman residue with the same Kabat positional numbering as the human residue of interest when the human and nonhuman sequences are aligned), but replacement with the nonhuman residue is not necessary. For example, a replacement FR residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable heavy FR residues which may be substituted include any one or more of FR residue numbers: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H, 94H (Kabat residue numbering employed here). Preferably at least two, or at least three, or at least four of these residues are substituted. A particularly preferred combination of FR substitutions is: 49H, 69H, 71H, 73H, 76H, 78H, and 94H.

With respect to the heavy chain hypervariable regions, these preferably have amino acid sequences as follows:

CDRH1

$GYX_1X_2X_3X_4YGX_5N$ (SEQ ID NO:117), wherein X_1 is D, T or E, but preferably is D or T; X_2 is F, W, or Y, but preferably is F; X_3 is T, Q, G or S, but preferably is T; X_4 is H or N; and X_5 is M or I, but preferably is M.

CDRH2

WINTX$_1$TGEPTYAADFKR (SEQ ID NO:118), wherein X_1 is Y or W, but preferably is Y.

CDRH3

YPX$_1$YX$_2$X$_3$X$_4$X$_5$HWYFDV (SEQ ID NO:119), wherein X_1 is H or Y; X_2 is Y, R, K, I, T, E, or W, but preferably is Y; X_3 is G, N, A, D, Q, E, T, K, or S, but preferably is G; X_4 is S, T, K, Q, N, R, A, E, or G, but preferably is S or T; and X_5 is S or G, but preferably is S.

The heavy chain variable domain optionally comprises what has been designated "CDR7" herein within (*i.e.* forming part of) FR3 (see Figs. 9B and 10B), wherein CDR7 may have the following amino acid sequence:

CDR7

$X_1SX_2DX_3X_4X_5X_6TX_7$ (SEQ ID NO:120), wherein X_1 is F, I , V, L, or A, but preferably is F; X_2 is A, L, V, or I, but preferably is L; X_3 is T, V or K, but preferably is T; X_4 is S or W, but preferably is S; X_5 is S, or K, but preferably is K; X_6 is N, or S, but preferably is S; and X_7 is V, A, L or I, but preferably is A.

The antibodies of the preferred embodiment herein have a light chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein "FR1-4" represent the four framework regions and "CDRL1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. In one preferred embodiment, the variable light FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human kappa light chains subgroup I (*e.g.* as in SEQ ID NO:12).

The human variable light FR sequence preferably has substitutions therein, *e.g.* wherein the

human FR residue is replaced by a corresponding mouse residue, but replacement with the nonhuman residue is not necessary. For example, a replacement residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable light FR residues which may be substituted include any one or more of FR residue numbers: 4L, 46L and 71L (Kabat residue numbering employed here). Preferably only 46L is substituted. In another embodiment, both 4L and 46L are substituted.

With respect to the CDRs, these preferably have amino acid sequences as follows:

CDRL1

$X_1AX_2X_3X_4X_5SNYLN$ (SEQ ID NO:121), wherein X_1 is R or S, but preferably is S; X_2 is S or N, but preferably is S; X_3 is Q or E, but preferably is Q; X_4 is Q or D, but preferably is D; and X_5 is I or L, but preferably is I.

CDRL2

FTSSLHS (SEQ ID NO:122).

CDRL3

$QQYSX_1X_2PWT$ (SEQ ID NO:123), wherein X_1 is T, A or N, but preferably is T; and X_2 is V or T, but preferably is V.

Preferred humanized anti-VEGF antibodies are those having the heavy and/or light variable domain sequences of F(ab)-12 in Example 1 and variants thereof such as affinity matured forms including variants Y0317, Y0313-1 and Y0238-3 in Example 3, with Y0317 being the preferred variant. Methods for generating humanized anti-VEGF antibodies of interest herein are elaborated in more detail below.

A. Antibody Preparation

Methods for humanizing nonhuman VEGF antibodies and generating variants of anti-VEGF antibodies are described in the examples below. In order to humanize an anti-VEGF antibody, the nonhuman antibody starting material is prepared. Where a variant is to be generated, the parent antibody is prepared. Exemplary techniques for generating such nonhuman antibody

starting material and parent antibodies will be described in the following sections.

(i) Antigen preparation

The VEGF antigen to be used for production of antibodies may be, *e.g.*, intact VEGF or a fragment of VEGF (*e.g.* a VEGF fragment comprising "epitope A4.6.1"). Other forms of VEGF useful for generating antibodies will be apparent to those skilled in the art. The VEGF antigen used to generate the antibody, is preferably human VEGF, *e.g.* as described in Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991).

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard

methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) Humanization and amino acid sequence variants

Examples 1-2 below describe procedures for humanization of an anti-VEGF antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of these humanized antibodies, particularly where these improve the binding affinity or other biological properties of the humanized antibody. Example 3 describes methodologies for generating amino acid sequence variants of an anti-VEGF antibody with enhanced affinity relative to the parent antibody.

Amino acid sequence variants of the anti-VEGF antibody are prepared by introducing appropriate nucleotide changes into the anti-VEGF antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-VEGF antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-VEGF antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-VEGF antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with VEGF antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-VEGF antibody variants are screened for the desired activity. Alanine scanning mutagenesis is described in Example 3.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-VEGF antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-VEGF antibody molecule include the fusion to the N- or C-terminus of the anti-VEGF antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-VEGF antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Pref rred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-VEGF antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display (see Example 3 herein). Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis (see Example 3) can be performed

to identified hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-VEGF antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-VEGF antibody.

(v) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and US Patents 5,591,669, 5,589,369 and 5,545,807. Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); and US Patents 5,565,332 and 5,573,905). As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275)

(vi) Antibody fragments

In certain embodiments, the humanized or variant anti-VEGF antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al., Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vii) Multispecific antibodies

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized or variant anti-VEGF antibodies having binding specificities for at least two different

epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the VEGF protein. Alternatively, an anti-VEGF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the VEGF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express VEGF. These antibodies possess an VEGF-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to

thionitrobenzoate(TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from *E. coli* can be chemically coupled *in vitro* to form bispecific antibodies. Shalaby *et al., J. Exp. Med.* 175:217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al., J. Immunol.* 147:60 (1991).

(viii) Other modifications

Other modifications of the humanized or variant anti-VEGF antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain

disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al., Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al., Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.,* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.,* a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-VEGF antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al., Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an

exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionuclide).

The anti-VEGF antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al., J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al., J. National Cancer Inst.*81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases,

such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-VEGF antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.*, by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain

of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence: PKNSSMISNTP (SEQ ID NO:17), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:18), HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s): HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21) and the sequence: PKNSSMISNTP (SEQ ID NO:17).

Covalent modifications of the humanized or variant anti-VEGF antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Exemplary covalent modifications of polypeptides are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized or variant anti-VEGF antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. In another embodiment, the antibody may be produced by homologous recombination, *e.g.* as described in US Patent 5,204,244, specifically incorporated herein by reference. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components

generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, *e.g.*, as described in US Patent 5,534,615 issued July 9, 1996 and specifically incorporated herein by reference.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-VEGF antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated anti-VEGF antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains

for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-VEGF antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the anti-VEGF antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and

phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzeneallow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin

(J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*,from about 0-0.25M salt).

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other (see Section F below). Such molecules are suitably present in

combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

D. Non-therapeutic Uses for the Antibody

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing

the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish

peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-VEGF antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc.

1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

E. Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided

as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

F. Therapeutic Uses for the Antibody

For therapeutic applications, the anti-VEGF antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies also are suitably administered by intra tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodys of the present invention are expected to be especially useful in reducing the severity of AMD.

Depending on the type and severity of the disease, about 1 μg/kg to about 50 mg/kg (*e.g.*, 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 μg/kg to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak *et al.*, PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics,

pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy. The antibody and one or more other anti-VEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of anti-VEGF antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF antibody. Treatment with anti-VEGF antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

G. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials,

syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

EXAMPLE 1

This example describes the production of humanized anti-VEGF antibodies with desirable properties from a therapeutic standpoint.

MATERIALS AND METHODS

Cloning of Murine A4.6.1 MAb and Construction of Mouse-Human Chimeric Fab: The murine anti-VEGF mAb A4.6.1 has been previously described by Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993). Total RNA was isolated from hybridoma cells producing the anti-VEGF Mab A.4.6.1 using RNAsol (TEL-TEST) and reverse-transcribedto cDNA using Oligo-dT primer and the SuperScript II system (GIBCO BRL, Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the N-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup kV and heavy chain subgroup II (Kabat *et al. Sequences of Proteins of Immunological Interest.* 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). After polymerase chain reaction (PCR) amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned respectively into the pEMX1 vector containing the human CL and CH1 domains (Werther *et al. J. Immunol.* 157:4986-4995 (1996)), thus generating a

mouse-human chimera. This chimeric F(ab) consisted of the entire murine A4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113 and the entire murine A4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to that of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

Computer Graphics Models of Murine and Humanized F(ab): Sequences of the VL and VH domains (Figs.1A and 1B) were used to construct a computer graphics model of the murine A4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (Carter *et al. Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992) and Eigenbrot *et al. J.Mol. Biol.* 229:969-995 (1993)).

Construction of Humanized F(ab)s: The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *E. coli* has been described previously (Werther *et al.*, *supra*). Briefly, the plasmid contains a DNA fragment encoding a consensus human k subgroup I light chain (VLkI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (Carter *et al.*, *supra*).

To construct the first F(ab) variant of humanized A4.6.1, F(ab)-1, site-directed mutagenesis (Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488-492 (1985)) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs according to Kabat *et al.*, *supra*, were changed to the murine A4.6.1 sequence . F(ab)-1 therefore consisted of a complete human framework (VL k subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml Luria broth-100 mg/ml carbenicillin for

5-8 h at 37°C. The 5 ml culture was added to 500 ml AP5-50 µg/ml carbenicillin and allcwed to grow for 20 h in a 4 L baffled shake flask at 30°C. AP5 media consists of: 1.5 g glucose, 11.0 g Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO4 (anhydrous), 1.07 g NH4Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 mm Sealkeen filter. Cells were harvested by centrifugation in a 1 L centrifuge bottle at 3000x*g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM Tris-1 mM EDTA-20% sucrose, pH 8.0. 250 ml of 0.1 M benzamidine (Sigma, St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000x*g* for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (0.5 ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

Construction and Expression of Chimeric and Humanized IgG: For generation of human IgG1 variants of chimeric (chIgG1) and humanized (huIgG1) A4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12, Table 2) domains were subcloned into separate, previously described, pRK vectors (Eaton *et al.*, *Biochemistry* 25:8343-8347 (1986)). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were co-transfected into human 293 cells (Graham *et al.*, *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media was changed to serum-free and harvested daily for up to five days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore, Bedford, MA) and stored at 4°C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (Lucas *et al.*, *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line developed by L. Chasin (Columbia University), via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover,DM, Hames, BD. *DNA Cloning 4. Mammalian systems.* Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening. One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after this step was ~99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was < 0.10 eu/mg.

***F(ab) and IgG Quantitation*:** For quantitating F(ab) molecules, ELISA plates were coated with 2 µg/ml goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% bovine serum albumin (blocking buffer) at room temperature for 1 h. Standards (0.78 - 50 ng/ml human F(ab)) were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% bovine serum albumin-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a

Vmax plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program. Data points which fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples. The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

VEGF Binding Assay: For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 µg/ml rabbit $F(ab')_2$ to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (Park *et al., J. Biol. Chem.* 269:25646-25645 (1994)) in blocking buffer were incubated on the plate for 1 h. Standards (6.9 - 440 ng/ml chimeric F(ab)) and two-fold serial of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma, St. Louis, MO) followed by 3,3',5,5'-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

BIAcore™ Biosensor Assay: VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore™ biosensor (Karlsson *et al. Methods: A Comparison to Methods in Enzymology* 6:97-108 (1994)). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) (35 µl of 2 µM F(ab) at a flow rate of 20 µl/min) and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0 - 4500 sec were used for off-rate kinetic analysis. The dissociation rate constant (k_{off}) was obtained from the slope of the plot of ln(R0/R) versus time, where R0 is the signal at t=0 and R is the signal at each time point.

On-rate kinetics were measured using two-fold serial dilutions of F(ab) (0.0625 - 2 mM). The slope, K_s, was obtained from the plot of ln(-dR/dt) versus time for each F(ab) concentration using the BIAcore™ kinetics evaluation software as described in the Pharmacia Biosensor manual. R is the signal at time t. Data between 80 and 168, 148, 128, 114, 102, and 92 sec were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant (k_{on}) was obtained from the slope of the plot of K_s versus F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5 µl of 50 mM HCl at a flow rate of 20 µl/min to regenerate the chip.

Endothelial Cell Growth Assay: Bovine adrenal cortex-derived capillary endothelial cells were cultured in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as previously described (Leung *et al. Science* 246:1306-1309 (1989)). For mitogenic assays, endothelial cells were seeded at a density of 6 x 10^3 cells per well, in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF was then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified *E.coli*-expressed rhVEGF165 was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells were dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

In Vivo Tumor Studies: Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female BALB/c nude mice, 6-10 weeks old, were injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF or a control MAb directed against the gp120 protein (Kim *et al. Nature* 362:841-844 (1993)). Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks

after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

RESULTS

Humanization: The consensus sequence for the human heavy chain subgroup III and the light chain subgroup k I were used as the framework for the humanization (Kabat *et al.*, *supra*) (Figs. 1A and 1B). This framework has been successfully used in the humanization of other murine antibodies (Werther *et al., supra*; Carter *et al., supra*; Presta *et al. J. Immunol.* 151:2623-2632 (1993); and Eigenbrot *et al. Proteins* 18:49-62 (1994)). CDR-H1 included residues H26-H35. The other CDRs were according to Kabat *et al., supra.* All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli.* Typical yields from 500 ml shake flasks were 0.1-0.4 mg F(ab).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in human) was changed to the murine Gly. In addition, F(ab)s which consisted of the chimeric heavy chain/F(ab)-1 light chain (F(ab)-2) and F(ab)-1 heavy chain/chimeric light chain (F(ab)-3) were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 2). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered in order to increase binding.

Table 2: Binding of Humanized Anti-VEGF F(ab) Variants to VEGF[a]

Variant	Template	Changes[b]	Purpose	EC50 F(ab)-X		
				EC50 chimeric F(ab)[c]		
				Mean	S.D.	N
chim-F(ab)	Chimeric F(ab)			1.0		
F(ab)-1	Human FR		Straight CDR swap AlaH49Gly	>1350		2
F(ab)-2			Chimera Light Chain F(ab)-1 Heavy Chain	>145		3
F(ab)-3			F(ab)-1 Light Chain Chimera Heavy Chain	2.6	0.1	2
F(ab)-4	F(ab)-1	ArgH71Leu AsnH73Thr	CDR-H2 conformation Framework	>295		3
F(ab)-5	F(ab)-4	LeuL46Val	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78Ala	CDR-H1 conformation	36.4	4.2	2
F(ab)-7	F(ab)-5	IleH69Phe	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69Phe LeuH78Ala	CDR-H2 conformation CDR-H1 conformation	9.6	0.9	4
F(ab)-9	F(ab)-8	GlyH49Ala	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76Ser	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75Ala	Framework	3.3	0.4	2
F(ab)-12	F(ab)-10	ArgH94Lys	CDR-H3 conformation	1.6	0.6	4

[a]Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, *supra*).

[b]Murine residues are underlined; residue numbers are according to Kabat *et al.*, *supra*.

[c]Mean and standard deviation are the average of the ratios calculated for each of the independent assays; the EC50 for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

Changing human residues H71 and H73 to their murine counterparts in F(ab)-4 improved binding by 4-fold (Table 2). Inspection of the models of the murine and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 (F(ab)-5), the binding affinity increased by almost 4-fold (Table 2). Three other buried framework residues were evaluated based on the molecular models: H49, H69 and H78. Position H69 may affect the conformation of CDR-H2 while position H78 may affect the conformation of CDR-H1 (Figure 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case (F(ab)-6 and F(ab)-7, Table 2). When both were simultaneously changed, the improvement in binding was 8-fold (F(ab)-8, Table 2). Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala the binding was reduced by 15-fold (F(ab)-9, Table 2).

In F(ab)-10 and F(ab)-11 two residues in framework loop 3, FR-3, were changed to their murine counterparts: AsnH76 to murine Ser (F(ab)-10) and LysH75 to murine Ala (F(ab)-11). Both effected a relatively small improvement in binding (Table 2). Finally, at position H94 human and murine sequences most often have an Arg (Kabat *et al.*, *supra*). In F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig.1A) and this resulted in binding which was less than 2-fold from the chimeric F(ab) (Table 2). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore™ system (Pharmacia). Using this technique the K_d of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower k_{on} and faster k_{off} (Table 3).

Table 3: Binding of Anti-VEGF F(ab) Variants to VEGF Using the BIAcore™ System[a]

Variant	Amount of (Fab) bound (RU)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
chim-F(ab)[b]	4250	$5.9x10^{-5}$	$6.5x10^{4}$	0.91
F(ab)-12	3740	$6.3x10^{-5}$	$3.5x10^{4}$	1.8

[a] The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore™ system when 2 µg F(ab) was injected onto a chip containing 2480 RU immobilized VEGF. Off-rate kinetics (k_{off}) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics (k_{on}) were measured using two-fold serial dilutions of F(ab). K_d, the equilibrium dissociation constant, was calculated as k_{off}/k_{on}.
b chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

Full length mAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human k light chain and human IgG1 heavy chain. The full length 12-IgG1 (F(ab)-12 fused to human IgG1) exhibited binding which was 1.7-fold weaker than the chimeric IgG1 (Table 4). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original murine mAb A4.6.1 (Table 4).

Table 4: Binding of Anti-VEGF IgG Variants to VEGF[a]

IgG1/chIgG1[b]			
Variant	**Mean**	**S.D.**	**N**
chIgG1	1.0		2
murIgG1[c]	0.759	0.001	2
12-IgG1[d]	1.71	0.03	2

[a] Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, (1994), *supra*).
[b] chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC50 for chIgG1 was 0.113 ± 0.013 µg/ml (0.75 nM).
[c] murIgG1 is muMAbVEGF A461 purified from ascites.
[d] 12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

Biological Studies: rhuMAb VEGF and muMAb VEGF A.4.6.1. were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). As illustrated in Figure 3, the two MAbs were essentially equivalent, both in potency and efficacy. The ED50 values were respectively 50 ± 5 ng/ml and 48 ± 8 ng/ml (~0.3 nM). In both cases 90% inhibition was achieved at the concentration of 500 ng/ml (~3 nM). Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any

effect on basal or bFGF-stimulated proliferation of capillary endothelial cells, confirming that the inhibition is specific for VEGF.

To determine whether such equivalency applies also to an *in vivo* system, the two antibodies were compared for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res* 56:4032-4039 (1996)). As shown in Figure 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements four weeks after cell inoculation. The decreases in tumor weight compared to the control group were respectively 85% and 93% at each dose in the animals treated with muMAb VEGF A.4.6.1. versus 90% and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435.

EXAMPLE 2

In this example, the murine anti-VEGF antibody A4.6.1 discussed above was humanized by randomizing a small set of framework residues and by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage in order to identify high affinity framework sequences via affinity-based selection.

MATERIALS AND METHODS

Construction of Anti-VEGF Phagemid Vector, pMB4-19: The murine anti-VEGF mAb A4.6.1 is discussed above in Example 1. The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 (Carter *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:4285-4289 (1992)) which codes for a human $V_L\kappa I$-$C\kappa_1$ light chain and human $V_H III$-$C_H 1\gamma_1$ heavy chain Fd fragment The transplanted A4.6.1 CDR sequences were chosen according to the sequence definition of Kabat *et al.*, *supra*, except for CDR-H1 which included residues 26-35. The Fab encoding sequence was subcloned into the phagemid vector phGHamg3 (Bass *et al. Proteins* 8:309-314 (1990) and Lowman *et al. Biochemistry* 30:10832-10838 (1991)). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion

of the M13 gene III coat protein. pMB4-19 is similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments (Garrard *et al. Biotechnology* 9:1373-1377 (1991)). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

Expression and Purification of Humanized A4.6.1 Fab Fragment: *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB4-19, or variants thereof. Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium (Chang *et al. Gene* 55:189-196 (1987)) containing 20 µg/mL carbenicillin and incubated for 26 hr at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 h. Cell pellets were then resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 min and centrifuged at 10,000 x g for 15 min. The supernatant was applied to a 1 mL streptococcal protein G-sepharose column (Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M Tris-HCl, pH 8.0. Fab preparations were buffer-exchanged into PBS and concentrated using Centricon-30 concentrators (Amicon). Typical yields of Fab were ~1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

Construction of the Anti-VEGF Fab Phagemid Library: The humanized A4.6.1 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel *et al. Methods Enzymol.* 204:125-139 (1991)). A derivative of pMB4-19 containing TAA stop triplets at V_H codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat *et al.*, *supra*). This modification was to prevent subsequent background contamination by wild type sequences. The codons targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

In order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60

and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol of 5' phosphorylated oligonucleotide 503-1 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:22)) or 503-2 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:23)) were combined with 1.5 nmol of 503-3 (5'-AGC CTG CGC GCT GAG GAC ACT GCC GTC TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG-3' (SEQ ID NO:24)) (randomized codons underlined; N=A/G/T/C; W=A/T; B=G/T/C; D=G/A/T; R=A/G; Y=C/T). Then, 1.5 nmol of template oligonucleotide (5'-CTC AGC GCG CAG GCT GTT CAT CTG CAG GTA-3' (SEQ ID NO:25)), with complementary sequence to the 5' ends of 503-1/2 and the 3' end of 503-3, was added to hybridize to each end of the ligation junction. *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95° C 1.25 min; 50° C for 5 min) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10mM Tris-HCl, 1mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons (V_L 4, 71; V_H 24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was effected in two steps. Firstly, V_L randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides 5'-GCT GAT ATC CAG TTG ACC CAG TCC CCG-3' (SEQ ID NO:26) 5'-and TCT GGG ACG GAT TAC ACT CTG ACC ATC-3' (SEQ ID NO:27). Deoxyuridine-containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (Table 5).

Oligonucleotides 504-1, a mixture of two 126-mer oligonucleotides (see above), and 5'-CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG-3' (SEQ ID NO:28) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 Blue cells (Stratagene) and combined. The total number of

independent transformants was estimated at >1.2 x 10^8, approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter *et al.*, *Ann. Rev. Immunol.* 12,433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard *et al.*, *Biotechn*, 9,1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 7). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8:309 (1990) and Lowman *et al.*, *Biochemistry* 30:10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37° C in 25 mL 2YT medium containing 50µg/mL carbenicillin and approximately 10^{10} M13KO7 helper phage (Vieira & Messing *Methods Enzymol.* 153:3-11 (1987)). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 µL PBS (~10^{14} phagemid/mL)

Selection of Humanized A4.6.1 Fab Variants: Purified $VEGF_{121}$ (100 µL at 10µg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well, in addition to an uncoated well, were blocked with 6% skim milk for 1 h and washed with PBS containing 0.05% TWEEN 20™ (detergent). Then, 10 µL of phagemid stock, diluted to 100 µL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05%TWEEN 20™, was added to each well. After 2 hours the wells were washed and the bound phage eluted with 100 µL of 0.1 M glycine (pH 2.0), and neutralized with 25 µL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467 (1977)).

Determinati n of VEGF Binding Affinities: Association (k_{on}) and dissociation (k_{off}) rate constants for binding of humanized A4.6.1 Fab variants to $VEGF_{121}$ were measured by surface plasmon resonance (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)) on a Pharmacia BIAcore instrument. $VEGF_{121}$ was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN 20™ over the chip at a flow rate of 20 µL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 µL of 50 mM aqueous HCl at 3 µL/min. Binding profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

RESULTS

Construction of Humanized A4.6.1: An initial humanized A4.6.1 Fab fragment was constructed (hu2.0, Figs. 5A and 5B), in which the CDRs from A4.6.1 were grafted onto a human V_LKI-V_HIII framework. All other residues in hu2.0 were maintained as the human sequence. Binding of this variant to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants, the K_D for binding of hu2.0 was estimated at >7 µM. This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact V_L and V_H domains from murine A4.6.1 and human constant domains. Thus binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

Design of Antibody Library: The group of framework changes to the human framework sequence herein is shown in Table 5 and Fig. 6.

Table 5: Key Framework Residues Important for Antigen Binding and Targeted for Randomization

Framework residue		Human V_KI, V_HIII consensus residue	Murine A4.6.1 residue	Randomization[a]
V_L:	4	Met	Met	Met, Leu
	71	Phe	Tyr	Phe, Tyr
V_H:	24	Ala	Ala	Ala, Val, Thr
	37	Val	Val	Val, Ile

	67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
	69	Ile	Phe	Ile, Phe
	71	Arg	Leu	Arg[b], Leu[b]
	73	Asp	Thr	Asp[b], Thr[b]
	75	Lys	Ala	Lys[b], Ala[b]
	76	Asn	Ser	Asn[b], Ser[b]
	78	Leu	Ala	Leu, Ala, Val, Phe
	93	Ala	Ala	Ala, Val, Leu, Ser, Thr
	94	Arg	Lys	Arg, Lys

[a]Amino acid diversity in phagemid library
[b]V_H71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) V_HIII tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V_L and V_H sequences. Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V_L framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126-base oligonucleotides were preassembled from smaller synthetic fragments. This made possible randomization of V_H codons 67, 69, 71, 73, 75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

Selection of Tight Binding Humanized A4.6.1 Fab's: Variants from the humanized A4.6.1 Fab phagemid library were selected based on binding to VEGF. Enrichment of functional

phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results, summarized in Table 6, revealed strong consensus amongst the clones selected. Ten out of the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 (V_L 71; V_H 37, 71, 73, 75, 76, 78 and 94). Interestingly, residues V_H 37 (Ile) and 78 (Val) were selected neither as the human V μI or murine A4.6.1 sequence. This result suggests that some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

Table 6: Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library

Variant	**Residue substitutions**												
	V_L		V_H										
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	F	A	V	F	I	R	N	K	N	L	A	R
Phage-selected clones:													
hu2.1(2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.2(2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.6(1)	L	-	-	I	T	-	L	T	A	S	V	-	K
hu2.7(1)	L	-	-	I	-	-	-	-	-	-	V	-	K
hu2.10(10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human V_LKI-V_HIII framework sequence (*i.e.* as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human V_HIII consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA assay (Cunningham *et al. EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

Expression and Binding Affinity of Humanized A4.6.1 Variants: Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes (K_D >7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

Table 7: VEGF Binding Affinity of Humanized A4.6.1 Fab Variants

Variant	**k_{on} $M^{-1}s^{-1}/10^4$**	**k_{off} 10^4s^{-1}**	**K_D nM**	**$\frac{K_D(A4.6.1)}{K_D(mut)}$**
A4.6.1 chimera	5.4	0.85	1.6	›4000
hu2.0	ND	ND	›7000**	
Phage selected clones:				
hu2.1	0.70	18	260	170

hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

*hu2.10V = hu2.10 with mutation V_L Leu->Val
Estimated errors in the Biacore binding measurements are +/-25%.
**Too weak to measure; estimate of lower bound

Additional Improvement of Humanized Variant hu2.1: Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human V_LKI-V_HIII framework (Figs. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V_L 46 sits at the V_L-V_H interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always leucine in most V_LK frameworks (Kabat *et al.*, *supra*), but is valine in A4.6.1. Accordingly, a Leu -> Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding, demonstrating the importance of valine at position V_L 46 in antibody A4.6.1. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

EXAMPLE 3

In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

C nstructi n of Humaniz d Antib dy pY0101: Phage-displayed antibody vector phMB4-19-1.6 (see Figs. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity for VEGF with oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

Table 8: Oligos for Directed Mutations

Oligo Number	Region	Substitution/ Comments	Sequence
HL-242	VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
HL-243	VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
HL-245	VH	CDR-7	5'-CGTCGTTTCACTTTTTCTGCAGACACCT CCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
HL-246	VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
HL-254	VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
HL-256	VH	I37V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
HL-257	VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTTCTTTAGACACCT CCAAAAGCACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

The resulting variant was termed Y0101 (Figs. 9A and 9B).

Construction of the First Generation of Antibody-Phage Libraries: To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis

with oligonucleotides using the degenerate NNS codon (where N is an equal mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement (Figs. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.

Table 9: Template Oligos for Mutagenesis

Oligo Number	Region Comments	Sequence
HL-248	VL1	5'-GGGTCACCATCACCTGCTAAGCATAATAATAATAAAGCAACT ATTTAAACTGG-3' (SEQ ID NO:36)
HL-249	VL1	5'-GCGCAAGTCAGGATATTTAATAATAATAATAATGGTATCAAC AGAAACCAGG-3' (SEQ ID NO:37)
HL-250	VH3	5'-GTCTATTACTGTGCAAAGTAATAACACTAATAAGGGAGCAG CCACTGG-3' (SEQ ID NO:38)
HL-251	VH3	5'-GGTACCCCCACTATTATTAATAATAATAATGGTATTTCGACG TCTGGGG-3' (SEQ ID NO:39)
HL-252	VH3	5'-CACTATTATGGGAGCAGCCACTAATAATAATAAGTCTGGGT CAAGGAACCCTG-3' (SEQ ID NO:40)
HL-263	VH1	5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATAATAAGGTATG AACTGGGTCCG-3' (SEQ ID NO:41)
HL-264	VH2	5'-GAATGGGTTGGATGGATTAACTAATAATAAGGTTAACCGAC CTATGCTGCGG-3' (SEQ ID NO:42)
YC-80	VH3	5'-CTGTGCAAAGTACCCGTAATATTAATAATAATAACACTGGTA TTTCGAC-3' (SEQ ID NO:43)
YC-100	CDR7	5'-CGTTTCACTTTTTCTTAAGACTAATCCAAATAAACAGCATAC CTGCAG-3' (SEQ ID NO:44)
YC-102	VH2	5'-GAATGGGTTGGATGGATTTAATAATAATAAGGTGAACCGAC CTATG-3' (SEQ ID NO:45)

Tabl 10: Rand m Oligos for Library Constructi n

Oligo Number	Region Comment	Sequence
HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNSNNSNNSNNSAGC AACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNSNNSNNSNNSTGGTATCAACA GAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSGGGAGCAGC CACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-GGTACCCCCACTATTATNNSNNSNNSNNSTGGTATTTCGACGT CTGGGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNSNNSNNSGTCTGGGGT CAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNNSNNSTTCNNSNNSNNSGGTATGA ACTGGGTCCG-3' (SEQ ID NO:51)
HL-266	VH2	5'-GAATGGGTTGGATGGATTAACNNSNNSNNSGGTNNSCCGACC TATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNSNNSNNSCACTGGTAT TTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTTCTNNSGACNNSTCCAAANNSACAGCATACCT GCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNSNNSNNSGGTGAACCGACC TATG-3' (SEQ ID NO:55)

The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from 2 x 10^7 to 1.5 x 10^8, was estimated based upon plating of the initial transformation onto carbenicillin plates.

Initial Affinity Selections: For each round of selection, approximately 10^9-10^{10} phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 µg/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEEN™ (0.05% TWEEN 20™

in PBS buffer). Typically, to select for enhanced affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEEN™ buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEEN™ buffer was removed, the remained phages were eluted with 0.1 M HCl and immediately neutralized with 1/3 volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

Phage-ELISA Assay of Binding Affinities: From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 µg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, *o*-phenylenediamine (Sigma). The reaction was stopped with addition of ½ volume of 2.5 M H_2SO_4. Optical density at 492nm was measured on a spectrophotometric plate reader.

Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R, S26N, Q27E, D28Q, and I29L in the VL region (Fig. 9A). In addition, this variant was found to have a spurious

mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

Ala-Scanning the CDRs of Anti-VEGF: To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to > 150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

Table 11: Relative VEGF Affinities of Ala-Scan Fab Variants

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
R24A	1	G26A	2
A25S	1	Y27A	34
N26A	1	T28A	1
E27A	1	F29A	16
Q28A	1	T30A	1
L29A	1	N31A	>150
S30A	2	Y32A	>150
N31A	2	G33A	6
Y32A	2	I34A	6
L33A	2	N35A	66
N34A	4		
		W50A	>150
F50A	1	I51A	4
T51A	1	N52A	>150

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
S52A	1	T53A	9
S53A	1	Y54A	9
L54A	1	T55A	4
H55A	1	G56A	1
S56A	1	E57A	2
		P58A	1
Q89A	4	T59A	3
Q90A	3	Y60A	2
Y91A	14	A61S	1
S92A	1	A62S	1
T93A	1	D63A	1
V94A	2	F64A	1
P95A	3	K65A	1
W96A	>150	R66A	1
T97A	1		
		Y99A	>150
		P100A	38
		H101A	4
		Y102A	4
		Y103A	5
		G104A	2
		S105A	1
		S106A	>150
		H107A	2
		W108A	>150
		Y109A	19
		F110A	25
		D111A	2

All variants are in the background of pY0192 ("wt"; see Figs. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction) and F110A (25-fold reduction). In contrast, only one VL substitution had a large impact on binding affinity, W96A

(>150-fold reduction). These results point to the three VH CDRs as the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

Design of Second-Generation CDR Mutation Libraries: Two additional libraries which randomized existing residues in anti-VEGF version Y0192 were designed based upon inspection of the crystal structure. In VH2, residues 52-55 were randomized because they lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see Fig. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

Second-Generation Selections of Anti-VEGF Libraries: Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from 6×10^7 to 5×10^8 which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

Table 12: Conditions for Secondary Selections of Fab Variants

Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (°C)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 μM VEGF/ELISA	room temp.

4	18	1 μM VEGF/ELISA	room temp.
5	37	1 μM VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37°C	1 μM VEGF/ELISA	room temp./37°C
7	63	1 μM VEGF/ELISA	37°C
8	121	1 μM VEGF/ELISA	37°C

ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

Phage-ELISA Assays of Second Generation Clones: After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

Table 13: Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries

Variants from library YC-81	
Name	**VH3 sequence (residues 99-111)**
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO:56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO:57)
Y0238-3	YPYYYGTSHWYFD (SEQ ID NO:58)
Y0238-4	YPYYYNQSHWYFD (SEQ ID NO:59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO:60)
Y0238-6	YPYYRDNSHWYFD (SEQ ID NO:61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO:62)

Y0238-8	YPYYRQNSHWYFD (SEQ ID NO:63)
Y0238-9	YPYYRQSSHWYFD (SEQ ID NO:64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO:65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO:66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO:67)
Y0228-21	YPYYRNASHWYFD (SEQ ID NO:68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO:69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO:70)
Y0228-24	YPYYRQRGHWYFD (SEQ ID NO:71)
Y0228-26	YPYYTGRSHWYFD (SEQ ID NO:72)
Y0228-27	YPYYTNTSHWYFD (SEQ ID NO:73)
Y0228-28	YPYYRKGSHWYFD (SEQ ID NO:74)
Y0228-29	YPYYTGSSHWYFD (SEQ ID NO:75)
Y0228-30	YPYYRSGSHWYFD (SEQ ID NO:76)
Y0229-20	YPYYTNRSHWYFD (SEQ ID NO:77)
Y0229-21	YPYYRNSSHWYFD (SEQ ID NO:78)
Y0229-22	YPYYKESSHWYFD (SEQ ID NO:79)
Y0229-23	YPYYRDASHWYFD (SEQ ID NO:80)
Y0229-24	YPYYRQKGHWYFD (SEQ ID NO:81)
Y0229-25	YPYYKGGSHWYFD (SEQ ID NO:82)
Y0229-26	YPYYYGASHWYFD (SEQ ID NO:83)
Y0229-27	YPYYRGESHWYFD (SEQ ID NO:84)
Y0229-28	YPYYRSTSHWYFD (SEQ ID NO:85)
Variants from library HL-265	
Name	**VH1 sequence (residue 26-35)**
Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO:86)
Y0243-2	GYEFQHYGMN (SEQ ID NO:87)

Y0243-3	GYEFTHYGMN (SEQ ID NO:88)
Y0243-4	GYDFGHYGMN (SEQ ID NO:89)
Y0243-5	GYDFSHYGMN (SEQ ID NO:90)
Y0243-6	GYEFSHYGMN (SEQ ID NO:91)
Variants from library YC-101	
Name	**VH "CDR7" sequence (residues 70-79)**
Y0244-1	FSVDVSKSTA (SEQ ID NO:92)
Y0244-2	FSLDKSKSTA (SEQ ID NO:93)
Y0244-3	FSLDVWKSTA (SEQ ID NO:94)
Y0244-4	FSIDKSKSTA (:95)

The sequence of the randomized region only is shown as deduced from DNA sequencing.

When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could be explained by the time-scale on which the assay was performed (< 3 hours).

In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants' DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

CDR Combination Variants: To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

Table 14: C mbination CDR Anti-VEGF Variants

Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTACTATTATGGGAC GAGCCACTGGTATTTC-3' (SEQ ID NO:96)
Y0317	Y0313-1	YC-108 (revert VL1 back to wild type)	5'-GTCACCATCACCTGCAGCGCAAGTCA GGATATTAGCAACTATTTAAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCAGCCACTG GTATTTC-3' (SEQ ID NO:98)

Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

BIAcore Analysis: The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25° C at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, Kd's from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

Table 15: Kinetics of Fab-VEGF binding from BIAc re™measurem nts				
Variant	**Kon (10^4/M/s)**	**koff (10^{-4}/s)**	**Kd (nM)**	**Kd (wt) / Kd (mut)**
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*
Y0313-3	1.2	0.081	0.65	4.5

* The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T28D and N31H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.15nM to 0.65 nM (see Table 15). The greater affinity enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

Cell-Based Assay of VEGF Inhibition: Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% diafiltered fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration),

and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

The results (Fig. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC50 for the mutant. For example, if the binding affinity (Kd) of the mutant is in fact < 0.2 nM, the IC50 in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*. Since the variant Y0317 differs from Y0313-1 only in the reversion of the VL1 sequence to wild-type (Fig. 10A), it is predicted that Y0317 will have similar activity to Y0313-1.

Variant Y0317 (Fab) and humanized variant F(ab)-12 from Example 1 (full length and Fab) were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF using the assay described in Example 1. As illustrated in Figure 12, Y0317 was markedly more effective at inhibiting bovine capillary endothelial cell proliferation than the full length and Fab forms of F(ab)-12 in this assay. The Y0317 affinity matured Fab demonstrated an ED50 value in this assay which was at least about 20 fold lower than F(ab)-12 Fab.

WHAT IS CLAIMED IS:

1. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M.

2. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 5×10^{-9}M.

3. A humanized anti-VEGF antibody which has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*.

4. A humanized anti-VEGF antibody which inhibits VEGF-induced angiogenesis *in vivo*.

5. The humanized anti-VEGF antibody of claim 4 wherein 5mg/kg of the antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

6. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129).

7. The humanized anti-VEGF antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:7.

8. The humanized anti-VEGF antibody of claim 6 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

10. The humanized anti-VEGF antibody of claim 9 comprising the amino acid sequence of SEQ ID NO:8.

11. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.

12. An anti-VEGF antibody light chain variable domain comprising the amino acid sequence: DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ KPGKAPKVLIYFTSSLHSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L.

13. An anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGYX $_1$FTX$_2$YGMNWVRQAPGKGLEWVGWINTYTGEPT YAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX $_3$YYGX$_4$SHWYFDVWGQGTLV TVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T.

14. A variant of a parent anti-VEGF antibody, wherein said variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of a heavy chain variable domain of said parent antibody.

15. The variant of claim 14 wherein said parent antibody is a human or humanized antibody.

16. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M.

17. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 5×10^{-9}M.

18. The variant of claim 14 wherein the substitution is in CDRH1 of the heavy chain variable domain.

19. The variant of claim 14 wherein the substitution is in CDRH3 of the heavy chain variable domain.

20. The variant of claim 14 which has amino acid substitutions in both CDRH1 and CDRH3.

21. The variant of claim 14 which binds human VEGF with a K_d value less than that of said parent antibody.

22. The variant of claim 14 which has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower than that of said parent antibody.

23. The variant of claim 18 wherein the CDRH1 comprises the amino acid sequence: GYDFTHYGMN (SEQ ID NO:126)

24. The variant of claim 19 wherein the CDRH3 comprises the amino acid sequence: YPYYYGTSHWYFDV (SEQ ID NO:127).

25. The variant of claim 14 wherein the heavy chain variable domain comprises the amino acid sequence of SEQ ID NO:116.

26. The variant of claim 25 further comprising the light chain variable domain amino acid sequence of SEQ ID NO:124.

27. The variant of claim 26 comprising the light chain variable domain amino acid sequence of SEQ ID NO:115.

28. The humanized anti-VEGF antibody of claim 1 which is a full length antibody.

29. The humanized anti-VEGF antibody of claim 28 which is a human IgG.

30. The humanized anti-VEGF antibody of claim 1 which is an antibody fragment.

31. The antibody fragment of claim 30 which is a Fab.

32. A composition comprising the humanized anti-VEGF antibody of claim 1 and a pharmaceutically acceptable carrier.

33. A composition comprising the variant anti-VEGF antibody of claim 14 and a pharmaceutically acceptable carrier.

34. Isolated nucleic acid encoding the antibody of claim 1.

35. A vector comprising the nucleic acid of claim 34.

36. A host cell comprising the vector of claim 35.

37. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 36 so that the nucleic acid is expressed.

38. The process of claim 37 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

39. A method for inhibiting VEGF-induced angiogenesis in a mammal comprising administering a therapeutically effective amount of the humanized anti-VEGF antibody of claim 1 to the mammal.

40. The method of claim 39 wherein the mammal is a human.

41. The method of claim 39 wherein the mammal has a tumor.

42. The method of claim 39 wherein the mammal has a retinal disorder.

ANTI-VEGF ANTIBODIES

Abstract of the Disclosure

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and inhibit tumor growth *in vivo*.

Express Mail No.: EL599584388US
Atty. Docket No.: P1093P1D1
Date Mailed: November 27, 2000

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY FOR CONTINUING APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTI-VEGF ANTIBODIES

the specification of which (check one) _ is attached hereto or X was filed on 06 August 1997 as Application Serial No. 08/908,469 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed Yes	No
Number	Country	Day/Month/Year Filed		

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in th manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/833,504	April 7, 1997	Pending
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: To Be Assigned Filed: Herewith For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on November 27, 2000 Eileen Ly

ASSOCIATE POWER OF ATTORNEY (37 CFR 1.34)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please recognize as Associate Attorney in this case:

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Respectfully submitted,
GENENTECH, INC.

Date: November 27, 2000

By: Janet E. Hasak
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09157
PATENT TRADEMARK OFFICE

#83625

PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2000

Application or Docket Number
09723,152

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	17 minus 20=	*
INDEPENDENT CLAIMS	1 minus 3 =	*
MULTIPLE DEPENDENT CLAIM PRESENT		☐

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
BASIC FEE	355.00	OR	BASIC FEE	710.00
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL		OR	TOTAL	710

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	* 16	Minus	**	= 0
Independent	* 1	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	0
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Ind pendent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
***If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

(FOR USE WITH FORM PTO-875)

SERIAL NO.	FILING DATE
APPLICANT(S)	

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT		*		*		*	
	IND.	DEP.	IND.	DEP.	IND.	DEP.	IND.	DEP.	IND.	DEP.	IND.	DEP.
1							51					
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43			1		1		93					
44						1	94					
45						1	95					
46						1	96					
47						1	97					
48						1	98					
49						1	99					
50						1	100					
TOTAL IND.							TOTAL IND.			1		1
TOTAL DEP.							TOTAL DEP.			16		15
TOTAL CLAIMS	4						TOTAL CLAIMS			17		16

* *MAY BE USED FOR ADDITIONAL CLAIMS OR ADMENDMENTS*

FORM PTO-1360 (REV. 3-78)

U.S.DEPARTMENT OF COMMERCE
Patent and Trademark Office

BIOTECHNOLOGY SYSTEMS BRANCH

1642

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CRF Problem Report

The Scientific and Technical Information Center (STIC) experienced a problem when processing the following computer readable form (CRF):

Application Serial Number: 09/723,752A
Filing Date: 11/27/2000
Date Processed by STIC: 11/19/02

STIC Contact: **Mark Spencer, 703-308-4212**

Nature of Problem:

The CRF (was):

✓ (circle one) Damaged (melted) or Unreadable (for Unreadable, see attached)
____ Blank (no files on CRF) (see attached)
____ Empty file (filename present, but no bytes in file) (see attached)
____ Virus-infected. Virus name: _____ The STIC will not process the CRF.
____ Not saved in ASCII text
____ Sequence Listing was embedded in the file. According to Sequence Rules, submitted file should **only** be the Sequence Listing.
____ Did not contain a Sequence Listing. (see attached sample)
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Revised 01/29/2002

#3 04co 01/10/01 #

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on February 28, 2001 Eileen Ly

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INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached revised Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement is filed in accordance with the provisions of:

[X] **37 CFR §1.97(b)**

- within three months of the filing date of the application other than a continued prosecution application under 37 CFR §1.53(d); **or**
- within three months of the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491, **or**
- before the mailing of the first Office action on the merits; **or**
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[] **37 CFR §1.97(c)**

- by the applicant after the period specified in 37 CFR §1.97(b), but prior to the mailing date of any of a final action under 37 CFR §1.113, or a notice of allowance under 37 CFR §1.311, or an action that otherwise closes prosecution in the application, and is accompanied by either the fee set forth in 37 CFR §1.17(p) **or** a statement as specified in 37 CFR §1.97(e), as checked below.

[] **37 CFR §1.97(d)**

- after the period specified in CFR §1.97(c), and is accompanied by the fee set forth in 37 CFR §1.17(p) **and** a statement as specified in 37 CFR §1.97(e), as checked below.

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[If either of boxes 37 CFR §1.97(c) or 37 CFR §1.97(d) is checked above, the following statement under 37 CFR §1.97(e) may need to be completed.]

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[] The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $180.00 to cover the cost of this Information Disclosure Statement under 37 CFR §1.17(p). Any deficiency or overpayment should be charged or credited to this deposit account.

A list of the patent(s) or publication(s) is set forth on the attached revised Form PTO-1449 (Modified). Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. 08/908,469, filed August 6, 1997 and relied upon in this application for an earlier filing date under 35 USC §120.

[] BLAST results enclosed:

The undersigned also wishes to bring to the attention of the Examiner BLAST results of computerized alignments of the against sequences contained in the nucleotide and protein databases. The BLAST results are provided in paper form and are identified as reference "BLAST Results A-1- A-()" (nucleotide) and "BLAST Results B-1 - B-()" (protein) on the PTO Form 1449. Applicant requests that these references also be considered and that the Form 1449 be initialed to indicate the Examiner's consideration of the references.

A concise explanation of relevance of the items listed on PTO-1449 is:

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[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

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In accordance with 37 CFR §1.97(g), the filing of this information disclosure statement shall not be construed as a representation that a search has been made.

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In the event that the Office determines a fee to be due where none is specifically authorized in this paper, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $180.00 to cover the cost of this Information Disclosure Statement under 37 CFR §1.17(p).

Respectfully submitted,

GENENTECH, INC.

Date: February 28, 2001

By: Steven X. Cui

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

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LIST OF DISCLOSURES CITED BY APPLICANT

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Atty Docket No.	Serial No.
P1093P1D1	09/723752
Applicant: Baca et al.	
Filing Date: 27 Nov 2000	Group:

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
	* 1	4,816,567	28.03.89	Cabilly et al.			
	* 2	5,530,101	25.06.96	Queen et al.			19.12.90
	* 3	5,580,723	01.12.96	Wells et al.			

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Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
	* 4	0,451,216	01.01.96	EPO				
	* 5	WO 91/09967	11.07.91	PCT				
	* 6	WO 92/22653	23.12.92	PCT				
	* 7	WO 94/04679	03.03.94	PCT				
	* 8	WO 94/10202	11.05.94	PCT				
	* 9	WO 96/30046	03.10.96	PCT				
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Examiner	Date Considered

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Atty Docket No.	Serial No.
P1093P1D1	09/723752
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(54) Title: HUMANISED ANTIBODIES

(57) Abstract

CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positons (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

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HUMANISED ANTIBODIES

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAbs by CDR-grafting was carried out on MAbs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3×10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor),
and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, $(Fab')_2$ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and $(Fab')_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.
For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-graft d chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	-	CDR1:	residues 26-35
	-	CDR2:	residues 50-65
	-	CDR3:	residues 95-102
Light chain	-	CDR1:	residues 24-34
	-	CDR2:	residues 50-56
	-	CDR3:	residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).

2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 72, 76
iii. If 48 is different between donor and acceptor sequences, consider 69
iv. If at 48 the donor residue is chosen, consider 38 and 46
v. If at 69 the donor residue is chosen, consider 80 and then 20
vi. 67
vii. If at 67 the donor residue is chosen, consider 82 and then 18
viii. 91
ix. 88
x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63

iii. 60, if 60 and 54 are able to form potential saltbridge

iv. 70, if 70 and 24 are able to form potential saltbridge

v. 73, and 21 if 47 is different between donor and acceptor

vi. 37, and 45 if 47 is different between donor and acceptor

vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C r gions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;

Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;

Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;

Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;

Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;

Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'

Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;

Figure 9 shows a similar graph of blocking assay results;

Figure 10 shows similar graphs for both binding assay and blocking assay results;

Figure 11 shows further similar graphs for both binding assay and blocking assay results;

Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and

Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction. Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL ($5x10^5$) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with $5x10^5$ HPB-ALL in 200 Ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

bodies were calculated from the equation [X]-[OKT3] = (1/Kx) - (1/Ka), where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2 x 10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5′ phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:
5′ TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5′ CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5′ untranslated regions, signal sequences, variable regions and 3′ untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.
The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3′ end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an AvaI site near the 3′ end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-AvaI fragment. An oligonucleotide adapter was designed to replace the remainder of the 3′

region of the variable region from the AvaI site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the V_L fragment and the 413 bp EcoR1-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-BamH1 fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoR1/BamH1/C1P pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala

VARIABLE CONSTANT

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal Hindlll site was not included in this adapter, to differentiate the two chimeric light chain genes.
The variable region fragment was isolated as a 376 bp EcoR1-AvaI fragment. The oligonucleotide linker was ligated to Nar1 cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoR1. The variable region fragment and the modified constant region fragment were ligated directly into EcoR1/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a Ban1 site near the 3′ end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoR1/C1P/Ban1 fragment. An oligonucleotide adapter was designated to replace the remainder of the 3′ region of the variable region from the Ban1 site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.
The linker was ligated to the V_H fragment and the EcoR1-Hindlll adapted fragment was purified from the ligation mixture.

The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bc11/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sa11/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS

and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl11/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT 78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS

Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

(a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.

(b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c).
Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis.
The key to this residue type designation is as follows:

N - near to CDR (From X-ray Structures)

P - Packing B - Buried Non-Packing

S - Surface E - Exposed

I - Interface * - Interface

- Packing/Part Exposed

? - Non-CDR Residues which may require to be left as Mouse sequence.

Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5′ of the signal

sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE -	KOZAK SEQUENCE +
LIGHT CHAIN	ALL HUMAN FRAMEWORK RE1			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d.	not done
SDM	Site directed mutagenesis
Gene assembly	Variable region assembled entirely from oligonucleotides
Partial gene assembly	Variable region assembled by combination of restriction fragments either fr m other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstructi n with restriction fragments from other genes originally created by SDM and gene assembly

14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody.

Insertion of the Kozak consensus sequence at a position 5′ to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level.

When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/cH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed. In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and

those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 are the same between OKT3 and RE1 (Fig. 3). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W,

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was

being degraded internally. In some experiments trace amounts of antibody could be detected in ^{35}S labelling studies.
As no net antibody was produced, analysis of these constructs was not pursued further.
When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5′ to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to

improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of othe other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	Q	K	A	I	G	F	T	K	S	A	A	Y	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	Q	K	A	I	G	V	T	K	S	A	A	Y	JA185
gH341E	Q	K	A	I	G	V	T	K	S	A	G	G	JA198
gH341*	Q	K	A	I	G	V	T	K	N	A	G	F	JA207
gH341*	Q	K	A	I	G	V	R	N	N	A	G	F	JA209
gH341D	Q	K	A	I	G	V	T	K	N	L	G	F	JA197
gH341*	Q	K	A	I	G	V	R	N	N	L	G	F	JA199
gH341C	Q	K	A	V	A	F	R	N	N	L	G	F	JA184
gH341*	Q	S	A	I	G	V	T	K	S	A	A	Y	JA203
gH341*	E	S	A	I	G	V	T	K	S	A	A	Y	JA205
gH341B	E	S	S	I	G	V	T	K	S	A	A	Y	JA183
gH341*	Q	S	A	I	G	V	T	K	S	A	G	F	JA204
gH341*	E	S	A	I	G	V	T	K	S	A	G	F	JA206
gH341*	Q	S	A	I	G	V	T	K	N	A	G	F	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3v1	Q	V	R	W	
GL221	D	Q	L	L	DA221
gL221A	Q	V	R	W	DA221A
gL221B	Q	V	L	L	DA221B
GL221C	D	Q	R	W	DA221C
RE1	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has neglibible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNFα(61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2

CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90..... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90 ..... is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention.

A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.

The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.

Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.

Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1. The regions transferred were:

CDR Number	Residues
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:

B72.3 cH/B72.3 cL

and B72.3 cH/B72.3 gL

Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and REI light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48.

Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?

For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised.

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.

On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

CDR Number	Residues
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody.

In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.