Exemption under FDCA § 505(i) became effective	October 7, 1999
Patent was granted	June 18, 2002
Biologics License Application (BLA) under PHSA § 351 was filed	December 29, 2005
BLA was approved	June 30, 2006

(ii) The '213 patent was granted during the period specified in § 156(g)(1)(B)(i) (*i.e.*, the period from the date of the grant of the exemption under § 505(i) of the FDCA until the date of submission of the BLA). Pursuant to § 156(b) and (c)(2), the calculated regulatory review period therefore includes a component of time between when the patent was granted and when the BLA was submitted (1/2 of 1289 days or 644 days).

(iii) The patent was granted prior to the start of the period specified in § 156(g)(1)(B)(ii) (*i.e.*, the period from the date of submission of the BLA until the date of approval). The regulatory review period under § 156(b) therefore includes a component equal to the total number of days in that period that are after the BLA was submitted (184 days).

(iv) The period determined according to § 156(b), (c)(2), and (g)(1) for the approved product (*i.e.*, the number of days following the date of issue of the patent between the dates of submission and of approval of the BLA for LUCENTIS™) is 828 days.

(v) The '213 patent will expire on June 18, 2019.

(vi) The date of approval of the approved product is June 30, 2006.

(vii) The date that is fourteen years from the date of approval of the approved product is June 30, 2020.

(viii) The period measured from the date the patent expires (*i.e.*, June 18, 2019) until the end of the fourteen-year period specified in §156 (c)(3) (*i.e.*, June 30, 2020) is approximately 1 year and 13 days or 378 days.

(ix) The number of days in the regulatory review period determined pursuant to § 156(g)(1)(B)(ii) (i.e., 828 days) exceeds the number of days that the

patent may be extended pursuant to §156(c)(3). As such, the period by which the patent may be extended is limited by the fourteen-year rule of §156(c)(3) to **378 days**.

(x) The '213 patent issued after the effective date of Public Law No. 98-417. As such, the two- or three-year limit of 35 U.S.C. § 156(g)(6)(C) does not apply.

13. Statement Pursuant to 37 C.F.R. § 1.740(a)(13)

Pursuant to 37 C.F.R. § 1.740(a)(13), Applicant acknowledges its duty to disclose to the Director of the PTO and to the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, particularly as that duty is defined in 37 C.F.R. § 1.765.

14. Applicable Fee [§ 1.740(a)(14)]

Our check in payment of the fee prescribed in 37 C.F.R. § 1.20(j) for a patent term extension application under 35 U.S.C. § 156 accompanies this application. Please deduct any additional required fees from, or credit any overpayments to our deposit account no. 18-1260.

15. Name and Address for Correspondence [§ 1.740(a)(14)]

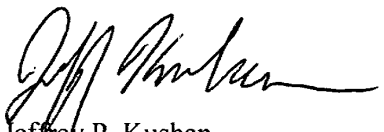
Please direct all inquiries, questions, and communications regarding this application for term extension to:

Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, D.C. 20005
Phone: 202-736-8914
Fax: 202-736-8111
email: jkushan@sidley.com

The correspondence address for U.S. Patent No. 6,407,213 is unchanged for all other purposes. A Power of Attorney granted to the undersigned by the patent assignee, a copy of which is included with this application as Attachment K, accompanies this communication.

Two additional copies of this application are enclosed, in compliance with 37 C.F.R. § 1.740(b). Applicant also provides herewith two further copies of the application for the convenience of the Office, pursuant to M.P.E.P. § 2763.

Sincerely,



Jeffrey P. Kushan
Attorney for Applicant
Registration No. 43,401

Sidley Austin LLP
1501 K Street, N.W.
Washington, D.C. 20005

Dated: August 25, 2006

INDEX OF ATTACHMENTS

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use LUCENTIS safely and effectively. See full prescribing information for LUCENTIS.

LUCENTIS™ (ranibizumab injection)

Initial U.S. Approval: 2006

----------INDICATIONS AND USAGE----------

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration (1).

----------DOSAGE AND ADMINISTRATION----------

- FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY (2.1)
- LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month (2.2).
- Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly (2.2).

----------DOSAGE FORMS AND STRENGTHS----------

- 10 mg/mL single-use vial (3)

----------CONTRAINDICATIONS----------

- Ocular or periocular infections (4.1)
- Hypersensitivity (4.2)

----------WARNINGS AND PRECAUTIONS----------

- Endophthalmitis and retinal detachments may occur following intravitreal injections. Patients should be monitored during the week following the injection (5.1).
- Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection (5.2).

----------ADVERSE REACTIONS----------

The most common adverse reactions (reported ≥ 6% higher in LUCENTIS-treated subjects than control subjects) are conjunctival hemorrhage, eye pain, vitreous floaters, increased intraocular pressure, and intraocular inflammation (6.2).

To report SUSPECTED ADVERSE REACTIONS, contact Genentech at 1-888-835-2555 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See Section 17 for PATIENT COUNSELING INFORMATION.

FULL PRESCRIBING INFORMATION: CONTENTS*

* Sections or subsections omitted from the Full Prescribing Information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

2 DOSAGE AND ADMINISTRATION

2.1 General Dosing Information

FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY.

2.2 Dosing

LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month.

Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly [*see Clinical Studies (14.2)*].

2.3 Preparation for Administration

Using aseptic technique, all (0.2 mL) of the LUCENTIS vial contents are withdrawn through a 5-micron 19-gauge filter needle attached to a 1-cc tuberculin syringe. The filter needle should be discarded after withdrawal of the vial contents and should not be used for intravitreal injection. The filter needle should be replaced with a sterile 30-gauge × 1/2-inch needle for the intravitreal injection. The contents should be expelled until the plunger tip is aligned with the line that marks 0.05 mL on the syringe.

2.4 Administration

The intravitreal injection procedure should be carried out under controlled aseptic conditions, which include the use of sterile gloves, a sterile drape, and a sterile eyelid speculum (or equivalent). Adequate anesthesia and a broad-spectrum microbicide should be given prior to the injection.

Following the intravitreal injection, patients should be monitored for elevation in intraocular pressure and for endophthalmitis. Monitoring may consist of a check for perfusion of the optic nerve head immediately after the injection, tonometry within 30 minutes following the injection, and biomicroscopy between two and seven days following the injection. Patients should be instructed to report any symptoms suggestive of endophthalmitis without delay.

Each vial should only be used for the treatment of a single eye. If the contralateral eye requires treatment, a new vial should be used and the sterile field, syringe, gloves, drapes, eyelid speculum, filter, and injection needles should be changed before LUCENTIS is administered to the other eye.

No special dosage modification is required for any of the populations that have been studied (e.g., gender, elderly).

2.5 Stability and Storage

LUCENTIS should be refrigerated at 2°-8°C (36°-46°F). DO NOT FREEZE. Do not use beyond the date stamped on the label. LUCENTIS vials should be protected from light. Store in the original carton until time of use.

3 DOSAGE FORMS AND STRENGTHS

Single-use glass vial designed to deliver 0.05 mL of 10 mg/mL.

4 CONTRAINDICATIONS

4.1 Ocular or Periocular Infections

LUCENTIS is contraindicated in patients with ocular or periocular infections.

4.2 Hypersensitivity

LUCENTIS is contraindicated in patients with known hypersensitivity to ranibizumab or any of the excipients in LUCENTIS.

5 WARNINGS AND PRECAUTIONS

5.1 Endophthalmitis and Retinal Detachments

Intravitreal injections, including those with LUCENTIS, have been associated with endophthalmitis and retinal detachments. Proper aseptic injection technique should always be used when administering LUCENTIS. In addition, patients should be monitored during the week following the injection to permit early treatment should an infection occur [*see Dosage and Administration (2.3, 2.4) and Patient Counseling Information (17)*].

5.2 Increases in Intraocular Pressure

Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection with LUCENTIS. Therefore, intraocular pressure as well as the perfusion of the optic nerve head should be monitored and managed appropriately [*see Dosage and Administration (2.4)*].

5.3 Thromboembolic Events

Although there was a low rate (<4%) of arterial thromboembolic events observed in the LUCENTIS clinical trials, there is a theoretical risk of arterial thromboembolic events following intravitreal use of inhibitors of VEGF [*see Adverse Reactions (6.3)*].

6 ADVERSE REACTIONS

6.1 Injection Procedure

Serious adverse events related to the injection procedure have occurred in < 0.1% of intravitreal injections, including endophthalmitis [*see Warnings and Precautions (5.1)*], rhegmatogenous retinal detachments, and iatrogenic traumatic cataracts.

6.2 Clinical Trials Experience – Ocular Events

Other serious ocular adverse events observed among LUCENTIS-treated patients occurring in <2% of patients

included intraocular inflammation and increased intraocular pressure [*see Warnings and Precautions (5.1, 5.2)*].

The available safety data include exposure to LUCENTIS in 874 patients with neovascular age-related macular degeneration in three double-masked, controlled studies with dosage regimens of 0.3 mg (375 patients) or 0.5 mg (379 patients) administered monthly by intravitreal injection (Studies 1 and 2) [*see Clinical Studies (14.1)*] and dosage regimens of 0.3 mg (59 patients) or 0.5 mg (61 patients) administered once a month for 3 consecutive doses followed by a dose administered once every 3 months (Study 3) [*see Clinical Studies (14.2)*].

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in one clinical trial of a drug cannot be directly compared with rates in the clinical trials of the same or another drug and may not reflect the rates observed in practice.

Table 1 shows the most frequently reported ocular adverse events that were reported with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

Table 1

Adverse Event	LUCENTIS	Control
Conjunctival hemorrhage	77%-43%	66%-29%
Eye pain	37%-17%	33%-11%
Vitreous floaters	32%-3%	10%-3%
Retinal hemorrhage	26%-15%	56%-37%
Intraocular pressure increased	24%-8%	7%-3%
Vitreous detachment	22%-7%	18%-13%
Intraocular inflammation	18%-5%	11%-3%
Eye irritation	19%-4%	20%-6%
Cataract	16%-5%	16%-6%
Foreign body sensation in eyes	19%-6%	14%-6%
Lacrimation increased	17%-3%	16%-0%
Eye pruritis	13%-0%	12%-3%
Visual disturbance	14%-0%	9%-2%
Blepharitis	13%-3%	9%-4%
Subretinal fibrosis	13%-0%	19%-10%
Ocular hyperemia	10%-5%	10%-1%
Maculopathy	10%-3%	11%-3%
Visual acuity blurred/decreased	17%-4%	24%-10%
Detachment of the retinal pigment epithelium	11%-1%	15%-3%
Dry eye	10%-3%	8%-5%
Ocular discomfort	8%-0%	5%-0%
Conjunctival hyperemia	9%-0%	7%-0%
Posterior capsule opacification	8%-0%	5%-0%
Retinal exudates	9%-1%	11%-3%

6.3 Clinical Trials Experience – Non-Ocular Events

Table 2 shows the most frequently reported non-ocular adverse events with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

Table 2

Adverse Event	LUCENTIS	Control
Hypertension/elevated blood pressure	23%-5%	23%-8%
Nasopharyngitis	16%-5%	13%-5%
Arthralgia	11%-3%	9%-0%
Headache	15%-2%	10%-3%
Bronchitis	10%-3%	8%-2%
Cough	10%-3%	7%-2%
Anemia	8%-3%	8%-0%
Nausea	9%-2%	6%-4%
Sinusitis	8%-2%	6%-4%
Upper respiratory tract infection	15%-2%	10%-4%
Back pain	10%-1%	9%-0%
Urinary tract infection	9%-4%	8%-5%
Influenza	10%-2%	5%-1%
Arthritis	8%-0%	8%-2%
Dizziness	8%-2%	10%-2%
Constipation	7%-3%	8%-2%

The rate of arterial thromboembolic events in the three studies in the first year was 2.1% of patients (18 out of 874) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 1.1% of patients (5 out of 441) in the control arms of the studies. In the second year of Study 1, the rate of arterial thromboembolic events was 3.0% of patients (14 out of 466) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 3.2% of patients (7 out of 216) in the control arm [*see Warnings and Precautions (5.3)*].

6.4 Immunogenicity

The pre-treatment incidence of immunoreactivity to LUCENTIS was 0%-3% across treatment groups. After monthly dosing with LUCENTIS for 12 to 24 months, low titers of antibodies to LUCENTIS were detected in approximately 1%-6% of patients. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to LUCENTIS in an electrochemiluminescence assay and are highly dependent on the sensitivity and specificity of the assay. The clinical significance of immunoreactivity to LUCENTIS is unclear at this time, although some patients with the highest levels of immunoreactivity were noted to have iritis or vitritis.

7 DRUG INTERACTIONS

Drug interaction studies have not been conducted with LUCENTIS.

LUCENTIS intravitreal injection has been used adjunctively with verteporfin photodynamic therapy (PDT). Twelve of 105 (11%) patients developed serious intraocular inflammation; in 10 of the 12 patients, this occurred when LUCENTIS was administered 7 days (± 2 days) after verteporfin PDT.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy
Pregnancy Category C. Animal reproduction studies have not been conducted with ranibizumab. It is also not known whether ranibizumab can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. LUCENTIS should be given to a pregnant woman only if clearly needed.

8.3 Nursing Mothers
It is not known whether ranibizumab is excreted in human milk. Because many drugs are excreted in human milk, and because the potential for absorption and harm to infant growth and development exists, caution should be exercised when LUCENTIS is administered to a nursing woman.

8.4 Pediatric Use
The safety and effectiveness of LUCENTIS in pediatric patients has not been established.

8.5 Geriatric Use
In the controlled clinical studies, approximately 94% (822/879) of the patients randomized to treatment with LUCENTIS were ≥ 65 years of age and approximately 68% (601/879) were ≥ 75 years of age. No notable difference in treatment effect was seen with increasing age in any of the studies. Age did not have a significant effect on systemic exposure in a population pharmacokinetic analysis after correcting for creatinine clearance.

8.6 Patients with Renal Impairment
No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with renal impairment. Sixty-eight percent of patients (136 of 200) in the population pharmacokinetic analysis had renal impairment (46.5% mild, 20% moderate, and 1.5% severe). Reduction in ranibizumab clearance is minimal in patients with renal impairment and is considered clinically insignificant. Dose adjustment is not expected to be needed for patients with renal impairment.

8.7 Patients with Hepatic Dysfunction
No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with hepatic impairment. Dose adjustment is not expected to be needed for patients with hepatic dysfunction.

10 OVERDOSAGE
Planned initial single doses of ranibizumab injection 1.0 mg were associated with clinically significant intraocular inflammation in 2 of 2 patients injected. With an escalating regimen of doses beginning with initial doses of ranibizumab injection 0.3 mg, doses as high as 2.0 mg were tolerated in 15 of 20 patients.

11 DESCRIPTION
LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

LUCENTIS is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS aqueous solution with 10 mM histidine HCl, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action
Ranibizumab binds to the receptor binding site of active forms of VEGF-A, including the biologically active, cleaved form of this molecule, $VEGF_{110}$. VEGF-A has been shown to cause neovascularization and leakage in models of ocular angiogenesis and is thought to contribute to the progression of the neovascular form of age-related macular degeneration (AMD). The binding of ranibizumab to VEGF-A prevents the interaction of VEGF-A with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells, reducing endothelial cell proliferation, vascular leakage, and new blood vessel formation.

12.2 Pharmacodynamics
Neovascular AMD is associated with foveal retinal thickening as assessed by optical coherence tomography (OCT) and leakage from CNV as assessed by fluorescein angiography.

In Study 3, foveal retinal thickness was assessed by OCT in 118/184 patients. OCT measurements were collected at baseline, Months 1, 2, 3, 5, 8, and 12. In patients treated with LUCENTIS, foveal retinal thickness decreased, on average, more than the sham group from baseline through Month 12. Retinal thickness decreased by Month 1 and decreased further at Month 3, on average. Foveal retinal thickness data did not provide information useful in influencing treatment decisions [*see Clinical Studies (14.2)*].

In patients treated with LUCENTIS, the area of vascular leakage, on average, decreased by Month 3 as assessed by fluorescein angiography. The area of vascular leakage for an individual patient was not correlated with visual acuity.

12.3 Pharmacokinetics
In animal studies, following intravitreal injection, ranibizumab was cleared from the vitreous with a half-life of approximately 3 days. After reaching a maximum at approximately 1 day,

the serum concentration of ranibizumab declined in parallel with the vitreous concentration. In these animal studies, systemic exposure of ranibizumab is more than 2000-fold lower than in the vitreous.

In patients with neovascular AMD, following monthly intravitreal administration, maximum ranibizumab serum concentrations were low (0.3 ng/mL to 2.36 ng/mL). These levels were below the concentration of ranibizumab (11 ng/mL to 27 ng/mL) thought to be necessary to inhibit the biological activity of VEGF-A by 50%, as measured in an in vitro cellular proliferation assay. The maximum observed serum concentration was dose proportional over the dose range of 0.05 to 1.0 mg/eye. Based on a population pharmacokinetic analysis, maximum serum concentrations of 1.5 ng/mL are predicted to be reached at approximately 1 day after monthly intravitreal administration of LUCENTIS 0.5 mg/eye. Based on the disappearance of ranibizumab from serum, the estimated average vitreous elimination half-life was approximately 9 days. Steady-state minimum concentration is predicted to be 0.22 ng/mL with a monthly dosing regimen. In humans, serum ranibizumab concentrations are predicted to be approximately 90,000-fold lower than vitreal concentrations.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No carcinogenicity or mutagenicity data are available for ranibizumab injection in animals or humans.

No studies on the effects of ranibizumab on fertility have been conducted.

14 CLINICAL STUDIES

The safety and efficacy of LUCENTIS were assessed in three randomized, double-masked, sham- or active-controlled studies in patients with neovascular AMD. A total of 1323 patients (LUCENTIS 879, Control 444) were enrolled in the three studies.

14.1 Study 1 and Study 2

In Study 1, patients with minimally classic or occult (without classic) CNV lesions received monthly LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or monthly sham injections. Data are available through Month 24. Patients treated with LUCENTIS in Study 1 received a mean of 22 total treatments out of a possible 24 from Day 0 to Month 24.

In Study 2, patients with predominantly classic CNV lesions received one of the following: 1) monthly LUCENTIS 0.3 mg intravitreal injections and sham PDT; 2) monthly LUCENTIS 0.5 mg intravitreal injections and sham PDT; or 3) sham intravitreal injections and active verteporfin PDT. Sham PDT (or active verteporfin PDT) was given with the initial LUCENTIS (or sham) intravitreal injection and every 3 months thereafter if fluorescein angiography showed persistence or recurrence of leakage. Data are available through Month 12. Patients treated with LUCENTIS in Study 2 received a mean of 12 total treatments out of a possible 13 from Day 0 through Month 12.

In both studies, the primary efficacy endpoint was the proportion of patients who maintained vision, defined as losing fewer than 15 letters of visual acuity at 12 months compared with baseline. Almost all LUCENTIS-treated patients (approximately 95%) maintained their visual acuity. 34%-40% of LUCENTIS-treated patients experienced a clinically significant improvement in vision, defined as gaining 15 or more letters at 12 months. The size of the lesion did not significantly affect the results. Detailed results are shown in the tables below.

Table 3
Outcomes at Month 12 and Month 24 in Study 1

Outcome Measure	Month	Sham n = 238	LUCENTIS 0.5 mg n = 240	Estimated Difference (95% CI)[a]
Loss of < 15 letters in visual acuity (%)[b]	Month 12	62%	95%	32% (26%, 39%)
	Month 24	53%	90%	37% (29%, 44%)
Gain of ≥ 15 letters in visual acuity (%)[b]	Month 12	5%	34%	29% (22%,-35%)
	Month 24	4%	33%	29% (23%, 35%)
Mean change in visual acuity (letters) (SD)[b]	Month 12	-10.5 (16.6)	+7.2 (14.4)	17.5 (14.8, 20.2)
	Month 24	-14.9 (18.7)	+6.6 (16.5)	21.1 (18.1, 24.2)

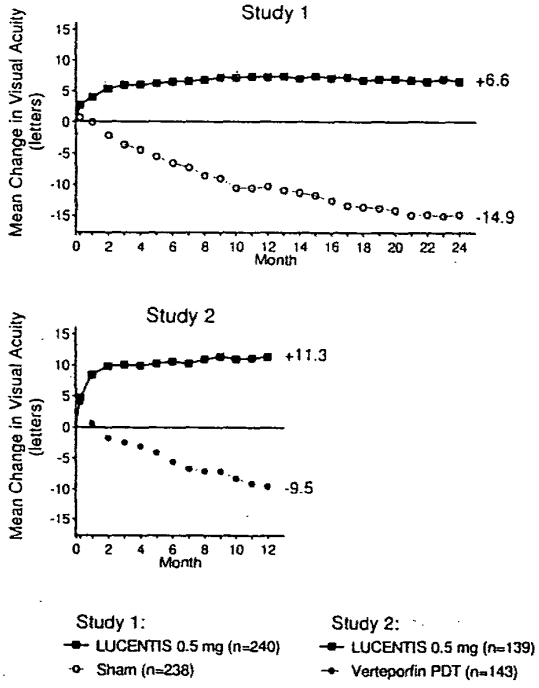
[a] Adjusted estimate based on the stratified model.
[b] $p<0.01$.

Table 4
Outcomes at Month 12 in Study 2

Outcome Measure	Verteporfin PDT n = 143	LUCENTIS 0.5 mg n = 140	Estimated Difference (95% CI)[a]
Loss of < 15 letters in visual acuity (%)[b]	64%	96%	33% (25%, 41%)
Gain of ≥ 15 letters in visual acuity (%)[b]	6%	40%	35% (26%, 44%)
Mean change in visual acuity (letters) (SD)[b]	-9.5 (16.4)	+11.3 (14.6)	21.1 (17.5, 24.6)

[a] Adjusted estimate based on the stratified model.
[b] $p<0.01$.

Figure 1
Mean Change in Visual Acuity from Baseline to Month 24 in Study 1 and to Month 12 in Study 2



Patients in the group treated with LUCENTIS had minimal observable CNV lesion growth, on average. At Month 12, the mean change in the total area of the CNV lesion was 0.1-0.3 DA for LUCENTIS versus 2.3-2.6 DA for the control arms.

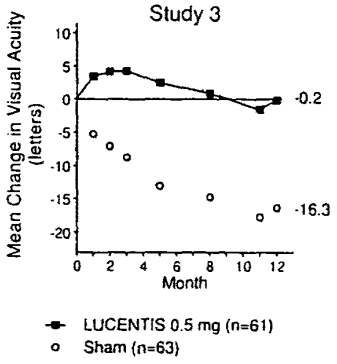
The use of LUCENTIS beyond 24 months has not been studied.

14.2 Study 3

Study 3 was a randomized, double-masked, sham-controlled, two-year study designed to assess the safety and efficacy of LUCENTIS in patients with neovascular AMD (with or without a classic CNV component). Data are available through Month 12. Patients received LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or sham injections once a month for 3 consecutive doses, followed by a dose administered once every 3 months. A total of 184 patients were enrolled in this study (LUCENTIS 0.3 mg, 60; LUCENTIS 0.5 mg, 61; sham, 63); 171 (93%) completed 12 months of this study. Patients treated with LUCENTIS in Study 3 received a mean of 6 total treatments out of possible 6 from Day 0 through Month 12.

In Study 3, the primary efficacy endpoint was mean change in visual acuity at 12 months compared with baseline (see Figure 2). After an initial increase in visual acuity (following monthly dosing), on average, patients dosed once every three months with LUCENTIS lost visual acuity, returning to baseline at Month 12. In Study 3, almost all LUCENTIS-treated patients (90%) maintained their visual acuity at Month 12.

Figure 2
Mean Change in Visual Acuity from Baseline to Month 12 in Study 3



16 HOW SUPPLIED/STORAGE AND HANDLING

Each LUCENTIS carton, NDC 50242-080-01, contains one 2-cc glass vial of ranibizumab, one 5-micron, 19-gauge × 1-1/2-inch filter needle for withdrawal of the vial contents, one 30-gauge × 1/2-inch injection needle for the intravitreal injection, and one package insert [*see Dosage and*

Administration (2.4)]. VIALS ARE FOR SINGLE EYE USE ONLY.

17 PATIENT COUNSELING INFORMATION

In the days following LUCENTIS administration, patients are at risk of developing endophthalmitis. If the eye becomes red, sensitive to light, painful, or develops a change in vision, the patient should seek immediate care from an ophthalmologist [*see Warnings and Precautions (5.1)*].

LUCENTIS™ [ranibizumab injection]

Manufactured by:
Genentech, Inc.
1 DNA Way
South San Francisco, CA 94080-4990

8277700
LL1404
4833801
FDA Approval Date:
June 2006

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

BLA 125156

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality & Compliance
1 DNA Way
South San Francisco, California 94080-4990

Dear Dr. Garnick:

We have approved your biologics' license application for Lucentis (ranibizumab injection) effective this date. You are hereby authorized to introduce or deliver for introduction into interstate commerce, ranibizumab injection under your existing Department of Health and Human Services U.S. License No. 1048. Lucentis (ranibizumab injection) is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

Under this license, you are approved to manufacture ranibizumab drug substance at Genentech, Inc., South San Francisco, California; fill the final formulated product at [redacted] and label and package filled vials at Genentech, Inc., South San Francisco, California. You may label your product with the proprietary name Lucentis and market it in 10 mg/mL single use glass vials.

We acknowledge receipt of your submissions dated December 29, 2005, and January 31, February 10, 17, 21, and 24, March 17, 23, and 31, April 10, and 28, May 5, 10, 25 (2), 26 (2), and 31, and June 1, 5 (2), 6, 9, 13, 16, 23, 26, 27, 28 (3), and 29, 2006.

The final printed labeling (FPL) must be identical in content to the enclosed labeling text for the package insert, submitted June 28, 2006; the immediate vial container submitted March 31, 2006; and the carton labels submitted June 5, 2006. The statement "No U.S. standard of potency" should be added with the next printing of carton labels. Marketing this product with FPL that is not identical in content to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for formulated drug product shall be 18 months from the date of manufacture when stored at 2°-8°C (36°-46°F). The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for ranibizumab drug substance shall be [redacted] when stored at -20 °C.

You currently are not required to submit samples of future lots of Lucentis to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of Lucentis, or in the manufacturing facilities.

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We are waiving the pediatric study requirement for this application.

The following are Postmarketing Studies that are subject to reporting requirements of 21 CFR 601.70:

1. Submit the final Clinical Study Report from Study FVF3689g by June 30, 2008.

2. Provide safety and efficacy data from a 2-year adequate and well-controlled clinical trial of a mutually acceptable design exploring multiple dosing frequencies of Lucentis.

   Date of submission of protocol: November 14, 2008.

   Date of start of study: September 21, 2009.

   Date of final clinical study report: April 1, 2013.

3. To detect and characterize immune responses to ranibizumab:

   a. Develop and validate a confirmatory assay capable of detecting both IgG and IgM isotype responses.

   b. Develop and validate an assay to detect neutralizing anti-ranibizumab antibodies.

   The assay methodology and validation reports: September 28, 2007.

4. To characterize further the immune response to ranibizumab, serum samples collected in studies FVF2587g, FVF2598g, FVF3192g will be assayed using the validated methods described above in Postmarketing Commitment #3. The data obtained will be analyzed to discover and evaluate any association between immunoreactivity and dosing frequency as well as any potential impact of immunoreactivity on efficacy or safety outcomes.

   The need for an additional clinical study will be determined based on the results from the analysis described above.

   Date of submission of protocol and statistical analysis plan: February 28, 2007.

   Date of submission of final study report: September 30, 2008.

The following are Postmarketing Studies that are not subject to reporting requirements of 21 CFR 601.70:

5. To revise release specifications, shelf-life specifications and in-process limits for ranibizumab drug substance and drug product after [redacted] commercial manufacturing runs to reflect increased manufacturing experience.

   These revisions to the Quality control system, the corresponding data from the [redacted] commercial manufacturing runs and the analysis plan used to create the revisions will be submitted as a supplement on or before June 30, 2008.

6. To perform additional Lucentis stability studies at 40°C using Ion Exchange Chromatography (IEC) to demonstrate that the corrective actions taken at [redacted] to address the atypical accelerated stability profile observed in the Lucentis 2005 qualification campaign have been sufficient.

   Specifically, a one time stability study consisting of [redacted] Lucentis Drug Product launch lots are placed at 40°C and tested by IEC at [redacted] months. These [redacted] Lucentis Drug Product lots are derived from the following:

   - [redacted] of these Lucentis Drug Product lots are manufactured from distinct lots of [redacted].
   - At least [redacted] these [redacted] lots are aliquoted and used to manufacture [redacted] Lucentis drug product lots.

   Data will be submitted as a supplement on or before March 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this application. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Protocol**
- **Postmarketing Study Final Report**
- **Postmarketing Study Correspondence**
- **Annual Report on Postmarketing Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),

- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the April 2001 Draft Guidance for Industry: Reports on the Status of Postmarketing Studies – Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cber/gdlns/post040401.htm) for further information.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Biological product deviations sent by courier or overnight mail should be addressed to Food and Drug Administration, CDER, Office of Compliance, Division of Compliance Risk Management and Surveillance, HFD-330, Montrose Metro 2, 11919 Rockville Pike, Rockville, MD 20852.

Please submit all FPL at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions.

If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Mark J. Goldberger, M.D., M.P.H.
Director
Office of Antimicrobial Products
Center for Drug Evaluation and Research

Enclosure

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US006407213B1

(12) **United States Patent**
Carter et al.

(10) **Patent No.: US 6,407,213 B1**
(45) **Date of Patent: Jun. 18, 2002**

(54) **METHOD FOR MAKING HUMANIZED ANTIBODIES**

(75) Inventors: **Paul J. Carter**; **Leonard G. Presta**, both of San Francisco, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/146,206**

(22) PCT Filed: **Jun. 15, 1992**

(86) PCT No.: **PCT/US92/05126**

§ 371 (c)(1),
(2), (4) Date: **Nov. 17, 1993**

Related U.S. Application Data

(63) Continuation-in-part of application No. 07/715,272, filed on Jun. 14, 1991, now abandoned.

(51) **Int. Cl.**[7] **C07K 16/00**
(52) **U.S. Cl.** **530/387.3**; 435/69.6; 435/69.7; 435/70.21; 435/91; 536/23.53; 424/133.1
(58) **Field of Search** 435/69.6, 69.7, 435/70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1, 328; 536/23.53; 424/133.1; 530/387.3

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(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

Variant immunoglobulins, particularly humanized antibody polypeptides are provided, along with methods for their preparation and use. Consensus immunoglobulin sequences and structural models are also provided.

82 Claims, 9 Drawing Sheets

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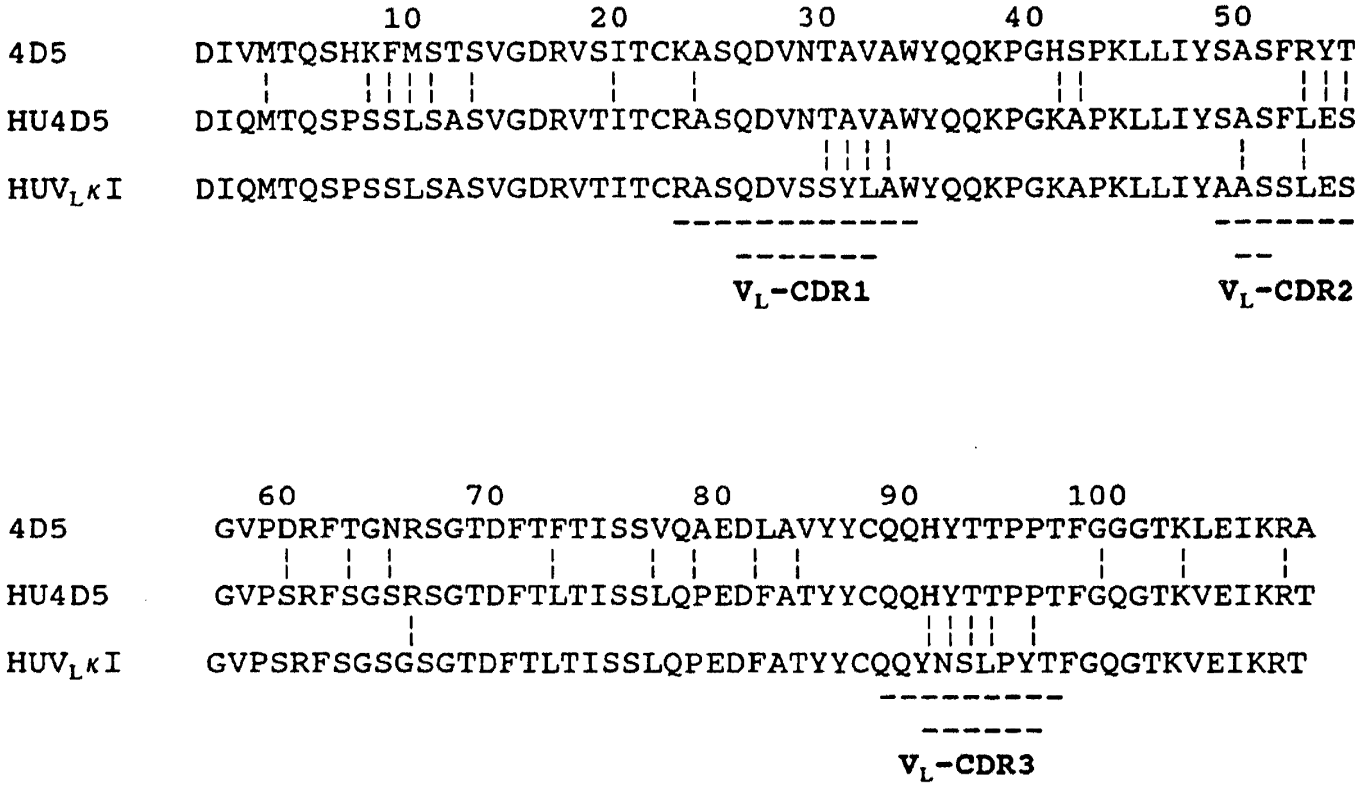
FIG. 1A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HU4D5 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES
HUVLκI DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES
VL-CDR1
VL-CDR2
60 70 80 90 100
4D5 GVPDRFTGNRSGTDFTFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA
HU4D5 GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT
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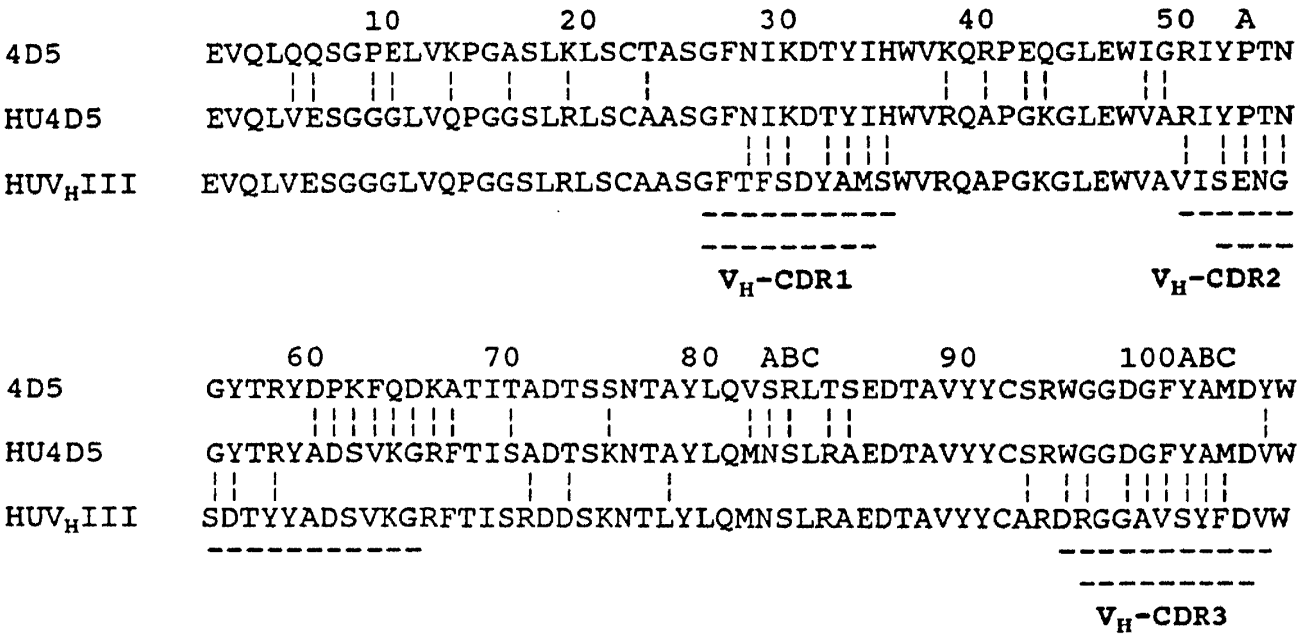
VL-CDR3

FIG. 1B


10 20 30 40 50 A
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HU4D5 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
HUV$_H$III EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENG
V$_H$-CDR1
V$_H$-CDR2
60 70 80 ABC 90 100ABC
4D5 GYTRYDPKFQDKATITADTSSNTAYLQVSRLTSEDTAVYYCSRWGGDGFYAMDYW
HU4D5 GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVW
HUV$_H$III SDTYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCARDRGGAVSYFDVW
V$_H$-CDR3
110
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HUV$_H$III GQGTLVTVSS

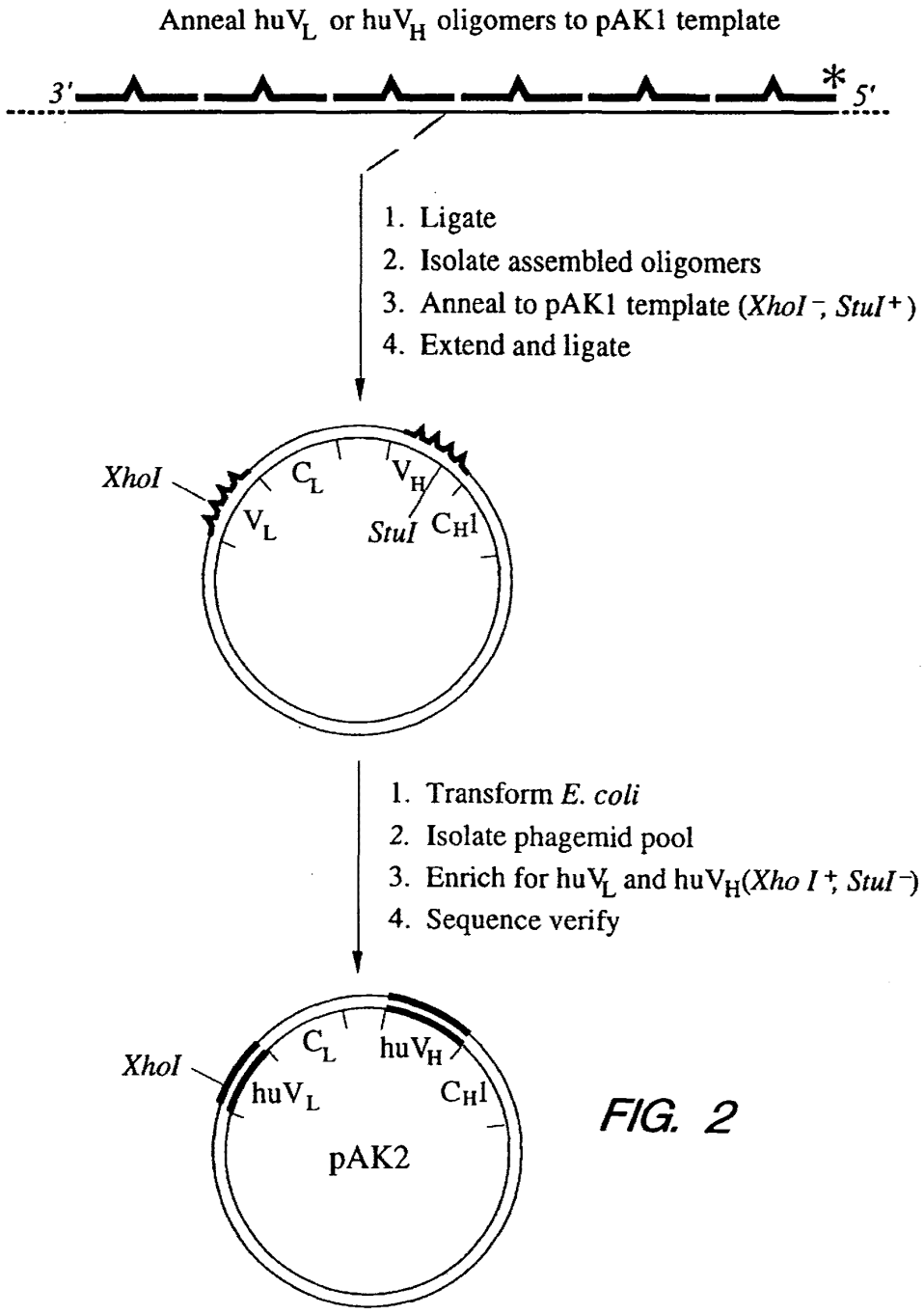

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
*
5'
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I*$^+$, *StuI*$^-$)
4. Sequence verify
XhoI
C$_L$
huV$_H$
huV$_L$
C$_H$1
pAK2

FIG. 2

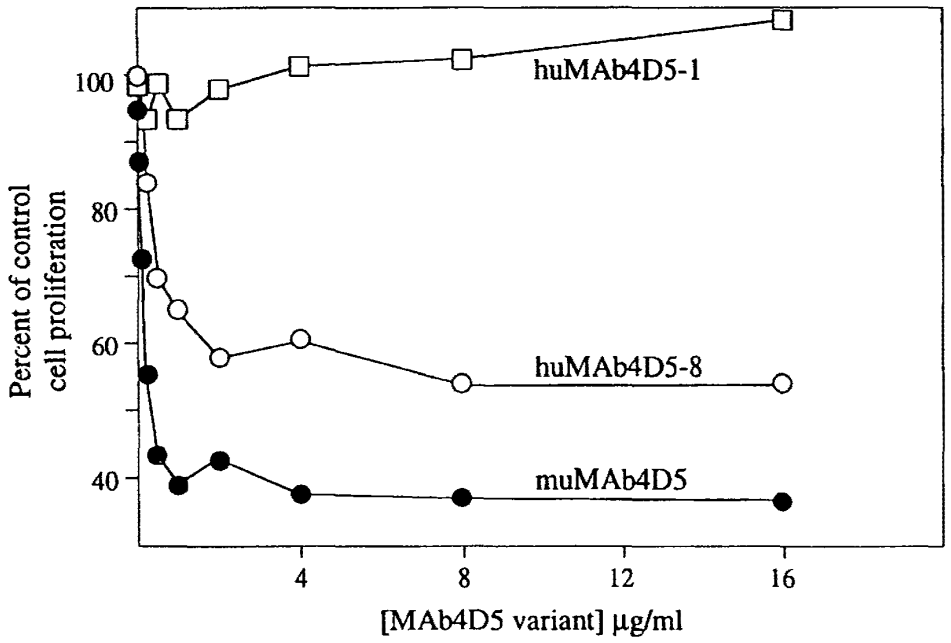

100
80
60
40
Percent of control cell proliferation
huMAb4D5-1
huMAb4D5-8
muMAb4D5
4
8
12
16
[MAb4D5 variant] µg/ml

FIG. 3

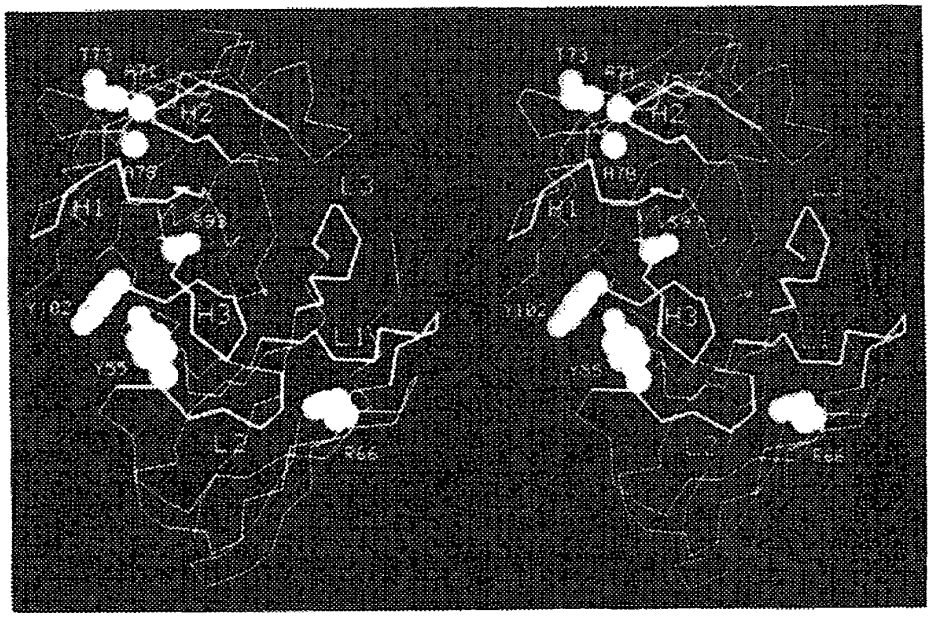


FIG. 4

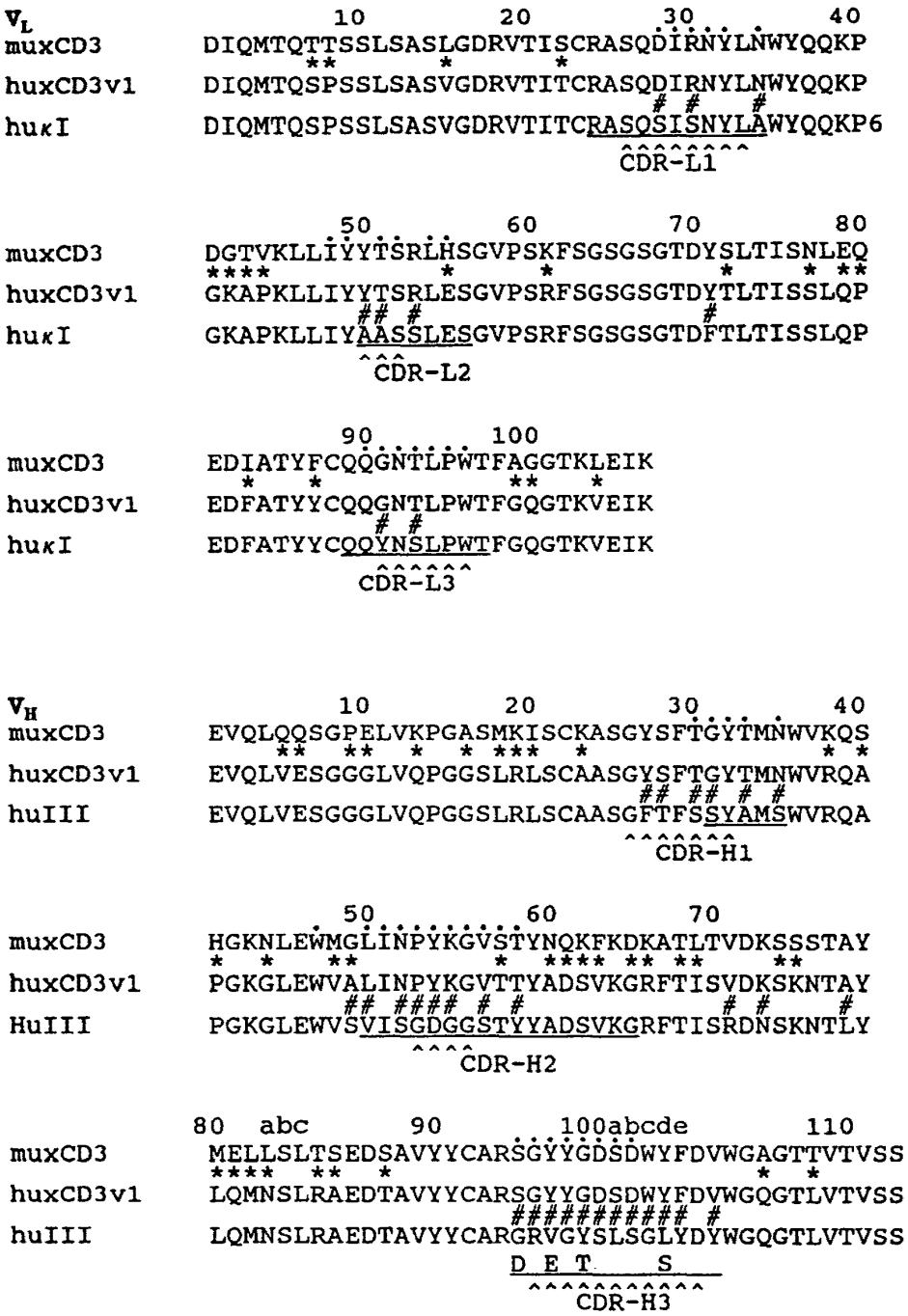

V_L
muxCD3 DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKP
huxCD3v1 DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKP
huκI DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP6
CDR-L1
muxCD3 DGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQ
huxCD3v1 GKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQP
huκI GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
CDR-L2
muxCD3 EDIATYFCQQGNTLPWTFAGGTKLEIK
huxCD3v1 EDFATYYCQQGNTLPWTFGQGTKVEIK
huκI EDFATYYCQQYNSLPWTFGQGTKVEIK
CDR-L3
V_H
muxCD3 EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQS
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQA
huIII EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
CDR-H1
muxCD3 HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALINPYKGVTTYADSVKGRFTISVDKSKNTAY
HuIII PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDNSKNTLY
CDR-H2
muxCD3 MELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSS
huxCD3v1 LQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSS
huIII LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWGQGTLVTVSS
D E T S
CDR-H3

FIG. 5

FIG. 6A-1

```
                                        10        20        30
H52H4-160                       QVQLQQSGPELVKPGASVKISCKTSGYTFTE
                                .*** .**  **.**.*...** ********
pH52-8.0    MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
                    10        20        30        40        50

                   40        50        60        70        80
H52H4-160   YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
            ******.*. **.***..*.******.********. *..**********
pH52-8.0    YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
                    60        70        80        90        100

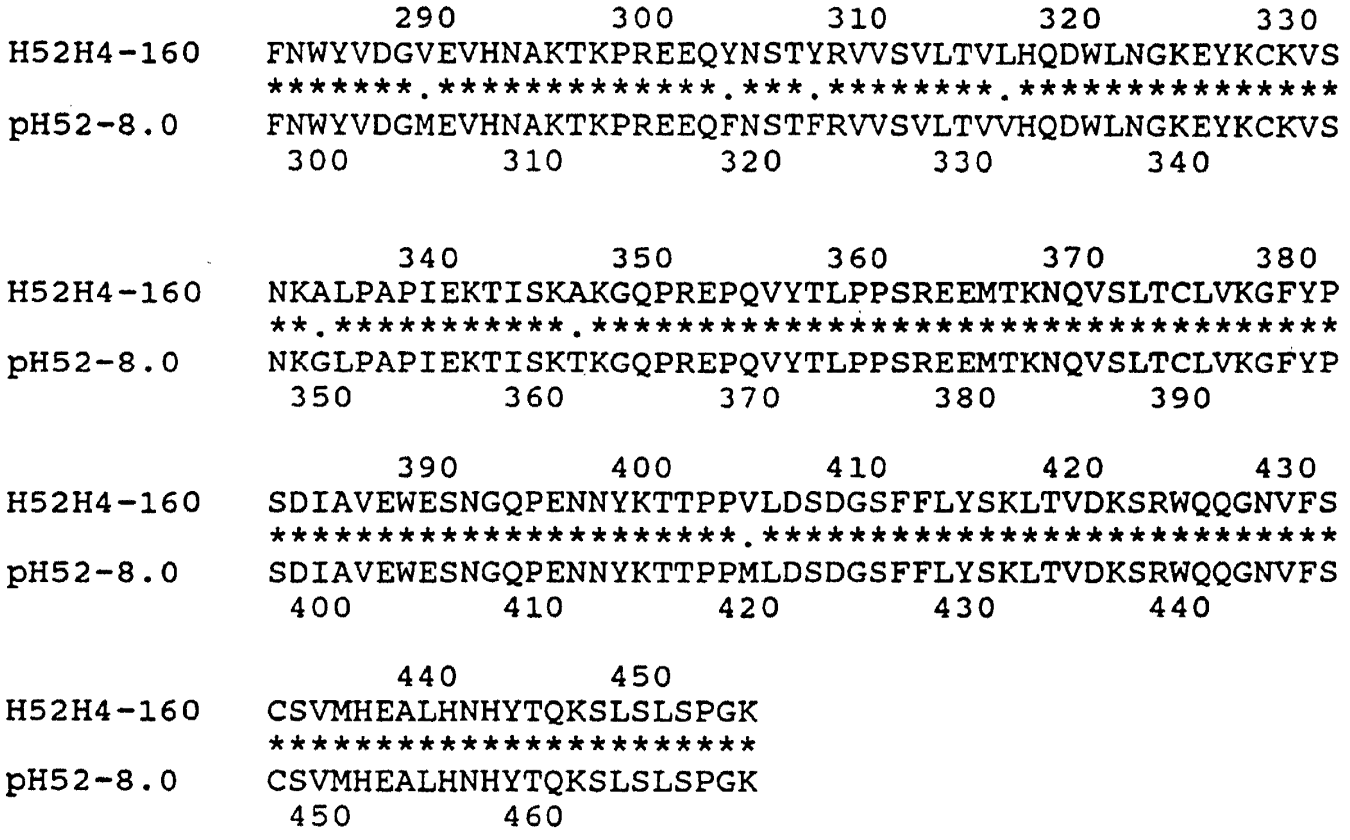
                   90       100       110       120       130
H52H4-160   ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
            .. ** .**...********************** ** ************
pH52-8.0    QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
                   110       120       130       140       150

                  140       150       160       170       180
H52H4-160   VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
            ****** *.*** .************************************
pH52-8.0    VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                   160       170       180       190       200

                  190       200       210       220       230
H52H4-160   QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
            ************** **..***** ***.********** ** * *
pH52-8.0    QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
                   210       220       230       240

                  240       250       260       270       280
H52H4-160   TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
             *******  ..**********************************.
pH52-8.0    ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
            250       260       270       280       290
```

FIG. 6A-2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pH52-8.0 FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS
300 310 320 330 340
340 350 360 370 380
H52H4-160 NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
pH52-8.0 NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
350 360 370 380 390
390 400 410 420 430
H52H4-160 SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
pH52-8.0 SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
400 410 420 430 440
440 450
H52H4-160 CSVMHEALHNHYTQKSLSLSPGK
pH52-8.0 CSVMHEALHNHYTQKSLSLSPGK
450 460

FIG. 6B

```
                                        10        20        30
H52L6-158                          DVQMTQTTSSLSASLGDRVTINCRASQDINN
                                   *.****. ******.****** *********
pH52-9.0    MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                     10        20        30        40        50

                    40        50        60        70        80
H52L6-158   YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
            *********  . **************************.****.*. *
pH52-9.0    YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                     60        70        80        90        100

                   90       100       110       120       130
H52L6-158   DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
            *.***.************ ********************************
pH52-9.0    DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                    110       120       130       140       150

                  140       150       160       170       180
H52L6-158   VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
            **************************************************
pH52-9.0    VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                    160       170       180       190       200

                  190       200       210
H52L6-158    SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
             ********************************
pH52-9.0     SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                    210       220       230
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METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of U.S. application Ser. No. 07/715,272 filed Jun. 14, 1991 (abandoned) which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

FIELD OF THE INVENTION

This invention relates to methods for the preparation and use of variant antibodies and finds application particularly in the fields of immunology and cancer diagnosis and therapy.

BACKGROUND OF THE INVENTION

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the heavy chains by disulfide bonds. 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 (V_L) at one end 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, see e.g. Chothia et al., *J. Mol. Biol.* 186:651–663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* 82:4592–4596 (1985).

The constant domains are not involved directly in binding the antibody to an antigen, but are involved in various effector functions, such as participation of the antibody in antibody-dependent cellular cytotoxicity. The variable domains of each pair of light and heavy chains are involved directly in binding the antibody to the antigen. The domains of natural light and heavy chains have the same general structure, and each domain comprises four framework (FR) regions, whose sequences are somewhat conserved, connected by three hyper-variable or complementarity determining regions (CDRs) (see Kabat, E. A. et a., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda, Md., (1987)). The four framework regions largely adopt a β-sheet conformation and the CORs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

Widespread use has been made of monoclonal antibodies, particularly those derived from rodents including mice, however they are frequently antigenic in human clinical use. For example, a major limitation in the clinical use of rodent monoclonal antibodies is an anti-globulin response during therapy (Miller, R. A. et al., *Blood* 62:988–995 (1983); Schroff, R. W. et al., *Cancer Res.* 45:879–885 (1985)).

The art has attempted to overcome this problem by constructing "chimeric" antibodies in which an animal antigen-binding variable domain is coupled to a human constant domain (Cabilly et al., U.S. Pat. No. 4,816,567; Morrison, S. L. et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984); Boulianne, G. L. et al., *Nature* 312:643–646 (1984); Neuberger, M. S. et al., *Nature* 314:268–270 (1985)). The term "chimeric" antibody is used herein to describe a polypeptide comprising at least the

antigen binding portion of an antibody molecule linked to at least part of another protein (typically an immunoglobulin constant domain).

The isotype of the human constant domain may be selected to tailor the chimeric antibody for participation in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (see e.g. Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988); Love et al., *Methods in Enzymology* 178:515–527 (1989); Bindon et al., *J. Exp. Med.* 168:127–142 (1988).

In the typical embodiment, such chimeric antibodies contain about one third rodent (or other non-human species) sequence and thus are capable of eliciting a significant anti-globulin response in humans. For example, in the case of the murine anti-CD3 antibody, OKT3, much of the resulting anti-globulin response is directed against the variable region rather than the constant region (Jaffers, G. J. et a., *Transplantation* 41:572–578 (1986)).

In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterologous sequences in human antibodies, Winter and colleagues (Jones, P. T. et al., *Nature* 321:522–525 (1986); Riechmann, L. et al., *Nature* 332:323–327 (1988); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)) have substituted rodent CDRs or CDR sequences for the corresponding segments of a human antibody. As used herein, the term "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The therapeutic promise of this approach is supported by the clinical efficacy of a humanized antibody specific for the CAMPATH-1 antigen with two non-Hodgkin lymphoma patients, one of whom had previously developed an anti-globulin response to the parental rat antibody (Riechmann, L. et al., *Nature* 332:323–327 (1988); Hale, G. et al., *Lancet i:*1394–1399 (1988)). A murine antibody to the interleukin 2 receptor has also recently been humanized (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) as a potential immunosuppressive reagent. Additional references related to humanization of antibodies include Co et al., *Proc. Natl. Acad. Sci. USA* 88:2869–2873 (1991); Gorman et al., *Proc. Natl. Acad. Sci. USA* 88:4181–4185 (1991); Daugherty et al., *Nucleic Acids Research* 19(9):2471–2476 (1991); Brown et al., *Proc. Natl. Acad. Sci. USA* 88:2663–2667 (1991); Junghans et al., *Cancer Research* 50:1495–1502 (1990).

In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones, P. T. et al., *Nature* 321:522–525 (1986); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)), whereas in other cases it has been necessary to additionally replace one (Riechmann, L. et al., *Nature* 332:323–327 (1988)) or several (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) framework region (FR) residues. See also Co et al., supra.

For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. et al., *Ann. Rev. Biochem.* 59:439–473 (1990)).

Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies et al., *Proc. Natl. Acad. Sci. USA* 72:2180–2184 (1975).

It is also known that, in a few instances, an antibody variable domain (either V_H or V_L) may contain glycosylation sites, and that this glycosylation may improve or abolish antigen binding, Pluckthun, *Biotechnology* 9:545–51 (1991); Spiegelberg et al., *Biochemistry* 9:4217–4223 (1970); Wallic et al., *J. Exp. Med.* 168:1099–1109 (1988); Sox et al., *Proc. Natl. Acad. Sci. USA* 66:975–982 (1970); Margni et al., *Ann. Rev. Immunol* 6:535–554 (1988). Ordinarily, however, glycosylation has no influence on the antigen-binding properties of an antibody, Pluckthun, supra, (1991).

The three-dimensional structure of immunoglobulin chains has been studied, and crystal structures for intact immunoglobulins, for a variety of immunoglobulin fragments, and for antibody-antigen complexes have been published (see e.g., Saul et al., *Journal of Biological Chemistry* 25:585–97 (1978); Sheriff et al., *Proc. Natl. Acad. Sci. USA* 84:8075–79 (1987); Segal et al., *Proc. Natl. Acad. Sci. USA* 71:4298–4302 (1974); Epp et al., *Biochemistry* 14(22) :4943–4952 (1975); Marquart et al., *J. Mol. Biol.* 141:369–391 (1980); Furey et al., *J. Mol. Biol.* 167:661–692 (1983); Snow and Amzel, *Protein: Structure, Function, and Genetics* 1:267–279, Alan R. Liss, Inc. pubs. (1986); Chothia and Lesk, *J. Mol. Bol.* 196:901–917 (1987); Chothia et al., *Nature* 342:877–883 (1989); Chothia et al., *Science* 233:755–58 (1986); Huber et al., *Nature* 264:415–420 (1976); Bruccoleri et al., *Nature* 335:564–568 (1988) and *Nature* 336:266 (1988); Sherman et al., *Journal of Biological Chemistry* 263:4064–4074 (1988); Amzel and Poljak, *Ann. Rev. Biochem.* 48:961–67 (1979); Silverton et al., *Proc. Natl. Acad. Sci. USA* 74:5140–5144 (1977); and Gregory et al., *Molecular Immunology* 24:821–829 (1987). It is known that the function of an antibody is dependent on its three dimensional structure, and that amino acid substitutions can change the three-dimensional structure of an antibody, Snow and Amzel, supra. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)).

Humanizing an antibody with retention of high affinity for antigen and other desired biological activities is at present difficult to achieve using currently available procedures. Methods are needed for rationalizing the selection of sites for substitution in preparing such antibodies and thereby increasing the efficiency of antibody humanization.

The proto-oncogene HER2 (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase ($p185^{HER2}$) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132–1139 (1985); Yamamoto, T. et al., *Nature* 319:230–234 (1986); King, C. R. et al., *Science* 229:974–976 (1985)). HER2 is also known in the field as c-erbB-2, and sometimes by the name of the rat homolog, neu. Amplification and/or overexpression of HER2 is associated with multiple human malignancies and appears to be integrally involved in progression of 25–30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science* 235:177–182 (1987), Slamon, D. J. et al., *Science* 244:707–712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, supra, Science 1989).

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)), directed against the extracellular domain (ECD) of $p185^{HER2}$, specifically inhibits the growth of tumor cell lines overexpressing $p185^{HER2}$ in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol* 9:1165–1172 (1989); Lupu, R. et al., *Science* 249:1552–1555 (1990)). MuMAb4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, supra, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333–395 (1988)). Thus muMAb4D5 has potential for clinical intervention in and imaging of carcinomas in which $p185^{HER2}$ is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

It is therefore an object of this invention to provide methods for the preparation of antibodies which are less antigenic in humans than non-human antibodies but have desired antigen binding and other characteristics and activities.

It is a further object of this invention to provide methods for the efficient humanization is of antibodies, i.e. selecting non-human amino acid residues for importation into a human antibody background sequence in such a fashion as to retain or improve the affinity of the non-human donor antibody for a given antigen.

It is another object of this invention to provide humanized antibodies capable of binding $p185^{HER2}$.

Other objects, features, and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

SUMMARY OF THE INVENTION

The objects of this invention are accomplished by a method for making a humanized antibody comprising amino acid sequence of an import, non-human antibody and a human antibody, comprising the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, the method of this invention comprises the additional steps of determining if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), retaining the consensus residue.

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In certain embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). If the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another embodiment of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

Certain alternate embodiments of the methods of this invention comprise obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and 103H.

In preferred embodiments, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody.

Optionally, this just-recited embodiment comprises the additional steps of following the method steps appearing at the beginning of this summary and determining whether a particular amino acid residue can reasonably be expected to have undesirable effects.

This invention also relates to a humanized antibody comprising the CDR sequence of an import, non-human antibody and the FR sequence of a human antibody, wherein an amino acid residue within the human FR sequence located at any one of the sites 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H has been substituted by another residue. In preferred embodiments, the residue substituted at the human FR site is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained. In other embodiments, no human FR residue other than those set forth in this group has been substituted.

This invention also encompasses specific humanized antibody variable domains, and isolated polypeptides having homology with the following sequences.

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMAb4D5:
DIQMTQSPSSLSASVGDRVTITCRASQD-VNTAVAWYQQKPGKAPKLLIYSASFLES-GVPSRFSGSRSGTDFTLTISSLQPEDFA-TYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5):
EVQLVESGGGLVQPGGSLRLSCAASGFNIK DTYIHWVRQAPGKGLEWVARIYPTNGYTRY ADSVKGRFTISADTSKNTAYLQMNSLRAED TAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

In another aspect, this invention provides a consensus antibody variable domain amino acid sequence for use in the preparation of humanized antibodies, methods for obtaining, using, and storing a computer representation of such a consensus sequence, and computers comprising the sequence data of such a sequence. In one embodiment, the following consensus antibody variable domain amino acid sequences are provided:

SEQ. ID NO. 3 (light chain): DDIQMTQSPSSLSAS-VGDRVTITCRASQDVSSYLAWYQQKPGKAPKLL IYAASSLESGVPSRFSGSGSGTDFTLTISSLQP EDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPG GSLRLSCAASGFTFSDYAMSWVRQAPGKGL EWVAVISENGGYTRYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAWYCSRWGGDGFYAMD VWGQGTLVTVSS

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (FIG. 1A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIG. 1B shows the comparison between the V_H domain amino acid residues of the muMAb4D5, huMAb4D5, and a consensus sequence (FIG. 1B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both FIGS. 1A and 1B use the generally accepted numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987)). In both FIG. 1A

and FIG. 1B, the CDR residues determined according to a standard sequence definition (as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are indicated by the first underlining beneath the sequences, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.

FIG. 2 shows a scheme for humanization of muMAb4D5 V_L and V_H by gene conversion mutagenesis.

FIG. 3 shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMAb4D5 (●), huMAb4D5-8 (○) and huMAb4D5-1 (□).

FIG. 4 shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_L and V_H. The CDR residues (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

FIG. 5 shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby et al., *J. Exp. Med.* 175, 217–225 (1992) with a humanized variant of this antibody (huxCD3v1). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. et al., *Sequences of Proteins of immunological Interest, 5th* edition, National Institutes of Health, Bethesda, Md., USA (1991)). The light chain sequences—muxCD3, huxCD3v1 and huKI—correspond to SEQ.ID. NOs 16, 17, and 18, respectively. The heavy chain sequences—muxCD3, huxCD3v1 and huxI—correspond to SEQ.ID.NOs 19, 26, and 21, respectively. Residues which differ between muxCD3 and huxCD3v1 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (•) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen complexes (Kabat et al., 1991; Mian, I. S. et al., *J. Mol. Biol* 217, 133–151 (1991)). The location of CDR residues according to a sequence definition (Kabat et al., 1991) and a structural definition (Chothia and Lesk, supra 1987) are shown by a line and carats (^) beneath the sequences, respectively.

FIG. 6A compares murine and humanized amino acid sequences for the heavy chain of an anti-CD18 antibody. H52H4-160 (SEQ. ID. NO. 22) is the murine sequence, and pH52–8.0 (SEQ. ID. NO. 23) is the humanized heavy chain sequence. pH52–8.0 residue 143S is the final amino acid in the variable heavy chain domain V_H, and residue 144A is the first amino acid in the constant heavy chain domain C_{H1}.

FIG. 6B compares murine and humanized amino acid sequences for the light chain of an anti-CD18 antibody. H52L6-158 (SEQ. ID. NO. 24) is the murine sequence, and pH52–9.0 (SEQ. ID. NO. 25) is the humanized light chain sequence. pH52–9.0 residue 128T is the final amino acid in the light chain variable domain V_L, and residue 129V is the first amino acid in the light chain constant domain C_L.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In general, the following words or phrases have the indicated definitions when used in the description, examples, and claims:

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. In this description and claims, the terms muMAb4D5, chMAb4D5 and huMAb4D5 represent murine, chimerized and humanized versions of the monoclonal antibody 4D5, respectively.

A humanized antibody for the purposes herein is an immunoglobulin amino acid sequence variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a FR region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin.

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are referred to herein as "import" residues, which are typically taken from an "import" antibody domain, particularly a variable domain. An import residue, sequence, or antibody has a desired affinity and/or specificity, or other desirable antibody biological activity as discussed herein.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR regions of the humanized antibody need not correspond precisely to the parental sequences, e.g., the import CDR or the consensus FR may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or FR residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences, more often 90%, and most preferably greater than 95%.

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those

skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

Residues that influence antigen binding are defined to be residues that are substantially responsible for the antigen affinity or antigen specificity of a candidate immunoglobulin, in a positive or a negative sense. The invention is directed to the selection and combination of FR residues from the consensus and import sequence so that the desired immunoglobulin characteristic is achieved. Such desired characteristics include increases in affinity and greater specificity for the target antigen, although it is conceivable that in some circumstances the opposite effects might be desired. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (although not all CDR residues are so involved and therefore need not be substituted into the consensus sequence). However, FR residues also have a significant effect and can exert their influence in at least three ways: They may noncovalently directly bind to antigen, they may interact with CDR residues and they may affect the interface between the heavy and light chains.

A residue that noncovalently directly binds to antigen is one that, by three dimensional analysis, is reasonably expected to noncovalently directly bind to antigen. Typically, it is necessary to impute the position of antigen from the spatial location of neighboring CDRs and the dimensions and structure of the target antigen. In general, only those humanized antibody residues that are capable of forming salt bridges, hydrogen bonds, or hydrophobic interactions are likely to be involved in non-covalent antigen binding, however residues which have atoms which are separated from antigen spatially by 3.2 Angstroms or less may also non-covalently interact with antigen. Such residues typically are the relatively larger amino acids having the side chains with the greatest bulk, such as tyrosine, arginine, and lysine. Antigen-binding FR residues also typically will have side chains that are oriented into an envelope surrounding the solvent oriented face of a CDR which extends about 7 Angstroms into the solvent from the CDR domain and about 7 Angstroms on either side of the CDR domain, again as visualized by three dimensional modeling.

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, Ca, C, O, Cβ) by more than about 0.2 Angstroms. Backbone atoms of CDR sequences are displaced for example by residues that interrupt or modify organized structures such as beta sheets, helices or loops. Residues that can exert a profound affect on the conformation of neighboring sequences include proline and glycine, both of which are capable of introducing bends into the backbone. Other residues that can displace backbone atoms are those that are capable of participating in salt bridges and hydrogen bonds.

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within about 2.5–3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

Immunoglobulin residues that affect the interface between heavy and light chain variable regions ("the V_L–V_H interface") are those that affect the proximity or orientation of the two chains with respect to one another. Certain residues involved in interchain interactions are already known and include V_L residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_H residues 35, 37, 39, 45, 47, 91, 93, 95, 100, and 103 (utilizing the nomenclature setforth in Kabat et al., *Sequences of Proteins of immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)). Additional residues are newly identified by the inventors herein, and include 43L, 85L, 43H and 60H. While these residues are indicated for IgG only, they are applicable across species. In the practice of this invention, import antibody residues that are reasonably expected to be involved in interchain interactions are selected for substitution into the consensus sequence. It is believed that heretofore no humanized antibody has been prepared with an intrachain-affecting residue selected from an import antibody sequence.

Since it is not entirely possible to predict in advance what the exact impact of a given substitution will be it may be necessary to make the substitution and assay the candidate antibody for the desired characteristic. These steps, however, are per se routine and well within the ordinary skill of the art.

CDR and FR residues are determined according to a standard sequence definition (Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), and a structural definition (as in Chothia and Lesk, *J. Mol. Biol.* 196:901–917 (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Throughout this description, reference is made to the numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. The Kabat numbering scheme is followed in this description.

For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab

sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in FIG. 5). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

Thus, in humanization of an import variable sequence, where one cuts out an entire human or consensus CDR and replaces it with an import CDR sequence, (a) the exact number of residues may be swapped, leaving the numbering the same, (b) fewer import amino acid residues may be introduced than are cut, in which case there will be a gap in the residue numbers, or (c) a larger number of amino acid residues may be introduced then were cut, in which case the numbering will involve the use of suffixes such as 100abcde.

The terms "consensus sequence" and "consensus antibody" as used herein refers to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass or subunit structure. The consensus sequence may be based on immunoglobulins of a particular species or of many species. A "consensus" sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

The subunit structures of the live immunoglobulin classes in humans are as follows:

Class	Heavy Chain Subclasses		Light Chain	Molecular Formula
IgG	γ	$\gamma 1, \gamma 2, \gamma 3, \gamma 4$	κ or λ	$(\gamma_2\kappa_2), (\gamma_2\lambda_2)$
IgA	α	$\alpha 1, \alpha 2$	κ or λ	$(\alpha_2\kappa_2)_n^8, (\alpha_2\lambda_2)_n^8$
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5, (\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2), (\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2), (\epsilon_2\lambda_2)$

(^8_n may equal 1, 2, or 3)

In preferred embodiments of an IgGγl human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence:

DIQMTQSPSSLSASVGDRVTITCRASQD-
VSSYLAWYQQKPGKAPKLLIYAASSLES-
GVPSRFSGSGSGTDFTLTISSLQPEDFA-
TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW
VRQAPGKGLEWVAVISENGGYTRYADSVKGRFT
ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD
GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 4).

These sequences include consensus CDRs as well as consensus FR residues (see for example in FIG. 1).

While not wishing to be limited to any particular theories, it may be that these preferred embodiments are less likely to be immunogenic in an individual than less abundant subclasses. However, in other embodiments, the consensus sequence is derived from other subclasses of human immunoglobulin variable domains. In yet other embodiments, the consensus sequence is derived from human constant domains.

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology. All sequence alignments called for in this invention are such maximal homology alignments. While such alignments may be done by hand using conventional methods, a suitable computer program is the "Align 2" program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed Dec. 9, 1991).

"Non-homologous" import antibody residues are those residues which are not identical to the amino acid residue at the analogous or corresponding location in a consensus sequence, after the import and consensus sequences are aligned.

The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel humanized anti-p185^{HER2} antibodies are provided. These novel anti-p185^{HER2} antibodies are sometimes collectively referred to herein as huMAb4D5, and also sometimes as the light or heavy chain variable domains of huMAb4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWY
QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT
DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK
VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW
VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT
ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD
GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

"Biological property", as relates for example to anti-p185^{HER2}, for the purposes herein means an in vivo effector or antigen-binding function or activity that is directly or indirectly performed by huMAb4D5 (whether in its native or denatured conformation). Effector functions include p185^{HER2} binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist or antagonist activity, any

cytotoxic activity. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAb4D5.

Biologically active huMAb4D5 is defined herein as a polypeptide that shares an effector function of huMAb4D5. A principal known effector function of huMAb4D5 is its ability to bind to $p185^{HER2}$.

Thus, the biologically active and antigenically active huMAb4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAb4D5; mature huMAb4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from muMAb4D5 plus residues from the human FR of huMAb4D5; amino acid sequence variants of huMAb4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAb4D5 or its fragment as defined above; amino acid sequence variants of huMAb4D5 or its fragment as defined above wherein an amino acid residue of huMAb4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-directed or PCR mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAb4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C. 102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide 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 polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein 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 gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant cells since at least one component of the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAb4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAb4D5, is complementary to nucleic acid sequence encoding such huMAb4D5, or hybridizes to nucleic acid sequence encoding such huMAb4D5 and remains stably bound to it under stringent conditions, and comprises nucleic acid from a muMAb4D5 CDR and a human FR region.

Preferably, the huMAb4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with the huMAb4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAb4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% $NaDodSO_4$ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C., with washes at 42 C. in 0.2×SSC and 0.1% SDS.

The term "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, a ribosome binding site, and possibly, other as yet poorly understood sequences. 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, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

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.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published May

4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., *Nucl. Acids Res.*, 14: 5399–5407 [1986]). They are then purified on polyacrylamide gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued Jul. 28, 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, 51: 263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, N.Y., 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

Suitable Methods for Practicing the Invention

Some aspects of this invention include obtaining an import, non-human antibody variable domain, producing a desired humanized antibody sequence and for humanizing an antibody gene sequence are described below. A particularly preferred method of changing a gene sequence, such as gene conversion from a non-human or consensus sequence into a humanized nucleic acid sequence, is the cassette mutagenesis procedure described in Example 1. Additionally, methods are given for obtaining and producing antibodies generally, which apply equally to native non-human antibodies as well as to humanized antibodies.

Generally, the antibodies and antibody variable domains of this invention are conventionally prepared in recombinant cell culture, as described in more detail below. Recombinant synthesis is preferred for reasons of safety and economy, but it is known to prepare peptides by chemical synthesis and to purify them from natural sources; such preparations are included within the definition of antibodies herein.

Molecular Modeling

An integral step in our approach to antibody humanization is construction of computer graphics models of the import and humanized antibodies. These models are used to determine if the six complementarity-determining regions (CDRs) can be successfully transplanted from the import framework to a human one and to determine which framework residues from the import antibody, if any, need to be incorporated into the humanized antibody in order to maintain CDR conformation. In addition, analysis of the sequences of the import and humanized antibodies and reference to the models can help to discern which framework residues are unusual and thereby might be involved in antigen binding or maintenance of proper antibody structure.

All of the humanized antibody models of this invention are based on a single three-dimensional computer graphics structure hereafter referred to as the consensus structure. This consensus structure is a key distinction from the approach of previous workers in the field, who typically begin by selecting a human antibody structure which has an amino acid sequence which is similar to the sequence of their import antibody.

The consensus structure of one embodiment of this invention was built in five steps as described below.

Step 1

Seven Fab X-ray crystal structures from the Brookhaven Protein Data Bank were used (entries 2FB4, 2RHE, 3FAB, and 1 REI which are human structures, and 2MCP, 1 FBJ, and 2HFL which are murine structures). For each structure, protein mainchain geometry and hydrogen bonding patterns were used to assign each residue to one of three secondary structure types: alpha-helix, beta-strand or other (i.e. non-helix and non-strand). The immunoglobulin residues used in superpositioning and those included in the consensus structure are shown in Table 1.

TABLE I

	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
				$V_L\kappa$ domain				
								2–11
	18–24	18–24	19–25	18–24	19–25	19–25	19–25	16–27
	32–37	34–39	39–44	32–37	32–37	32–37	33–38	33–39
								41–49
	60–66	62–68	67–72	53–66	60–65	60–65	61–66	59–77
	69–74	71–76	76–81	69–74	69–74	69–74	70–75	
	84–88	86–90	91–95	84–88	84–88	84–88	85–89	82–91
								101–105
RMS[c]		0.40	0.60	0.53	0.54	0.48	0.50	
				V_H domain				
								3–8
	18–25		18–25	18–25	18–25	18–25		17–23
	34–39		34–39	34–39	34–39	34–39		33–41
	46–52		46–52	46–52	46–52	46–52		45–51
	57–61		59–63	56–60	57–61	57–61		57–61
	68–71		70–73	67–70	68–71	68–71		66–71
	78–84		80–86	77–83	78–84	78–84		75–82
	92–99		94–101	91–98	92–99	92–99		88–94
								102–108

TABLE I-continued

	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
RMS[c]			0.43	0.85	0.62	0.91		
RMS[d]	0.91		0.73	0.77	0.92			

[a]Four-letter code for Protein Data Bank file.
[b]Residue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.
[c]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.
[d]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2HFL.

Step 2

Having identified the alpha-helices and beta-strands in each of the seven structures, the structures were superimposed on one another using the INSIGHT computer program (Biosym Technologies, San Diego, Calif.) as follows: The 2FB4 structure was arbitrarily chosen as the template (or reference) structure. The 2FB4 was held fixed in space and the other six structures rotated and translated in space so that their common secondary structural elements (i.e. alpha-helices and beta-strands) were oriented such that these common elements were as close in position to one another as possible. (This superpositioning was performed using accepted mathematical formulae rather than actually physically moving the structures by hand.)

Step 3

With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, is if all Cα-Cα distances for a given residue position were $\leqq$1.0 Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was >1.0 Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4

For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S. J. et. al., *J. Amer. Chem. Soc.*, 106: 765–784 (1984).

Step 5

In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S. J. et. al., *J. Amer. Chem. Soc.*, 106: 765–784 (1984)) parameter set with only the Ca coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

TABLE II

Average Bond Lengths and Angles for "Average" (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures

	$V_L\kappa$ before (Å)	$V_L\kappa$ after (Å)	V_H before (Å)	V_H after (Å)	Standard Geometry (Å)
N—Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα-C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C—N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα-Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(°)	(°)	(°)	(°)	(°)
C—N—Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N—Cα-C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα-C—N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C—N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N—Cα-Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ-Cα-C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)	111.1

Values in parentheses are standard deviations. Note that while some bond length and angle averages did not change appreciably after energy-minimization, the corresponding standard deviations are reduced due to deviant geometries assuming standard values after energy-minimization. Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

The consensus structure might conceivably be dependent upon which crystal structure was chosen as the template on which the others were superimposed. As a test, the entire procedure was repeated using the crystal structure with the worst superposition versus 2FB4, i.e. the 2HFL Fab structure, as the new template (reference). The two consensus structures compare favorably (root-mean-squared deviation of 0.11 Å for all N, Cα and C atoms).

Note that the consensus structure only includes mainchain (N, Cα, C, O, Cβ atoms) coordinates for only those residues which are part of a conformation common to all seven X-ray crystal structures. For the Fab structures, these include the common β-strands (which comprise two β-sheets) and a few non-CDR loops which connect these β-strands. The consensus structure does not include CDRs or sidechains, both of which vary in their conformation among the seven structures. Also, note that the consensus structure includes only the V_L and V_H domains.

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure

the model of any import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody V_L and V_H domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193: 775–791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

CDRs are added to the model (now having the backbone plus side chains) as follows. The size (i.e. number of amino acids) of each import CDR is compared to canonical CDR structures tabulated by Chothia et al., *Nature,* 342:877–883 (1989)) and which were derived from Fab crystals. Each CDR sequence is also reviewed for the presence or absence of certain specific amino acid residues which are identified by Chothia as structurally important: e.g. light chain residues 29 (CDR1) and 95 (CDR3), and heavy chain residues 26, 27, 29 (CDR1) and 55 (CDR2). For light chain CDR2, and heavy chain CDR3, only the size of the CDR is compared to the Chothia canonical structure. If the size and sequence (i.e. inclusion of the specific, structurally important residues as denoted by Chothia et al.) of the import CDR agrees in size and has the same structurally important residues as those of a canonical CDR, then the mainchain conformation of the import CDR in the model is taken to be the same as that of the canonical CDR. This means that the import sequence is assigned the structural configuration of the canonical CDR, which is then incorporated in the evolving model.

However, if no matching canonical CDR can be assigned for the import CDR, then one of two options can be exercised. First, using a program such as INSIGHT (Biosym Technologies), the Brookhaven Protein Data Bank can be searched for loops with a similar size to that of the import CDR and these loops can be evaluated as possible conformations for the import CDR in the model. Minimally, such loops must exhibit a conformation in which no loop atom overlaps with other protein atoms. Second, one can use available programs which calculate possible loop conformations, assuming a given loop size, using methods such as described by Bruccoleri et al., *Nature* 335: 564–568 (1988).

When all CDRs and sidechains have been added to the consensus structure to give the final model (import, human or humanized), the model is preferably subjected to energy minimization using programs which are available commercially (e.g. DISCOVER, Biosym Technologies). This technique uses complex mathematical formulae to refine the model by performing such tasks as checking that all atoms are within appropriate distances from one another and checking that bond lengths and angles are within chemically acceptable limits.

Models of a humanized, import or human antibody sequence are used in the practice of this invention to understand the impact of selected amino acid residues of the activity of the sequence being modeled. For example, such a model can show residues which may be important in antigen binding, or for maintaining the conformation of the antibody, as discussed in more detail below. Modeling can also be used to explore the potential impact of changing any amino acid residue in the antibody sequence.

Methods for Obtaining a Humanized Antibody Sequence

In the practice of this invention, the first step in humanizing an import antibody is deriving a consensus amino acid sequence into which to incorporate the import sequences. Next a model is generated for these sequences using the methods described above. In certain embodiments of this invention, the consensus human sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and have the sequences indicated in the definitions above.

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol Bol.* 196:901–917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

Differences between the non-human import and the human consensus framework residues are individually investigated to determine their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is desirably performed through modeling, by examination of the characteristics of the amino acids at particular locations, or determined experimentally through evaluating the effects of substitution or mutagenesis of particular amino acids.

In certain preferred embodiments of this invention, a humanized antibody is made comprising amino acid sequence of an import, non-human antibody and a human antibody, utilizing the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least

one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, one determines if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), one may retain the consensus residue.

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In preferred embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). if the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human sequence if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another preferred embodiment of the methods of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

In certain alternate embodiments, one need not utilize the modeling and evaluation steps described above, and may instead proceed with the steps of obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. fin the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

If after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one preferably reexamines the potential effects of the amino acids at the specific locations recited above. Additionally, it is desirable to reinvestigate any buried residues which are reasonably expected to affect the V_L–V_H interface but may not directly affect CDR conformation. It is also desirable to reevaluate the humanized antibody utilizing the steps of the methods claimed herein.

In certain embodiments of this invention, amino acid residues in the consensus human sequence are substituted for by other amino acid residues. In preferred embodiments, residues from a particular non-human import sequence are substituted, however there are circumstances where it is desired to evaluate the effects of other amino acids. For example, if after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one may compare the sequences of other classes or subgroups of human antibodies, or classes or subgroups of antibodies from the particular non-human species, and determine which other amino acid side chains and amino acid residues are found at particular locations and substituting such other residues.

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with the ATCC (as described below). Thus, the references throughout this description to the use of monoclonal antibodies are intended to include the use of natural or native antibodies as well as humanized and chimeric antibodies. As used herein, the term "antibody" includes the antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import antibodies) are isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. in certain embodiments, the antibodies of this invention are obtained by routine screening. Polyclonal antibodies to an antigen generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence 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 is anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

The route and schedule of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of 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 ⅕ to ⅒ the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen 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 agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodiesare prepared by recovering immune lymphoid cells—typically spleen cells or lymphocytes from lymph node tissue—from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J. Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in vitro in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored. and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, Ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM as the case may be that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g. ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the antigen in test samples.

While routinely rodent monoclonal antibodies are used as the source of the import antibody, the invention is not limited to any species. Additionally, techniques developed for the production of chimeric antibodies (Morrison et al., *Proc. Natl. Acad. Sci.,* 81:6851 (1984); Neuberger et al., *Nature* 312:604 (1984); Takeda et al., *Nature* 314:452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger ANA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the CDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional FAb fragments for those which bind the antigen. Such FAb fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

Amino Acid Sequence Variants

Amino acid sequence variants of the antibodies and polypeptides of this invention (referred to in herein as the target polypeptide) are prepared by introducing appropriate nucleotide changes into the DNA encoding the target polypeptide, or by in vitro synthesis of the desired target polypeptide. Such variants include, for example, humanized variants of non-human antibodies, as well as deletions from, or insertions or substitutions of, residues within particular amino acid sequences. Any combination of deletion, insertion, and substitution can be 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 target polypeptide, such as changing the number or position of glycosylation sites, altering any membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader sequence of the native target polypeptide.

In designing amino acid sequence variants of target polypeptides, the location of the mutation site and the nature of the mutation will depend on the target polypeptide characteristics) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1–3. In certain embodiments, these choices are guided by the methods for creating humanized sequences set forth above.

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesism" 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 the surrounding aqueous environment in or outside the cell. Those domains 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 optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the

expressed target polypeptide variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. In general, the location and nature of the mutation chosen will depend upon the target polypeptide characteristic to be modified.

Amino acid sequence deletions of antibodies are generally not preferred, as maintaining the generally configuration of an antibody is believed to be necessary for its activity. Any deletions will be selected so as to preserve the structure of the target antibody.

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. Intrasequence insertions (i.e., insertions within the target polypeptide sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include the target polypeptide with an N-terminal methionyl residue, an artifact of the direct expression of target polypeptide in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the target polypeptide molecule to facilitate the secretion of the mature target polypeptide from recombinant host cells. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli,* alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published Apr. 6, 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the target polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of the target polypeptide, and sites where the amino acids found in the target polypeptide from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites for substitution are described infra, considering the effect of the substitution of the antigen binding, affinity and other characteristics of a particular target antibody.

Other sites of interest are those in which particular residues of the target polypeptides obtained from various species are identical. These positions may be important for the biological activity of the target polypeptide. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. If such substitutions result in a change in biological activity, then other changes are introduced and the products screened until the desired effect is obtained.

Substantial modifications in function or immunological identity of the target polypeptide 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, lb) 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, gin, 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. Such substituted residues may be introduced into regions of the target polypeptide that are homologous with other antibodies of the same class or subclass, or, more preferably, into the non-homologous regions of the molecule.

Any cysteine residues not involved in maintaining the proper conformation of target polypeptide also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the target polypeptide is 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 target polypeptide. A particularly preferred method of gene conversion mutagenesis is described below in Example 1. These techniques may utilized target polypeptide nucleic acid (DNA or RNA), or nucleic acid complementary to the target polypeptide nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of target polypeptide DNA. This technique is well known in the art as described by Adelman et al., *DNA,* 2: 183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the target polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the target polypeptide DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci. USA,* 75: 5765 [1978]).

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase 1, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for

synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding target polypeptide variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, supra, the chapter by R. Higuchi, p. 61–70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.), and 25 pmole of each oligonucleotide primer, to a final volume of 50μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100° C., placed briefly on ice, and then 1 μl *Thermus aquaticus* (Taq) DNA polymerase (5 units/μl, purchased from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55° C., then 30 sec. at 72° C., then 19 cycles of the following: 30 sec. at 94° C., 30 sec. at 55° C., and 30 sec. at 72° C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene,* 34: 315 [1985]). The starting material is the plasmid (or other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the target polypeptide DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the target polypeptide DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. 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.

(a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(b) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using Bacillus species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. However, the recovery of genomic DNA encoding the target polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for Bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., *J. Molec. Appl. Genet.,* 1: 327 [1982]), mycophenolic acid (Mulligan et al., *Science:* 1422 [1980]) or hygromycin (Sugden et al., *Mol. Cell. Biol.,* 5: 410–413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants

under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the target polypeptide. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature,* 22: 39 [1979]; Kingsman et al., *Gene,* 7: 141 [1979]; or Tschemper et al., *Gene,* 10: 157 [1980]). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 5: 12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

(d) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the target polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding the target polypeptide, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the target polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native target polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target polypeptide as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature,* 275: 615 [1978]; and Goeddel et al., *Nature,* 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.,* 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the tao promoter (deBoer et al., *Proc. Natl. Acad. Sci, USA,* 80: 21–25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist et al., *Cell,* 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.,* 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.,* 2: 149 [1968]; and Holland, *Biochemistry,* 17: 4900 [1978]), such asenolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also ate advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that

also contains the SV40 viral origin of replication. Fiers et al., *Nature,* 273:113 (1978); Mulligan and Berg, *Science,* 209: 1422–1427 (1980); Pavlakis et al., *Proc. Natl. Acad. Sci. USA,* 78: 7398–7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene,* 18: 355–360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature,* 29: 503–508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes et al., *Nature,* 297: 598–601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA,* 79: 5166–5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Aced. Sci. USA,* 79: 6777–6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10–300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., *Proc. Natl. Acad. Sci. USA,* 78: 993 [1981]) and 3' (Lusky et al., *Mol. Cell Bio.* 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., *Cell,* 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., *Mol. Cell Bio.,* 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100–270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature,* 297: 17–18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target polypeptide. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced is by the method of Messing et al., *Nucleic Acids Res.,* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature,* 293: 620–625 [1981]; Mantei et al., *Nature,* 281: 40–46 [1979]; Levinson et al.,; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli* , Bacilli such as *B. subtilis*, Pseudomonas species such as *P. aeruginosa, Salmonella typhimurium,* or *Serratia marcescans*. 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. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for target polypeptide-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* [Beach and Nurse, *Nature,* 290: 140 (1981); EP 139,383 published May 2, 1985], Kluyveromyces hosts (U.S. Pat. No. 4,943,529) such as, e.g., *K. lactis* [Louvencourt et al., *J. Bacteriol.,* 737 (1983)], *K. fragilis, K. bulgaricus, K. thermotolerans,* and *K. marxianus,* yarrowia [EP 402,226], *Pichia pastoris* [EP 183,070; Sreekrishna et al., *J. Basic Microbiol.,* 28: 265–278 (1988)], Candida, *Trichoderma reesia* [EP 244,2341], *Neurospora crassa* [Case et al., *Proc. Natl. Acad. Sci. USA,* 76: 5259–5263 (1979)], and filamentous fungi such as, e.g, Neurospora, Penicillium, Tolypocladium [WO 91/00357 published Jan. 10, 1991], and Aspergillus hosts such as *A. nidulans* [Ballance et al., *Biochem. Biophys. Res. Commun.* 112: 284–289 (1983); Tilburn et al., *Gene,* 26: 205–221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA,* 81: 1470–1474 (1984)] and *A. niger* [Kelly and Hynes, *EMBO J.,* 4: 475–479 (1985)].

Suitable host cells for the expression of glycosylated target polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. 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* host cells have been identified. See, e.g., Luckow et al., *Bio/Technologvy* 6: 47–55 (1988); Miller et al., in *Genetic Engineering* Setlow, J. K. et a., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277–279; and Maeda et al., *Nature,* 315: 592–594 (1985). A variety of such viral strains 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 cutures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens,* which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens,* the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., *J. Mol. Appl. Gen.,* 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. 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 and Chasin, *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, HS 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 cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene,* 23: 315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30–16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.,* 130: 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA),* 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturina the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the target polypeptide 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 and Wallace, *Meth. Enz.,* 58: 44 (1979), Barnes and Sato, *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. 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), nucleosides (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.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field.

For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expression, as desired.

Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77: 5201–5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., *Am. J. Clin. Path.*, 75: 734–738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of the Target Polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to absorb the target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,3-nitro-2-pyridyl disulfide, methyl2-pyridyldisulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5–7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal;

chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in so introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'—N=C=N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis (diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modification include hydroxylation of proline and lysine, phophorylation of hydroxyl groups of seryl or threonyl resides, methylation of the α-amino groups of lysine, arginine, and histidine side chains, (T. E. Creighton, *Protein: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco, pp. 79–86 [1983]), acetylation of the N-terminal amine, and amidaatioon of any C-terminal carboxyl group.

Another type of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glucosylatuion pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or adding one or more glycosylation sites that are not present in the native target polypeptide.

Gylcosylation of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbonhydrate moiety to the side chain of an asparagine reisdue. The tri-peptide sequences asparagine-X-resine and asparagine-X-threonine, where X is any aminoe acid except proline, are the recoginition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgactosamine, 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 target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of hte above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or theonine resides to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequences is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that condons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling glycosides to the polypeptides. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the couple mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (*CRC Crit. Rev. Biochem., pp.* 259–306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (*Arch. Biochem. Biophys.,* 259:52 [1987]) and by Edge et al. (*Anal. Biochem.,* 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (*Meth. Enzymol.* 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (*J. Biol. Chem.,* 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the

manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate]microcapsules, respectively), in colloidal drug deliverysystems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Reminaton's Pharmaceutical Sciences,* 16th edition, Osol, A., Ed., (1980).

Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antigen or antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for the target polypeptide in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), 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, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotintavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalenily to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al., *Nature,* 144: 945 (1962); David et al., *Biochemistry,* 13: 1014–1021 (1974); Pain et al., *J. Immunol. Methods,* 40: 219–230 (1981); and Nygren, *J. Histochem. and Cytochem.,* 30: 407–412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in in Enzymology,* ed. J. J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, N.Y., 1981), pp. 147–166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-

response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. U.S. patent application Ser. No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection. The methods of this invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are 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. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., *Science* 238:1098 (1987).

When used to kill infected human cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver). In another embodiment, whole ricin (A chain plus B chain) is conjugated to antibody if the galactose binding property of B-chain can be blocked ("blocked ricin").

In a further embodiment toxin-conjugates are made with Fab or $F(ab')_2$ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uananue and Benacerraf,

Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and lgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and lgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or AOCC can be assayed. The cells of interest are grown and labeled in vitro; the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the in vitro test can then be used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody domains prepared and used according to the teachings of this invention.

Therapeutic and Other Uses of the Antibodies

When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1–2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

There is evidence that complement activation in vivo leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology,* 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom et al., *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski et al., *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients, as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are prepared for administration according to the description of preparation of polypeptides for administration, infra.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the American Type Culture Collection, 10801 University Blvd., Mauassas, Va., USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the international Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures' availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed

when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

Example 1

Humanization of muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for $p185^{HER2}$ ECD and anti-proliferative activity against $p185^{HER2}$ overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) as described by Orlandi et al. (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989); Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCC GATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT GGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), PstI and V_H anti-sense, 5'-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID. NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., *J. Mol. Biol.* 141:369–391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Siosym Technologies). The distance from the template Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα distances for a given residue were ≦1 Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, C, O and Cβ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., *J. Amer. Chem. Soc.* 106:765–784 (1984)) and Cα coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., *Nature* 342:877–883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775–791 (1987)) and packing considerations. Since V_H-COR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 model into the consensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but notas defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)):

V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (FIG. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the $p185^{HER2}$ ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing ihe human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990)). Briefly, gene segments encoding muMAb4D5 V_L (FIG. 1A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167–191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (FIG. 1B) and human γ1 constant region (Capon, D. J. et al., *Nature* 337:525–531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology,* Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323–327 (1988)). The PCR-generated V_L and V_H fragments (FIG. 1) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V_{104}L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071–4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) to create pAK1 and simultaneously humanized in a single step (FIG. 2). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (FIG. 1). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154: 382–403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100° C. to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14° C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. et al., *Methods Enzymol.* 154:367–382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71–18 mutL as previously described (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using XhoI and then for huV_H by restriction selection using StuI as described in Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. et al., *Phil. Trans. R. Soc. Lond., A* 317:415–423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. et al., *J. Gen. Virol.* 36:59–72 (1977)) using a high efficiency procedure (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990); Gorman, C., in *DNA Cloning,* vol II, pp 143–190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4° C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the $p185^{HER2}$ ECD prepared as described in Fendly, B. M. et al., *J. Biol. Resp. Mod.* 9:449–455 (1990). Briefly, antibody and $p185^{HER2}$ ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized $p185^{HER2}$ ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., *J. Immunol. Methods* 77:305–319 (1985)).

Results

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (FIG. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (FIG. 2). A 311-mer oligonucle-

otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: <3 nucleotide changes and <1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized $p185^{HER2}$ ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see FIG. 1) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) and CDR residues from muMAb4D5. Additional variants were constructed by replacing selected human residues in huMAb4D5-1 with their muMAb4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold more tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (FIG. 3). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but an arginine occupies this position in the muMAb4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68–69 (FIG. 4). Consistent with the importance of this residue, the mutation V_L G66R (huMAb4D5-3→huMAb4D5-5) increases the affinity for the $p185^{HER2}$ ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L–V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for $p185^{HER2}$ ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress $p185^{HER2}$ (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (Kd_d=0.1 μM) and its human IgG_1 subtype. Table 4 compares the ADCC mediated by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of $p185^{HER2}$ and on SK-BR-3, which expresses a high level of $p185^{HER2}$. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress $p185^{HER2}$.

Discussion

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded $p185^{HER2}$ receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to $p185^{HER2}$ ECD ($K_d \leq 1$ nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates ADCC against human tumor cell lines overexpressing $p185^{HER2}$ in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., *FEBS Lett.* 249: 379–382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the $p185^{HER2}$ ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a simple function of their binding affinity for $p185^{HER2}$ ECD. For example the huMAb4D5–8 variant binds $p185^{HER2}$ 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-SR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress $p185^{HER2}$ allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

TABLE 3

$p185^{HER2}$ ECD binding affinity and anti-proliferative activities of MAb4D5 variants

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K_d† nM	Relative
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thre	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

*Human and murine residues are shown in one letter and three letter amino acid code respectively.
†K_d values for the $p185^{HER2}$ ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is ≦ ± 10%.
‡Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., Molec. Cell. Biol. 9: 1165–1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see FIG. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≦ ± 15%.

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

Effector:Target ratio†	WI-38* muMAb4D5	WI-38* huMAb4D5-8	SK-BR-3 muMAb4D5	SK-BR-3 huMAb4D5-8
A.‡				
25:1	<1.0	9.3	7.5	40.6
12.5:1	<1.0	11.1	4.7	36.8
6.25:1	<1.0	8.9	0.9	35.2
3.13:1	<1.0	8.5	4.6	19.6
B.				
25:1	<1.0	3.1	6.1	33.4
12.5:1	<1.0	1.7	5.5	26.2
6.25:1	1.3	2.2	2.0	21.0
3.13:1	<1.0	0.8	2.4	13.4

*Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of $p185^{HER2}$ (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of $p185^{HER2}$ (64 pg $p185^{HER2}$ per μg cell protein), as determined by ELISA (Fendly et al., J. Biol. Resp. Mod. 9:449–455 (1990)).
†ADCC assays were carried out as described in Brüggemann et al., J. Exp. Med. 166:1351–1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37° C. Values given represent percent specific cell lysis as determined by ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≦±10%.
‡Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/ml (B).

Example 2
Schematic Method for Humanizing an Antibody Sequence

This example illustrates one stepwise elaboration of the methods for creating a humanized sequence described

above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
   a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
   b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
      i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.
      ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.
         a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.
            (i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L–V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.
7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.
8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.
   a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):
      i. Variable light domain: 36, 46, 49*, 63–70
      ii. Variable heavy domain: 2, 47*, 68, 70, 73–76.
   b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L=LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
      i. Variable light domain:
         a) CDR-1 (residues 24L–34L): 2L, 4L, 66L-69L, 71L
         b) CDR-2 (residues 50L–56L): 35L, 46L, 47L, 48L, 49L, 58L, 62L, 64L–66L, 71L, 73L
         c) CDR-3 (residues 89L–97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H
      ii. Variable heavy domain:
         a) CDR-1 (residues 26H–35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, 94H
         b) CDR-2 (residues 50H–55H): 49H, 69H, 69H, 71H, 73H, 78H
         c) CDR-3 (residues 95H–102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.
9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L–V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

Example 3

Engineering a Humanized Bisnecific F(ab')$_2$ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')$_2$v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')$_2$v1 (anti-CD3/anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human

CD3+CTL in vitro against the human breast tumor cell line, SK-BR-3, which overexpresses the p185^{HER2} product of the protooncogene HER2. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-p185^{HER2}arm of BsF(ab')$_2$v1. In contrast BsF(ab')$_2$ v1 binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')$_2$ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')$_2$ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Ss F(ab')$_2$v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')$_2$v1 with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')$_2$v9 binds to T cells (Jurkat) much more efficiently than does BsF(ab')$_2$v1 and almost as efficiently as the chimeric BsF(ab')$_2$. This improvement in the efficiency of T cell binding of the humanized BsF(ab')$_2$ is an important step in its development as a potential therapeutic agent for the treatment of p185^{HER2}-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both in vitro and in vivo (reviewed by Fanger, M. W. et al., *Immunol. Today* 10: 92–99 (1989); Fanger, M. W. et al., *Immunol. Today* 12: 51–54 (1991); and Nelson, H., *Cancer Cells* 3: 163–172 (1991)). BsF(ab')$_2$ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')$_2$ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* 79: 315–321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1–11 (1990)).

BsF(ab')$_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. et al., *Science* 229, 81–83 (1985) and Glennie, M. J. et al., *J. Immunol.* 139: 2367–2375 (1987)). One such BsF(ab')$_2$ fragment (anti-glioma associated antigen/anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. et al., *Lancet* 335: 368–371 (1990) and another BsF(ab')$_2$ (anti-indium chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. et al., *Antibody, Immunoconj. Radiopharm.* 2: 1–13 (1989)). Future SsF(ab')$_2$ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. et al., *Nature* 332: 323–327 (1988) to reduce their immunogenicity (Hale, G. et al., *Lancet i:* 1394–1399 (1988)).

Recently a facile route to a fully humanized BsF(ab')$_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. et al., *J. Exp. Med.* 175: 217–225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling in vitro to form the BsF(ab')$_2$. One arm of the BsF(ab')$_2$ was a humanized version (Carter, P. et al., *Proc. Natl. Aced. Sci. USA* (1992a) and Carter, P., et al., *Bio/Technology* 10: 163–167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the p185^{HER2} product of the protooncogene HER2 (c-erbB-2) (Fendly, B. M. et al. *Cancer Res.* 50: 1550–1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby et al. supra) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329–334 (1981)) into the humanized anti-p185^{HER2} antibody. The BsF(ab')$_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing p185^{HER2} and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF(ab')$_2$v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing p185^{HER2}. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

Materials and Methods

Construction of Mutations in the Anti-CD3 Variable Region Genes

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_1) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby et al. supra). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach,* (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest,* 5th edition, National Institutes of Health, Bethesda, Md., USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcA-gAaatTCAAGGatCGTTTCACgATAtc-CGTAGATAAATCC 3' (SEO.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_L E55H, v11.

Oliconucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad, Sci. USA* 74: 5463–5467 (1977)).

E. coli Expression of Fab' Fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-p185^{HER2} variant, HuMAb4D5-8, is described in Carter et al., 1992b, supre. Briefly, the Fab' expression unit is bicistronic with both chains under the

transcriptional control of the ohoA promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human k_1 C_L and IgG1C_H1 constant domain genes, respectively. The C_H1gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_o transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby et al.,supra; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37° C. in an aerated 10 liter fermentor. The final cell density was 120–150 OD_{550} and the titer of soluble and functional anti-p185^{HER2} Fab' was 1–2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, suora). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3 variants were 200 mgaliter and 700 mgaliter, respectively, as judged by total immunoglobulin ELISA.

Construction of $BsF(ab')_2$ Fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra). Thioether linked $BsF(ab')_2$ fragments (anti-p185^{HER2}/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (o-PDM) in dimethyl formamide for ~1.5 hr at 20° C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1\%}$=1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5,5' -dithiobis (2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach,* (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4° C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20 ° C. to reduce any unwanted disulfide-linked $F(ab')_2$ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. $BsF(ab')_2$ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm×100 cm) in the presence of PBS. The $BsF(ab')_2$ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at −70° C.

Flow Cytometric Analysis of $F(ab')_2$Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as. recommended by the ATCC. Aliquots of 10^6 Jurkat cells were incubated with appropriate concentrations of $BsF(ab')_2$ (anti-p185^{HER2}/anti-CD3 variant) or control mono-specific anti-p185^{HER2} $F(ab')_2$ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4° C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human $F(ab')_2$ (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8×10^3) were acquired by list mode and gated by forward light scatter versus side light scatter excluding dead cells and debris.

Results

Design of Humanized anti-CD3 Variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby et al.,supra) 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (SEQ ID NO:20). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v_1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of $BsF(ab')_2$ Fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75–100% Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b, supra). Thioether-linked $BsF(ab')_2$ fragments were then constructed by directed coupling using o-PDM as described by Glennie et al., supra. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with o-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. $F(ab')_2$ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation ($BsF(ab')_2$ v8) in data not shown. The $F(ab')_2$ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this $BsF(ab')_2$v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kD) as well as several very minor

bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L/V_H: D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for $BsF(ab')_2$. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that $F(ab')_2$ constructed by directed chemical coupling carry both anti-$p185^{HER2}$ and anti-CD3 antigen specificities (Shalaby et al., supra). The level of contamination of the $BsF(ab')_2$ with monospecific $F(ab')_2$ iS likely to be very low since mock coupling reactions with either anti-$p185^{HER2}$ w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of $F(ab')_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked $F(ab')_2$ that might be present. SDS-PAGE of the purified $F(ab')_2$ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a o-PDM coupled $F(ab')_2$ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect $F(ab')_2$ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of $BsF(ab')_2$ to Jurkat Cells

Binding of $BsF(ab')_2$ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). $BsF(ab')_2v9$ binds much more efficiently to Jurkat cells than does our starting molecule, $BsF(ab')_2v1$, and almost as efficiently as the chimeric $BsF(ab')_2$. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_HK75S:N76S) and v12 (V_HK75S:N76S plus V_L E55H) did not further improve binding of corresponding $BsF(ab')_2$to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_HK75S (v6), V_HN76S (v7), V_HK75S:N76S (V8), V_LE55H (v11) (not shown). $BsF(ab')_2v9$ was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-$p185^{HER2}$ $F(ab')_2$ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

Discussion

A minimalistic strategy was chosen to humanize the anti-$p185^{HER2}$ (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the $BsF(ab')_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might not be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-$p185^{HER2}$ antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the $p185^{HER2}$ antigen ~3-fold more tightly than the parent murine antibody (Carter et al., 1992a, supra). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60–65, were discarded in favor of human counterparts. In contrast, $BsF(ab')_2v1$ containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby et al., supra) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric $BsF(ab')_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, FIG. 5). It appears more likely that these murine residues enhance antigen binding indirectly by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by directly contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50–58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat et al., supra; and Mian, I. S. et al., *J. Mol. Biol* 217: 133–151 (1991), FIG. 5). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (FIG. 5). $BsF(ab')_2v9$ binds to SK-BR-3 breast tumor cells with equal efficiency to $BsF(ab')_2v1$ and chimeric $BsF(ab')_2$ as anticipated since the anti-$p185^{HER2}$ arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of $BsF(ab')_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter et al., 1992b, supra). Traditional directed chemical coupling of Fab'-SH fragments is then used to form $BsF(ab')_2$ in vitro (Brennan et al.,supra; and Glennie et al., supra). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in $BsF(ab')_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of $F(ab')_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie et al., supra with future in vivo testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked $F(ab')_2$ may be more stable than disulfide-linked $F(ab')_2$ in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked $BsF(ab')_2$ v1 has a 3-fold longer plasma residence time than $BsF(ab')_2$ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric $BsF(ab')_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the

$BsF(ab')_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked $BsF(ab')_2$ (murine anti-$p185^{HER2}$/murine anti-CD3) was recently shown by others (Nishimura et al., Int. *J. Cancer* 50: 800–804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby et al., supra) together with this one and that of Nishimura, T. et al., supra improve the potential for using $BsF(ab')_2$ in targeted immunotherapy of $p185^{HER2}$-overexpressing cancers in humans.

Example 4

Humanization of an anti-CD18 Antibody

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized following the methods described above. FIGS. 6A and 6B provide amino acid sequence comparisons for the murine and humanized antibody light chains and heavy chains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
             20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
             35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
             50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
             65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
             80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
             95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
             20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
             35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
             50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
             65                  70                  75
```

-continued

```
Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                95                 1 00                 1 05

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115                  120
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
                20                  25                  30

Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                20                  25                  30

Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gly Ala Val Ser
                95                 1 00                 1 05

Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115                  120
```

-continued

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
1 5 10 15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
20 25 30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys Arg Ala
109

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
35 40 45

Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
65 70 75

Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 1 00 1 05

Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCGATATCC AGCTGACCCA GTCTCCA 27

-continued

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

-continued

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50

ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
1 5 10 15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
35 40 45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
50 55 60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
65 70 75

Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
80 85 90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys
107

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys

-continued

```
                35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Gly Asn Thr Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                  1 00                1 05

Ile Lys
    107
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1                 5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                20                  25                  30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                  1 00                1 05

Ile Lys
    107
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
  1                 5                  10                  15

Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
                35                  40                  45

Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                50                  55                  60

Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
                65                  70                  75

Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
                80                  85                  90
```

-continued

```
Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                  1 00                1 05

Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val
               110                  115                 120

Ser Ser
    122
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                50                  55                  60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                  1 00                1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
               110                  115                 120

Ser Ser
    122
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                  1 00                1 05

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
               110                  115                 120
```

-continued

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
50 55 60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
80 85 90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 1 00 1 05

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140 145 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
320 325 330

-continued

```
Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                335                 340                 345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                350                 355                 360

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                365                 370                 375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                380                 385                 390

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
                395                 400                 405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                410                 415                 420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                425                 430                 435

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                440                 445                 450

Ser Pro Gly Lys
            454
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 469 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1                 5                  10                  15

Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu
                 20                  25                  30

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly
                 35                  40                  45

Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro
                 50                  55                  60

Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly
                 65                  70                  75

Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser
                 80                  85                  90

Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu
                 95                 1 00                 1 05

Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly
                110                 115                 120

Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln
                125                 130                 135

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
                140                 145                 150

Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
                155                 160                 165

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
                170                 175                 180

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
                185                 190                 195

Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
                200                 205                 210
```

-continued

```
Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
                215                 220                 225

Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr
                230                 235                 240

Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
                245                 250                 255

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
                260                 265                 270

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                275                 280                 285

Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
                290                 295                 300

Val Asp Gly Met Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                305                 310                 315

Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val
                320                 325                 330

Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
                335                 340                 345

Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                350                 355                 360

Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                365                 370                 375

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                380                 385                 390

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                395                 400                 405

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu
                410                 415                 420

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                425                 430                 435

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                440                 445                 450

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                455                 460                 465

Ser Pro Gly Lys
            469
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
                35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                65                  70                  75
```

-continued

```
Ser Asn Leu Asp Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
                95                 1 00                 1 05

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
            214
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1                 5                  10                  15

Gly Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
                20                  25                  30

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
                35                  40                  45

Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly
                50                  55                  60

Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser
                65                  70                  75

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
                80                  85                  90

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
                95                 1 00                 1 05

Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly
                110                 115                 120

Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe
                125                 130                 135

Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser
                140                 145                 150

Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val
                155                 160                 165

Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
                170                 175                 180

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                185                 190                 195
```

-continued

```
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                 205                 210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                 220                 225

Lys Ser Phe Asn Arg Gly Glu Cys
                230         233


(2) INFORMATION FOR SEQ ID NO:26:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 122 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                 1 00                1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
               110                 115                 120

Ser Ser
    122
```

We claim:

1. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

2. The humanized variable domain of claim 1 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

3. The humanized variable domain of claim 1 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

4. The humanized variable domain of claim 1 wherein the human antibody variable domain is a consensus human variable domain.

5. The humanized variable domain of claim 1 wherein the residue at site 4L has been substituted.

6. The humanized variable domain of claim 1 wherein the residue at site 38L has been substituted.

7. The humanized variable domain of claim 1 wherein the residue at site 43L has been substituted.

8. The humanized variable domain of claim 1 wherein the residue at site 44L has been substituted.

9. The humanized variable domain of claim 1 wherein the residue at site 58L has been substituted.

10. The humanized variable domain of claim 1 wherein the residue at site 62L has been substituted.

11. The humanized variable domain of claim 1 wherein the residue at site 65L has been substituted.

12. The humanized variable domain of claim 1 wherein the residue at site 66L has been substituted.

13. The humanized variable domain of claim 1 wherein the residue at site 67L has been substituted.

14. The humanized variable domain of claim 1 wherein the residue at site 68L has been substituted.

15. The humanized variable domain of claim 1 wherein the residue at site 69L has been substituted.

16. The humanized variable domain of claim 1 wherein the residue at site 73L has been substituted.

17. The humanized variable domain of claim 1 wherein the residue at site 85L has been substituted.

18. The humanized variable domain of claim 1 wherein the residue at site 98L has been substituted.

19. The humanized variable domain of claim 1 wherein the residue at site 2H has been substituted.

20. The humanized variable domain of claim 1 wherein the residue at site 4H has been substituted.

21. The humanized variable domain of claim 1 wherein the residue at site 36H has been substituted.

22. The humanized variable domain of claim 1 wherein the residue at site 39H has been substituted.

23. The humanized variable domain of claim 1 wherein the residue at site 43H has been substituted.

24. The humanized variable domain of claim 1 wherein the residue at site 45H has been substituted.

25. The humanized variable domain of claim 1 wherein the residue at site 69H has been substituted.

26. The humanized variable domain of claim 1 wherein the residue at site 70H has been substituted.

27. The humanized variable domain of claim 1 wherein the residue at site 74H has been substituted.

28. The humanized variable domain of claim 1 wherein the residue at site 92H has been substituted.

29. An antibody comprising the humanized variable domain of claim 1.

30. An antibody which binds $p185^{HER2}$ and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind $p185^{HER2}$ incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

31. The antibody of claim 30 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

32. The antibody of claim 30 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

33. The antibody of claim 30 wherein the human antibody variable domain is a consensus human variable domain.

34. The antibody of claim 30 wherein the residue at site 4L has been substituted.

35. The antibody of claim 30 wherein the residue at site 38L has been substituted.

36. The antibody of claim 30 wherein the residue at site 43L has been substituted.

37. The antibody of claim 30 wherein the residue at site 44L has been substituted.

38. The antibody of claim 30 wherein the residue at site 46L has been substituted.

39. The antibody of claim 30 wherein the residue at site 58L has been substituted.

40. The antibody of claim 30 wherein the residue at site 62L has been substituted.

41. The antibody of claim 30 wherein the residue at site 65L has been substituted.

42. The antibody of claim 30 wherein the residue at site 66L has been substituted.

43. The antibody of claim 30 wherein the residue at site 67L has been substituted.

44. The antibody of claim 30 wherein the residue at site 68L has been substituted.

45. The antibody of claim 30 wherein the residue at site 69L has been substituted.

46. The antibody of claim 30 wherein the residue at site 73L has been substituted.

47. The antibody of claim 30 wherein the residue at site 85L has been substituted.

48. The antibody of claim 30 wherein the residue at site 98L has been substituted.

49. The antibody of claim 30 wherein the residue at site 2H has been substituted.

50. The antibody of claim 30 wherein the residue at site 4H has been substituted.

51. The antibody of claim 30 wherein the residue at site 36H has been substituted.

52. The antibody of claim 30 wherein the residue at site 39H has been substituted.

53. The antibody of claim 30 wherein the residue at site 43H has been substituted.

54. The antibody of claim 30 wherein the residue at site 45H has been substituted.

55. The antibody of claim 30 wherein the residue at site 69H has been substituted.

56. The antibody of claim 30 wherein the residue at site 70H has been substituted.

57. The antibody of claim 30 wherein the residue at site 74H has been substituted.

58. The antibody of claim 30 wherein the residue at site 75H has been substituted.

59. The antibody of claim 30 wherein the residue at site 76H has been substituted.

60. The antibody of claim 30 wherein the residue at site 78H has been substituted.

61. The antibody of claim 30 wherein the residue at site 92H has been substituted.

62. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a consensus human variable domain, and further comprising an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a chronic disease in that patient, wherein the humanized antibody comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprises an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

64. A humanized variant of a non-human parent antibody which binds an antigen and comprises a human variable domain comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another.

65. The humanized variant of claim 63 which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.

66. A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining

Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 73H, 76H, 78H, and 93H, utilizing the numbering system set forth in Kabat.

67. The humanized variable domain of claim **66** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

68. The humanized variable domain of claim **66** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

69. The humanized variable domain of claim **66** wherein the human antibody variable domain is a consensus human variable domain.

70. The humanized variable domain of claim **66** wherein the residue at site 24H has been substituted.

71. The humanized variable domain of claim **66** wherein the residue at site 73H has been substituted.

72. The humanized variable domain of claim **66** wherein the residue at site 76H has been substituted.

73. The humanized variable domain of claim **66** wherein the residue at site 78H has been substituted.

74. The humanized variable domain of claim **66** wherein the residue at site 93H has been substituted.

75. The humanized variable domain of claim **66** which further comprises an amino acid substitution at site 71H.

76. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H and 73H.

77. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H, 73H and 78H.

78. An antibody comprising the humanized variable domain of claim **66**.

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region (CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

80. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

(c) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78H, 92H and 93H, utilizing the numbering system set forth in Kabat.

81. The humanized variable domain of claim **80** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

82. The humanized variable domain of claim **80** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

* * * * *

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,407,213 B1
DATED : June 18, 2002
INVENTOR(S) : Carter et al.

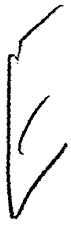
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 88,
Line 63, please delete "63" and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

JAMES E. ROGAN
Director of the United States Patent and Trademark Office



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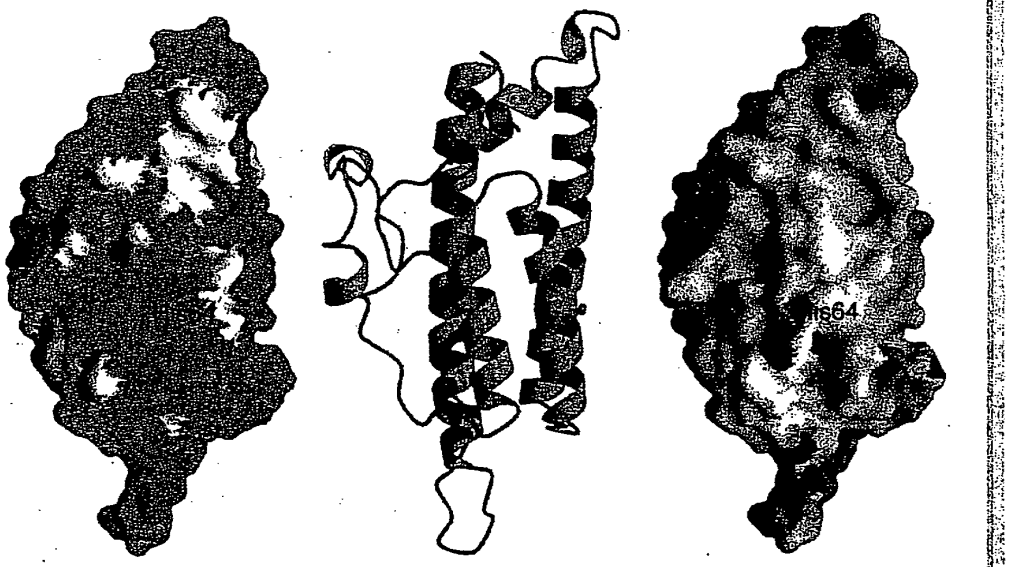

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Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-matured Fab in Complex with Antigen

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The Fab portion of a humanized antibody (Fab-12; IgG form known as rhuMAb VEGF) to vascular endothelial growth factor (VEGF) has been affinity-matured through complementarity-determining region (CDR) mutation, followed by affinity selection using monovalent phage display. After stringent binding selections at 37 °C, with dissociation (off-rate) selection periods of several days, high affinity variants were isolated from CDR-H1, H2, and H3 libraries. Mutations were combined to obtain cumulatively tighter-binding variants. The final variant identified here, Y0317, contained six mutations from the parental antibody. *In vitro* cell-based assays show that four mutations yielded an improvement of about 100-fold in potency for inhibition of VEGF-dependent cell proliferation by this variant, consistent with the equilibrium binding constant determined from kinetics experiments at 37 °C. Using X-ray crystallography, we determined a high-resolution structure of the complex between VEGF and the affinity-matured Fab fragment. The overall features of the binding interface seen previously with wild-type are preserved, and many contact residues are maintained in precise alignment in the superimposed structures. However, locally, we see evidence for improved contacts between antibody and antigen, and two mutations result in increased van der Waals contact and improved hydrogen bonding. Site-directed mutants confirm that the most favorable improvements as judged by examination of the complex structure, in fact, have the greatest impact on free energy of binding. In general, the final antibody has improved affinity for several VEGF variants as compared with the parental antibody; however, some contact residues on VEGF differ in their contribution to the energetics of Fab binding. The results show that small changes even in a large protein-protein binding interface can have significant effects on the energetics of interaction.

Keywords: angiogenesis; humanized antibody-antigen complex; affinity maturation; phage display; X-ray crystallography

Introduction

Angiogenic factors (Folkman & Klagsbrun, 1987), which stimulate endothelial cells leading to new vascularization, have roles in such disease states as cancer, rheumatoid arthritis, and macular degeneration (reviewed by Ferrara, 1995; Folkman, 1995; Iruela-Arispe & Dvorak, 1997). Vascular endothelial growth factor (VEGF), a heparin-binding protein initially identified from pituitary cells (Ferrara & Henzel, 1989), is clearly a key angio-

Abbreviations used: CDR, complementarity-determining region; FR, framework region; HuVEC, human umbilical vein endothelial cell; $K_d^{25°}$, equilibrium dissociation constant determined at 25 °C; mAb, IgG form of monoclonal antibody; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; VEGF, vascular endothelial growth factor; VEGF(109), receptor-binding fragment of VEGF with residues 8-109; VEGF(165), VEGF form with residues 1-165.

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genic factor in development as well as in certain disease states, including the growth of solid tumors (reviewed by Ferrara, 1999). A murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependent cell proliferation *in vitro* and to antagonize tumor growth *in vivo* (Kim *et al.*, 1993). The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12 (Presta *et al.*, 1997). Both chimeric and humanized antibodies retained high affinity binding to VEGF, with an apparent equilibrium dissociation constant, $K_d^{25°}$, of 0.9 to 3 nM (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a). The corresponding full-length IgG form of this antibody, rhumAb VEGF, is being developed as a possible therapeutic agent for the treatment of human solid tumors (Mordenti *et al.*, 1999).

We became interested in obtaining higher affinity variants of Fab-12 in order to test whether affinity improvements of this antibody might improve its potency and efficacy. Phage display of randomized libraries of antibodies and other proteins has been extensively used to engineer proteins with improved affinity and specificity (Lowman *et al.*, 1991; reviewed by Kay & Hoess, 1996; Rader & Barbas, 1997; Griffiths & Duncan, 1998). In particular, a phage-based *in vitro* affinity maturation process has been successful in improving the binding affinity of antibodies previously identified from traditional monoclonal or naive-library sources (e.g. Hawkins *et al.*, 1992; Marks *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Thompson *et al.*, 1996).

In previous work, the humanized anti-VEGF antibody Fab-12 was adapted for improved monovalent phage display through selection of a CDR-L1 variant, designated Y0192 (Muller *et al.*, 1998a). To select target residues for randomization and affinity optimization, we also previously screened all CDR residues, as defined by a combination of the hypervariable (Kabat *et al.*, 1987) and structurally defined (Chothia & Lesk, 1987) CDR residues. Fab variants of Y0192 generated by alanine scanning were analyzed for side-chain contributions to antigen binding (Muller *et al.*, 1998a). In addition, a crystal structure of Fab-12 in complex with the receptor-binding domain of VEGF, VEGF(109), was determined (Muller *et al.*, 1998a). The results of these studies showed that the antigen binding site is almost entirely composed of residues from the heavy chain CDRs, CDR-H1, H2, and H3. Therefore, these CDRs appeared most likely to provide the opportunity for improved binding interactions with antigen.

Here, we describe the selection of an affinity-improved anti-VEGF antibody by phage display and off-rate selection. We show that the affinity-matured antibody binds VEGF with at least 20-fold improved affinity and inhibits VEGF-induced cell proliferation with enhanced potency in a cell-based assay. We also report the crystal structure of an affinity-optimized antibody in complex with VEGF, to our knowledge, representing the first reported structure of an *in vitro* affinity-matured antibody:antigen complex. The structure, together with mutational analysis, shows that subtle changes in the antibody-antigen interface account for improved affinity.

Results

Library design

We used the results of an alanine-scanning analysis, combined with a crystal structure of the wild-type Fab fragment in complex with VEGF (Muller *et al.*, 1998a), to design targeted libraries within the antibody CDRs for random mutagenesis and affinity selection. This strategy enabled us to construct theoretically complete libraries with a small number of residues randomized in each CDR. Although sites remote from the antigen-combining region or buried within the protein could modulate antigen binding affinity indirectly and have in fact been exploited for affinity improvement (Hawkins *et al.*, 1993), clearly residues shown to be important by alanine scanning are useful starting points for binding-affinity optimization (Lowman *et al.*, 1991; Lowman & Wells, 1993). Furthermore, we reasoned that by making mutations at residues of the antibody CDRs which were known to affect antigen binding and were located at or near points of contact in the bound complex, we could minimize the possibility of other indirect effects which might alter stability, immunogenicity, or other properties of the antibody.

Both Ala-scanning and crystallography (Muller *et al.*, 1998a) identified CDR-H3 as the predominant contact segment for VEGF, consistent with the general observation that CDR-H3 is often key to antigen binding (Chothia & Lesk, 1987). Within CDR-H3, residues Y95, P96, H97, Y98, Y99, S100b, H100c, W100d, Y100e, and F100f (numbering is as described by Kabat *et al.* (1987)), all showed effects on binding over a range of twofold to >150-fold when mutated to Ala, and Ala substitution at S100a caused a slight improvement in binding. However, H100c, Y100e, and F100f were found to have little or no direct contact with VEGF and presumed to have indirect effects on binding. On the other hand, Y95 and W100d have significant contacts with VEGF, and Ala substitutions resulted in no detectable binding to VEGF. Therefore, these residues were excluded from optimization. Inspection of the complex structure suggested that substitutions at P96 and Y98 could be disruptive to the antibody structure, while G100, where Ala mutation had little effect, might tolerate further substitutions. We therefore constructed a library (YC81) which fully randomized positions H97, Y99, G100, S100a, and S100b, within CDR-H3.

Significant effects of Ala substitution were also found in CDR-H2. Here, W50, I51, N52, T52a, Y53, T54, T58 alanine mutants all showed >twofold loss in binding affinity, with the greatest residue surface area buried at positions W50, I51, Y53, and

T58 (Muller *et al.*, 1998a). Indeed, W50 along with other aromatic side-chains was observed to form a deep pocket into which a loop of VEGF inserts in the complex, and was excluded from further optimization. Residue I51, on the other hand, showed no direct contact with VEGF and was also excluded. Residue T58 had multiple interactions within the interface, including contacts with VEGF and with the critical W50 of the CDR pocket. Although E56 showed no contact with VEGF and little effect (<twofold) upon alanine substitution, its side-chain lies at the periphery of the interface, near several hydrophobic residues of VEGF. We reasoned that these might be exploited for additional binding interactions. Two CDR-H2 libraries were constructed: YC266, randomizing positions T52a, Y53, T54, and E56; and YC103, randomizing positions N52, T52a, Y53, and T54.

In CDR-H1 G26, Y27, F29, N31, Y32, G33, M34, and N35 were implicated by alanine mutagenesis as important for binding VEGF; however, only N31, Y32, and G33 had significant direct contacts with VEGF. Since Ala substitution of G33 showed reduced binding, larger side-chains seemed less desirable; for this reason, this position was not randomized. Residues 27 (buried in the antibody structure) and T28 and T30 (which are mutually contacting) were included at the end of the H1 loop as possible indirect determinants of binding. Residues 27, 28, and 30-32 were randomized in a library designated YC265.

Framework residues, especially heavy chain residues 71 and 93, normally outside the region of contact with antigen, have also been found to affect antibody binding affinity (Tramontano *et al.*, 1990; Foote & Winter, 1992; Hawkins *et al.*, 1993; Xiang *et al.*, 1995), and sometimes participate in antigen contacts (reviewed by Nezlin, 1998). Therefore, an additional region of the anti-VEGF Fab, within FR-H3 and including position 71, was also targeted for randomization. Since the residue 71-76 region has contacts with CDR-H1 (at F29) and CDR-H2 (at I51 and T52a), these represented potential sites for affinity improvement through secondary effects on the interface residues. Residues L71, T73, and S76 were randomized in this FR-H3 library.

Phage selections

Fab libraries were constructed using a fusion to the g3p minor coat protein in a monovalent phage display (phagemid) vector (Bass *et al.*, 1990; Lowman *et al.*, 1991). For each library, stop codons were introduced by mutagenesis into the Y0192 phage template (Muller *et al.*, 1998a) at each residue position to be randomized. Each stop-codon construct was then used for construction of a fully randomized (using NNS codons) library as described in Materials and Methods. Phage were precipitated from overnight *Escherichia coli* shake-flask cultures and applied to VEGF-coated immunosorbant plates for binding selections. Cycles of selection followed by amplification were carried out essentially as described (Lowman, 1998).

We used an off-rate selection process (see Materials and Methods) similar to previously described procedures (Hawkins *et al.*, 1992; Yang *et al.*, 1995), modified by gradually increasing the selective pressure for binding to antigen during successive cycles of enrichment. The enrichment factor (ratio of displaying phage to non-displaying phage eluted *versus* applied) was used to monitor the stringency of selection at each step (Table 1). As a control, and to obtain a relative measure of affinity improvement, Y0192-phage were subjected to the same procedure at each cycle.

Fab-phage clones were sequenced from several phage-binding selection rounds that showed enrichment for Fab-phage over non-displaying phage. From round 6 of the CDR-H1 library selections, a dominant clone, Y0243-1 was found, having wild-type residues at Y27, T30, and Y32, and substitutions T28D and N31H (Table 2). Additional clones had related sequences, with N31H found in all selectants; Asp or Glu substituting for T28; and Thr, Ser, Gln, or Gly found at position T30.

Table 1. Enrichment factors from phage-displayed Fab libraries

Round	Wash time (hours)	CDR-H1 YC265	CDR-H2 YC266	CDR-H2 YC103	CDR-H3 YC81	FR-H3 YC101	Control Y0192
1	0	8.2	1.7	1.3	3.3	4	1.5
2	1	1.6	25	0.7	10	110	90
3	2	340	880	100	570	2300	22000
4	18	6800	880	5200	3700	600	2700
5	37[a]	210	900	920	1300	480	32
6	47[a]	130	80	100	3500	30	20
7	63[a]	1	1	>3	>25	1	>8

Libraries are designated by CDR region and oligonucleotide label (see the text for details). Library Fab-phage (ampicillin-resistant) were mixed with non-displaying control phage (chloramphenicol-resistant) in each starting pool, and subjected to VEGF binding selection, washing, and elution as described in the text.

The enrichment factor for each library is reported here as the ratio of Amp/Cam colony-forming units in the eluted pool, divided by the ratio of Amp/Cam colony-forming units in the starting pool. Starting phage concentrations were about 10^{12}/ml, except 10^{13}/ml in round 1. The wild-type Fab-phage, Y0192 was included at each round for comparison of enrichment under the particular conditions used.

[a] In some cases, the wash-step included incubation at 37 °C.

Table 2. Anti-VEGF Fab variants selected from a CDR-H1 library (HL-265)

Variant	n	Y 27	T 28	T 30	N 31	Y 32	I 34[a]	K_d(Y0192)/ K_d(variant)
Round 6 (HCl)								
Y0243-1	5	Y	D	T	H	Y	M	3.1
Y0243-2	1	Y	E	Q	H	Y	M	
Y0243-3	1	Y	E	T	H	Y	M	
Y0243-4	1	Y	D	G	H	Y	M	
Y0243-5	1	Y	D	S	H	Y	M	
Y0243-6	1	Y	E	S	H	Y	M	
Consensus:		Y	D	T	H	Y	M	3.1

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987).

[a] Position 34 was not randomized, but was changed to Met (as in Fab-12) in this library. The consensus reported here, equivalent to clone Y0243-1, represents the most abundant amino acid residue at each position (including clones with multiple representation ($n > 1$)). K_d(Y0192)/K_d (variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

Clones from two independently constructed CDR-H2 libraries were remarkable in that all sequenced library clones conserved wild-type residues at virtually all positions mutated, except at position Y53, where all clones contained a Trp substitution (Table 3).

Because of the strong enrichment observed from the CDR-H3 library, a number of clones were sequenced from rounds 5 and 7 (Table 4). Of 39 sequenced clones, 37 retained the wild-type residue S100b, and all contained the mutation H97Y. The remaining positions showed greater diversity, even after seven cycles of selection. The dominant clone at round 7, Y0238-3, contained the mutation S100aT (in addition to H97Y), with wild-type residues Y99 and G100. Other substitutions observed included Lys or Arg for Y99 (in 18 of 39 clones), G100N (11 of 39 clones), and a variety of substitutions including Arg, Glu, Gln, and Asn at S100a. In this library, the consensus sequence is represented by the dominant clone, Y0238-1 (Table 4).

Clones from round 6 of the FR-H3 library (Table 5) showed conservation of wild-type residue S76, with wild-type residues or various substitutions at the remaining positions: Val or Ile substituting for L71, and Val or Lys substitutions at T73.

Binding affinity of selected variants

For measurements of binding affinity, we made use of an amber stop codon placed between the genes for the Fab heavy chain and the g3p C-terminal domain, and expressed soluble Fab variants from *E. coli* shake-flask or fermentation cultures. Fab variants purified from protein-G affinity chromatography were characterized for binding affinity using an SPR-based assay on a BIAcore™-2000 instrument. The binding-kinetics assay has been described (Muller *et al.*, 1998a).

Association kinetics (k_{on}) for the wild-type antibody binding to immobilized VEGF are slow (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a), and none of the variants tested had significantly improved on-rates. On the other hand, dissociation kinetics varied over a range of 10^{-4} s^{-1} to $\leq 4 \times 10^{-6}$ s^{-1} at 25 °C (Table 6). Based on measurements of instrumental drift, we could not accurately measure k_{off} (and consequently K_d)

Table 3. Anti-VEGF Fab variants selected from CDR-H2 libraries (HL-266, YC103)

Variant	n	N 52[a]	T 52a	Y 53	T 54	G 55[a,b]	E 56[a]	K_d(Y0192)/ K_d(variant)
Round 6 (HCl)								
HL266-A[b]	6	N	T	W	T	G	E	1.3
HL266-E	1	N	T	W	T	G	T	
HL266-I	1	N	T	W	T	G	Q	
YC103-A[b]	7	N	T	W	T	G	E	1.3
YC103-C	1	N	T	W	D	G	E	
Consensus		N	T	W	T	G	E	1.3

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clones HL266A and YC103A, represents the most abundant amino acid at each position (including clones with multiple representation; i.e. $n > 1$). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] Constant positions were position 52 in the HL-266 library and position 56 in the YC103 library.

[b] Equivalent clones are assumed to have equal affinity.

Table 4. Anti-VEGF Fab variants selected from a CDR-H3 library (YC81)

Variant	*n*	H 97	Y 99	G 100	S 100a	S 100b	K_d(Y0192)/K_d(variant)
Round 5 (VEGF)							
Y0228-21	1	Y	R	N	A	S	
Y0228-22	1	Y	T	T	R	S	
Y0228-23	1	Y	E	G	S	S	
Y0228-24	1	Y	R	Q	R	G	
Y0228-26	1	Y	T	G	R	S	
Y0228-27	1	Y	T	N	T	S	
Y0228-28	1	Y	R	K	G	S	
Y0228-29	1	Y	T	G	S	S	
Y0228-30	1	Y	R	S	G	S	
Round 5 (HCl)							
Y0229-20	1	Y	T	N	R	S	
Y0229-21	1	Y	R	N	S	S	
Y0229-22	1	Y	K	E	S	S	
Y0229-23	1	Y	R	D	A	S	
Y0229-24	1	Y	R	Q	K	G	
Y0229-25	1	Y	K	G	G	S	
Y0229-26	1	Y	Y	G	A	S	
Y0229-27	1	Y	R	G	E	S	
Y0229-28	1	Y	R	S	T	S	
Y0238-10[a]	1	Y	R	N	T	S	3.8
Round 7 (HCl)							
Y0238-3	6	Y	Y	G	T	S	≥9.4
Y0238-1	2	Y	R	G	T	S	7.3
Y0238-2	2	Y	I	N	K	S	
Y0238-10[a]	2	Y	R	N	T	S	3.8
Y0238-4	1	Y	Y	N	Q	S	
Y0238-5	1	Y	I	A	K	S	2.1
Y0238-6	1	Y	R	D	N	S	≥5.4
Y0238-7	1	Y	W	G	T	S	
Y0238-8	1	Y	R	Q	N	S	
Y0238-9	1	Y	R	Q	S	S	
Y0238-11	1	Y	K	N	T	S	
Y0238-12	1	Y	I	E	R	S	
Consensus		Y	R	G	T	S	7.3

All variants are in the background of Y0192 (Muller *et al.*, 1998a). The clones are grouped according to the round of selection (5 or 7) and the type of elution (VEGF competition or HCl elution) used for recovery of bound phage. *n*, indicates the number of clones found with identical DNA sequence within each group. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clone Y0238-1, represents the most abundant amino acid at each position (including clones with multiple representation (n > 1)). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] One clone was identified at both rounds 5 and 7. Equivalent clones are assumed to have equal affinity.

under these conditions, but instead used the kinetics data to place an upper limit on K_d.

The phage-derived Fab variants tested showed a range of small (within experimental error of about twofold) to significant (>fivefold) improvements in binding affinity over the wild-type (parental phage) antibody Y0192 (Table 6). From the CDR-H1 library, the dominant clone (Y0243-1) showed threefold improved affinity. Variant Y0242-1, the dominant clone in each of three CDR-H2 libraries, showed an affinity equivalent to wild-type within experimental error, and two clones derived from the FR-H3 library (Y0244-1 and Y0244-4) were equivalent or slightly weaker in affinity. Small

Table 5. Anti-VEGF Fab variants selected from a FR-H3 library

Variant	*n*	L 71	T 73	S 76	K_d(Y0192)/K_d(variant)
Round 6 (HCl)					
Y0244-1	1	V	V	S	0.3
Y0244-2	1	L	K	S	
Y0244-3[a]	1	L	V	S	
Y0244-4	1	I	K	S	0.9

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n*, indicates the number of clones found with identical DNA sequence. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] One variant contained a spontaneous mutations, S74W.

Table 6. Binding kinetics of anti-VEGF Fab variants at 25 °C

Variant	$k_{on}/10^4$ (M^{-1} s^{-1})	$k_{off}/10^{-1}$ (s^{-1})	K_d (nM)	K_d(Y0192)/K_d(variant)
Y0192[a]	4.1	1.2	2.9	1
A. *Library-derived*				
Y0238-1	2.6	0.09	0.4	7.3
Y0238-3	1.3	≤0.04[b]	≤0.3[b]	≥9.4[b]
Y0238-5	0.57	0.08	1.4	2.1
Y0238-7	1.1	≤0.06[b]	≤0.5[b]	≥5.4[b]
Y0238-10	1.2	0.09	0.8	3.8
Y0242-1	3.8	0.86	2.3	1.3
Y0243-1	4.8	0.45	0.9	3.1
Y0244-1	3.0	2.7	9.0	0.3
Y0244-4	5.2	1.7	3.3	0.9
B. *Engineered*				
Y0268-1	4.0	0.15	0.38	7.6
Y0313-1	3.5	≤0.05[b]	≤0.15[b]	≥20[b]
Y0192(T28D)	6.8	1.4	2.0	1.4
Y0192(N31H)	4.8	0.37	0.8	3.6
Y0192(H97Y)	2.5	0.045	0.2	14
Y0192(S100aT)	6.8	1.0	1.5	1.9
Y0317	3.6	≤0.05[b]	≤0.14[b]	≥20[b]

Kinetic constants were determined from measurements using a BIAcore™-2000 instrument with a biosensor chip containing immobilized VEGF(109). Measurements were performed at 25 °C. Fab concentrations were calculated from quantitative amino acid analysis. The equilibrium dissociation constant, K_d, is calculated form the ratio of the rate constants, k_{off}/k_{on}. The relative affinity, reported as K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity versus the wild-type humanized antibody Y0192. Errors in K_d were approximately ±25%. Variant Y0242-1 corresponds to the point mutations Y53W in CDR-H2 of Fab Y0192; for descriptions of the other variants, see Tables 2, 3, 4, 5, and 8.

[a] Data for Y0192 is from Muller *et al.* (1998a,).

[b] In some cases, the dissociation rate constant observed was at or near the limit of detection; therefore, the reported k_{off} and K_d are upper limits, and the relative affinities are an upper limit.

improvements were seen in CDR-H3 variants Y0238-5 and Y0238-10. However, larger improvements (exceeding the limits of measurement (>fivefold to >ninefold)) were observed for the CDR-H3 variants Y0238-1, Y0238-3, and Y0238-7.

All tested variants (in fact all sequenced clones) from the CDR-H3 library contained the mutation H97Y. In the higher affinity group, Gly was conserved at position 100, while the lower affinity variant contained Ala (known to cause 1.8-fold reduction in Y0192 binding; Muller *et al.*, 1998a) or Asn (Table 4). The S100a position, while quite varied among sequenced clones, was changed to Thr in the higher affinity CDR-H3 variants, and Thr or Lys in the lower affinity ones. Substitutions at Y99, though mostly confined to basic or aromatic residues, apparently had little effect since Y0238-1 (representing the consensus CDR-H3 sequence with Y99R) was not significantly different in affinity from Y0238-3, which retained Y99.

Affinity improvements from combinations of CDR mutations

To improve affinity further, several combinations of the phage-selected CDR-H1, H2, and H3 mutations were made by site-directed mutagenesis (Table 7). Among these, the highest affinity was obtained with pY0313-1 (i.e. pY0192 with mutations CDR-H1 (T28D/N31H/I34M) and CDR-H3 (H97Y/S100aT); note I34M is a reversion to Fab-12 wild-type). From BIAcore™ kinetics measurements carried out at 25 °C, this Fab variant had ≥20-fold higher affinity than Y0192 (Table 6).

Addition of the Y53W mutation, which alone produced little or no improvement over Y0192, to Y0313-1 (producing variant Y0268-1) actually reduced binding affinity by >twofold (Table 6).

The final Fab version was constructed by removing the phage-expression enhancing mutations in CDR-L1 from pY0313-1 by site-directed mutagen-

Table 7. Anti-VEGF CDR combination variants

	CDR-L1					CDR-H1			CDR-H2	CDR-H3	
Y0192: Variant	R 24	N 26	E 27	Q 28	L 29	T 28	N 31	I 34	Y 53	H 97	S 100a
Y0313-1	-	-	-	-	-	D	H	M	-	Y	T
Y0268-1	-	-	-	-	-	D	H	M	W	Y	T
Y0317	S	S	Q	D	I	D	H	M	-	Y	T
Fab-12	S	S	Q	D	I	-	-	-	-	-	-

Substitutions are shown relative to Y0192. Fab-12 also contains T221 in the heavy chain. Dashes (-) indicate no substitution. Numbering is according to Kabat *et al.* (1987) for both the light chain (CDR-L1) and heavy chain (CDR-H1, H2, H3).

esis. The M4L substitution was identified during phage-humanization experiments (Baca *et al.*, 1997), and the Leu residue was retained so as to preclude possible oxidation of the Met side-chain. The first libraries were constructed from a Fab-12 phagemid derivative, pY0101, which contained a buried framework mutation, V_L(M4L), as well as a mutation (T221L) at the junction to g3p. Thus the final version, Y0317 (Table 7 and Figure 1) differs from Fab-12 by the following six mutations: V_L(M4L), V_H(T28D/N31H/H97Y/S100aT/T221L).

Each of the CDR mutations in H1 and H3 was tested for its effect on VEGF binding affinity by introducing the corresponding point mutation into the parental Y0192 Fab and measuring binding kinetics. The results (Table 6) show a 14-fold and 3.6-fold improvement with substitution of H97Y or N31H, respectively, into the parental Fab. However, T28D or S100aT had identical affinity to Y0192, within experimental error.

We compared Fab-12 and Y0317 Fab affinities in a solution binding assay, using VEGF competition with [^{125}I]VEGF for binding to Fab. The results showed Fab-12 having $K_d^{25°}$ = 433 pM and Y0317 Fab having $K_d^{25°}$ = 20 pM, a 22-fold improvement in binding affinity (Figure 2).

Because dissociation kinetics in surface plasmon resonance (SPR) experiments exceeded instrumental capabilities at 25 °C, and in order to assess binding affinity under more physiological conditions, we compared binding affinities of the original humanized antibody Fab-12 with the final variant Y0317 in kinetics experiments at 37 °C. k_{on} and k_{off} were faster for both antibodies than at 25 °C, and k_{off} was clearly measurable above background. Using either immobilized VEGF(109) or immobilized VEGF(165), Y0317 was 120-fold to 140-fold improved in affinity over Fab-12, with a $K_d^{37°}$ of 80-190 pM (Table 8).

VEGF Ala-scan of the Y0317 binding epitope

In order to understand how mutations in the Fab affected binding affinity to VEGF, we also tested VEGF variants for binding to the affinity-improved antibody. For these experiments, we made use of the full-length IgG forms of Fab-12 (known as rhuMab VEGF) and Y0317 (termed Y0317-IgG) produced in CHO cells (V. Chisholm, unpublished results). These VEGF variants were previously used for mapping the parental antibody's binding site on VEGF (Muller *et al.*, 1998a).

In this assay, carried out at 37 °C, VEGF competed with biotin-VEGF with an IC_{50} of 9 nM in binding rhuMab VEGF, compared with an IC_{50} of 1 nM for Y0317-IgG (Table 9). SPR measurements have shown similar affinity improvement of Y0317-IgG over rhuMAb VEGF (H. Lowman, unpublished results).

Alanine mutations of VEGF that affected rhuMab VEGF binding also affected Y0317-IgG. For example, M81A, G88A, and G92A all caused large (100 to >500-fold) losses in binding affinity. And smaller reductions (3 to 30-fold) in binding affinity for both antibodies were seen for I80A, K84A, I91A, E93A, and M94A.

However, significant differences in the magnitude of the effect were observed at certain sites, including Y45A, fourfold reduced in affinity for rhuMAb VEGF *versus* 26-fold for Y0317-IgG; Q89A, 19-fold *versus* sixfold; and M94A, 11-fold versus 25-fold. Most surprisingly, two mutations that led to loss of detectable binding affinity for rhumAb VEGF (>500-fold) had only modest effects (four- to ninefold) on binding to Y0317-IgG. These differences might suggest a shift in the binding epitope of the antibody, and this possibility was addressed with receptor-inhibition assays and structural analysis, both described below.

Inhibition of VEGF activity

Cell-proliferation assays have been described (Fairbrother *et al.*, 1998) for the measurement of VEGF mitogenic activity on human umbilical vein endothelial cells. Here, we compared the potency of Fab-12 and the affinity-improved variants Y0238-3 and Y0313-1.

The results (Figure 3) show both variants Y0238-3 and Y0313-1 inhibit VEGF activity more potently than Y0192 Fab. Comparing the Fab forms, variant Y0313-1 appeared at least 30-fold to 100-fold more potent than the wild-type Fab. In additional experiments, Y0317 activity was similar to that of Y0313-1 (data not shown). It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC_{50} for the mutant. For example, if the bind-

Table 8. Binding kinetics of anti-VEGF Fab variants at 37 °C

Variant	Immobilized	$k_{on}/10^4$ (M^{-1} s^{-1})	$k_{off}/10^{-4}$ (s^{-1})	K_d (nM)	K_d(Fab-12)/ K_d(variant)
Fab-12	VEGF(109)	5.1	6.6	13 ± 2.2	1
Y0317	VEGF(109)	5.4	0.059	0.11 ± 0.02	120
Fab-12	VEGF(165)	5.5	11	20 ± 3.8	1
Y0317	VEGF(165)	5.3	0.074	0.14 ± 0.05	140

Kinetic constants were determined by injecting Fab solutions onto a BIAcore™-2000 instrument with a biosensor chip containing approximately 190 RU of immobilized VEGF(109) or VEGF(165), as indicated. The equilibrium dissociation constant, K_d, is calculated from the ratio of the rate constants, k_{off}/k_{on}. The relative affinity, reported as K_d(Fab-12)/K_d(variant) indicates the fold increase in binding affinity *versus* the original humanized antibody (Fab-12; Presta *et al.*, 1997) under the specified conditions.

```
Light chain:

          1       10        20        30        40        50
Fab-12    DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYF
Y0192     DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQKPGKAPKVLIYF
Y0317.    DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYF

          1       10        20        30        40        50
                  60        70        80        90       100
Fab-12    TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
Y0192     TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
Y0317     TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ

                  60        70        80        90       100
                 110       120       130       140       150
Fab-12    GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
Y0192     GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
Y0317     GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

                 110       120       130       140       150
                 160       170       180       190       200
Fab-12    DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
Y0192     DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
Y0317     DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

                 160       170       180       190       200
                 210
Fab-12    LSSPVTKSFNRGEC
Y0192     LSSPVTKSFNRGEC
Y0317     LSSPVTKSFNRGEC

                 210
```

Figure 1 (*legend shown apposite*)

ing affinity (K_d) of the mutant is in fact <0.2 nM, then the IC_{50} 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*.

Structure of the complex

In order to compare the structure and binding site of the affinity-improved antibody with that of the parental antibody, we determined the complex structure by X-ray crystallography. Crystals of the complex between the receptor binding domain of VEGF (residues 8 to 109) and the affinity-matured Fab Y0317 were grown as described (see Materials and Methods) and diffracted to a maximum resolution of 2.4 Å. The structure was refined starting from the coordinates of the complex between VEGF and the parent of Fab Y0317, Fab-12 (Muller *et al.*, 1998a), and refined to an R-value of 19.9% (R_{free} = 27.4%) for the reflections between 20 Å and 2.4 Å resolution.

Heavy chain:

```
          1       10        20        30        40        50
Fab-12    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGW
Y0192     EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVRQAPGKGLEWVGW
Y0317     EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGW

          1       10        20        30        40        50
                  60        70        80        90       100
Fab-12    INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
Y0192     INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
Y0317     INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP

            a       60        70        80  abc   90    96
                  110       120       130       140       150
Fab-12    HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
Y0192     HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
Y0317     YYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC

           100abcdef      110       120       130       140
                  160       170       180       190       200
Fab-12    LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
Y0192     LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
Y0317     LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG

                  150       160       170       180       190
                  210       220       230
Fab-12    TQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
Y0192     TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL
Y0317     TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL

                  200       210       220
```

Figure 1. Sequence alignment of the original humanized antibody (Fab-12; Presta *et al.*, 1997), the phage-displayed antibody (Y0192; Muller *et al.*, 1998a) and the affinity-improved antibody (Y0317). Sequential numbering of each chain is shown above the sequences; numbering according to Kabat *et al.* (1987) is shown below. CDR regions are underlined. Positions at which Y0317 differs from Fab-12 are indicated with double underlining.

The final model consists of two Fab fragments bound to the symmetrical poles of the VEGF dimer. Only residues 14–107 of each VEGF monomer are well defined in the electron density, and therefore the six N-terminal and the two C-terminal residues of each monomer were omitted from the model. Each Fab light chain comprises residues 1 to 213, with the C-terminal residue disordered, whereas for each heavy chain residues 138 to 143 as well as the six C-terminal residues are absent from the model. As in the parental Fab complex, two out of 1050 residues, namely T51 in the V_L chain of each Fab fragment, are located in the "disallowed regions" (Laskowski *et al.*, 1993) of the Ramachandran plot; 85% of all residues have their main-chain torsion angles in the "most favored"

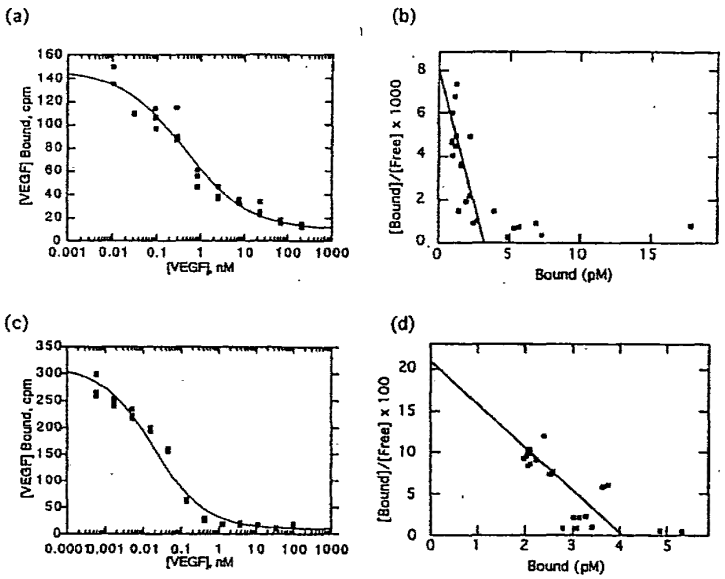

Figure 2. Radiolabeled VEGF binding assay. [^{125}I]VEGF was equilibrated (23 °C) with serial dilutions of unlabeled VEGF and (a) Fab-12 or (c) Y0317. Fabs were captured with an anti-Fab antibody-coated immunosorbant plate. Scatchard analysis (Munson & Rodbard, 1980) with a 1:1 binding model was used to calculate K_d of (b) 433 (±116) pM for Fab-12 and (d) 19.8(±4.3) pM for Y0317.

regions. The average *B*-factor of the model is 51.8 Å^2 and the mobility of the individual domains follows the pattern that was previously observed in the crystal structure of VEGF in complex with the Fab-12, with the constant domain dimer (C_L:C_H1) of one of the Fabs poorly ordered (Muller *et al.*, 1998a).

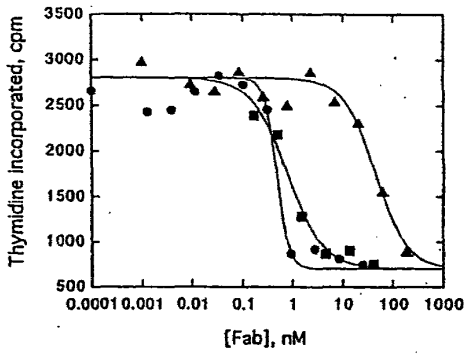

Figure 3. Human umbilical vein endothelial cell (HuVEC) assay of VEGF inhibition. Cells were cultured in the presence of 0.2 nM VEGF and serial dilutions of Fab Y0192 (triangles), Y0238-3 (squares), or Y0313-1 (circles). Cell proliferation was measured by incorporation of [^{3}H]thymidine. Curves show four-parameter fits to the data. Each point represents the mean of three treated wells.

Comparison of the final model with that of the parental Fab-VEGF complex (Muller *et al.*, 1998a) shows that the bound structures are very similar overall (Figure 4(a)) with Y0317 binding to the same site on VEGF as Fab-12 (Figure 4(b)). Side-chains show excellent overlap, and the main-chain structures show very little difference. The most prominent difference in contact residues is at H97Y (Figure 4(c); discussed below), where the tyrosine side-chain packs more favorably with VEGF and a buried water molecule from the parental Fab-VEGF complex is absent in the Y0317-Fab-VEGF complex.

Discussion

Antibody binding selections and affinity improvement

Here we made use of results from alanine-scanning and the previous structure of a humanized antibody-antigen complex to design Fab-phage libraries that randomized the three heavy-chain CDRs as well as one framework region (FR-H3) for improving the binding affinity of an anti-VEGF antibody. Affinity-improved Fab variants were obtained, with the largest effects seen in variants from the CDR-H3 library, although significant improvement was also obtained from mutation of CDR-H1. We therefore combined two mutations from H1 with two from H3, generating a further improved variant, Y0317. By making point mutations, we showed that the 20-fold (Figure 2)

Table 9. Alanine scan of VEGF by ELISA at 37 °C

VEGF(109) variant	IC_{50} (variant)/IC_{50} (VEGF)	
	Fab12-IgG	Y0317-IgG
VEGF(109)	1	1
F17A	1	1
Y21A	1	1
Y45A	4	26
K48A	2	1
Q79A	1	3
I80A	4	5
M81A	>500	930
R82A	>500	4
I83A	>500	9
K84A	3	10
H86A	1	1
Q87A	1	1
G88A	105	87
Q89A	19	6
H90A	1	1
I91A	2	6
G92A	>500	>900
E93A	4	7
M94A	11	25

ELISA assays were carried out using the full-length IgG form of Fab-12 or the IgG form of Y0317 and VEGF(109). Incubation of antibody with VEGF was at 37 °C for five hours. The IC_{50} for inhibition by each Ala mutant was evaluated using a four-parameter equation, and the relative affinities calculated as IC_{50}(mutant VEGF)/IC_{50}(wild-type VEGF). Under these conditions, Fab12-IgG and Y0317-IgG showed IC_{50} values of 9 nM and 1 nM, respectively.

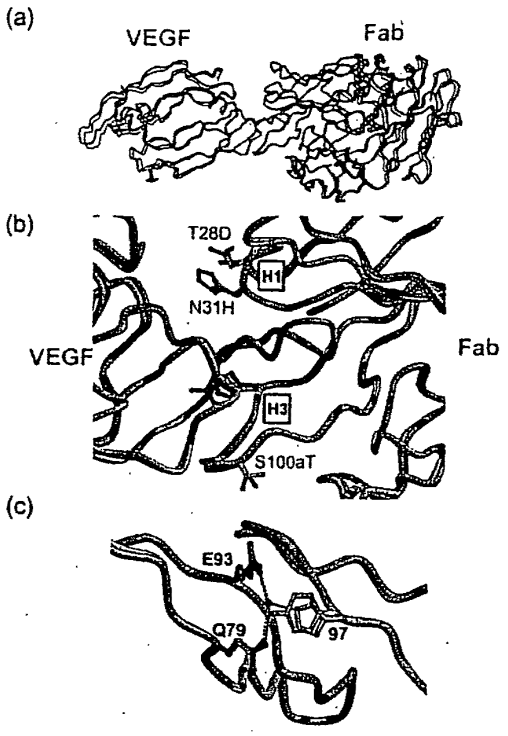

Figure 4. Structure of the affinity-improved Y0317 Fab in complex with VEGF. A superposition of the structure (Muller *et al.*, 1998a) of wild-type humanized antibody Fab-12 (gray) in complex with VEGF (gray) is shown with that of Fab Y0317 (green) in complex with VEGF (yellow). (a) Overall view of the complex, including one Fab molecule bound to one dimer of VEGF (a second Fab molecule is bound at left in the crystal) shows that the binding site for both antibody variants centers on the "80's loop" of VEGF. (b) A view of the four CDR changes between Fab-12 and Y0317 Fab shows that the new D28 and T100a side-chains do not directly contact antigen. However, H31 and Y97 form new contacts. (c) Interactions of H97 and an associated, buried water molecule in the Fab-12 complex, compared with those of Y97 in the Y0317 complex.

to >100-fold (Table 8) affinity improvement in Y0317 can be attributed to two CDR mutations: H97Y and N31H. In fact, H97Y alone improves binding affinty 14-fold.

Despite the relatively slow k_{on} and slow k_{off} of the parental antibody, binding selections described here yielded slower dissociation rates and improved equilibrium dissociation constants. Results of SPR measurements demonstrated that affinity is enhanced mainly through a slower dissociation rate (as opposed to faster association). These results are consistent with the idea of off-rate selection (Hawkins *et al.*, 1992) and with the progressively increased stringency in washing procedures used here (see Materials and Methods and Table 1). Previous binding-optimization efforts have also often yielded larger improvements in k_{off} than in k_{on} (see Lowman & Wells, 1993; Yang *et al.*, 1995; Schier *et al.*, 1996). This may suggest fundamental limitations to the improvements in k_{on} for a given binding site. Even if no conformational changes need occur between free and bound states, the on-rate is limited by the size of the binding interface and the translational and rotational diffusion rates of the binding components (reviewed by Delisi, 1983).

The association rate constants (k_{on}) for both the wild-type Y0192 and the final Y0317 antibodies are relatively slow (about 4×10^4 M^{-1} s^{-1} for both) compared to other antibodies of equal or weaker antigen binding affinity. In fact, the fastest k_{on} identified for any mutant was 6.8×10^4 M^{-1} s^{-1} (Table 6). Typically, k_{on} for antibodies binding to protein antigens, including affinity-matured antibodies, has fallen in the range of 3×10^4 to 1×10^6 M^{-1} s^{-1} (Karlsson *et al.*, 1991; Malmborg *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Wu *et al.*, 1998). In this particular protein-protein interaction, a likely explanation for the slow k_{on} is the high degree of flexibility associated with the binding site both on the Fab and on VEGF. In fact, crystallographic evidence suggests that the "80's loop" region is quite mobile (Muller *et al.*, 1997; Muller *et al.*, 1998b). We are pursuing other strategies to assess whether improvements to k_{on} can be obtained.

The contributions of point mutations in proteins to the free energy of binding or activation are often additive (Wells, 1990). This principle has been used to produce a variety of affinity-improved protein variants based on point or grouped mutations identified by phage display (Lowman & Wells, 1993; Yang *et al.*, 1995) or point-mutant screening (Wu *et al.*, 1998). Considering the initial library selectants Y0238-3 (>ninefold improved in affinity) and Y0243-1 (3.1-fold improved), we would have predicted an improvement of >27-fold for Y0313-1 or Y0317 (Table 7). In fact, a 22-fold improvement is observed (Figure 2) at 25 °C. Addition of the CDR-H1 mutation would be predicted to improve affinity slightly (1.3-fold), but in fact this mutation reduced affinity >twofold (Y0268-1 *versus* Y0313-1; Table 6). Certainly additivity does not always apply, particularly if interacting residues are involved (Wells, 1990). In this case, non-additivity probably results from steric interference between the new Trp in CDR-H2 and the new Tyr in CDR-H3.

To test the energetics of binding by the final Y0317 antibody to VEGF, we made use of a panel of alanine mutants that had been previously constructed for mapping the binding site of the original antibody (Muller *et al.*, 1998a). For these experiments, we made use of the full-length IgG forms of both antibodies. In view of the slow dissociation kinetics for both antibodies, ELISA assays were carried out at 37 °C with incubation for at least five hours to insure that equilibrium was reached. Under these conditions two dramatic differences appear in the Ala-scan of VEGF with respect to Y0317 *versus* Fab-12: both R82A and I83A have small effects on binding in Y0317, but result in large decreases in binding for Fab-12. The reasons for these differences are not clear, but R82 and I83 do have significant surface area (55 Å^2 and 32 Å^2, respectively) buried on binding to VEGF, and make contacts that include residues S100a of CDR-H3 and N52 of CDR-H2 in the wild-type antibody (Muller *et al.*, 1998a).

Structural analysis of the affinity-matured Fab

The structures of a number of antibodies derived from *in vivo* immunization and hybridoma techniques have been determined, in complex with their antigens (reviewed by Nezlin, 1998), and recently, crystallization and preliminary X-ray studies of a chain-shuffled anti-lysozyme scFv antibody in complex with antigen were reported (Küttner *et al.*, 1998). However, to our knowledge, the Y0317 Fab:VEGF structure is the first report of an *in vitro* affinity-matured Fab in complex with antigen. The structural basis of binding affinity improvement is therefore of interest

The Fab fragment of the affinity-matured anti-VEGF antibody Y0317 preserves the structure of the original humanized antibody, Fab-12. Superposition with Fab-12 results in an rmsd of only 0.38 Å for a total of 431 C^{α}-positions, demonstrating the absence of major structural changes between the two molecules. With a total of 1800 Å^2 of solvent-accessible surface buried in each VEGF-Fab interface, the contact area is about 50 Å^2 larger than in the Fab-12 complex. This small increase in buried surface area is mostly due to the exchange of H97 to a tyrosine residue. In the VEGF:Fab-12 complex, H97 buries a solvent-accessible area of 56 Å^2, while the larger tyrosine side-chain of the matured antibody accounts for 86 Å^2 of buried surface. The tyrosine side-chain also affects the hydrogen-bonding pattern and the number of ordered water molecules in the vicinity. In the parental antibody complex, a water molecule near H97 forms two hydrogen bonds to the side-chains of Q79 and E93 of VEGF (Figure 4(c)). In the complex with the affinity-matured Fab, this water molecule is replaced by the hydroxyl group of the newly introduced tyrosine side-chain at position 97. The H97Y mutation therefore not only increases the amount of buried surface area, but also introduces two additional hydrogen bonds between the ligand and Fab-0317 (Figure 4(c)). This is in good agreement with the observation that this single substitution improves VEGF binding affinity by 14-fold (Table 6). We therefore conclude that this single substiution is responsible for the majority of the improvement in binding affinity of Y0317 compared to the parent antibody.

In contrast, despite the availability of the crystal structures of both complexes, it remains uncertain what the structural basis is of the 3.6-fold enhanced binding caused by the N31H mutation. The side-chains of the asparagine and the histidine residues in this position adopt identical conformations in both crystal structures, and the amount of buried surface is not significantly increased in the VEGF:Fab-Y0317 complex. The only difference we can detect is a slight possible improvement in the hydrophobic interactions between the histidine side-chain and the phenyl group of VEGF residue F17, which has rotated slightly compared to the parent complex. It is unclear whether this could contribute to the increased affinity.

Neither of the remaining differences between Fab-12 and Fab-Y0317 has a significant effect on the binding affinity towards VEGF, and the structures show that these residues contribute only marginally to the interface. Some interactions are present between VEGF and the main-chain atoms of the serine and threonine residues in position 100a of the two Fabs, but the side-chains of these residues are not in contact with VEGF. Finally, no contact exist between VEGF and T28 (or D28) of the Fab fragments (the closest point on VEGF to this residue is more than 6 Å distant).

In summary, the analysis and comparison of the two crystal structures are in very good agreement with the results of the binding assays on the various single mutants of the Fab fragments. Although it is not possible to quantify the effects introduced by the amino acid exchanges solely based on the crystal structures, the detailed crystallographic

analysis supports and enables us to interpret the binding data.

Biological implications for antibody inhibition of VEGF

An inhibitory antibody of improved affinity may have improved potency or efficacy in treating diseases associated with VEGF expression. Preceding versions of the anti-VEGF antibody described here, including the murine A4.6.1 (Kim *et al.*, 1993), the humanized version Fab-12 (Presta *et al.*, 1997), as well as Y0192 (Muller *et al.*, 1998a), clearly demonstrated sufficient affinity to effect inhibition of VEGF activity. Here, we show that an affinity-improved variant, Fab Y0317, can inhibit endothelial cell proliferation *in vitro* with least 30-fold greater potency than the parental humanized Fab (Figure 3).

We have limited our optimization strategy to a subset of heavy-chain CDR residues implicated by alanine-scanning and crystallography (Muller *et al.*, 1998a). Furthermore, not all combinations of phage-derived mutations have been tested. One may therefore reasonably ask whether Y0317, with $K_d^{25°} = 20$ pM and $K_d^{37°} = 130$ pM, is the globally optimum variant for binding to this particular epitope (or others) on VEGF. Other affinity optimization efforts have resulted in protein-protein binding affinities in the low picomolar range, from $K_d = 6$ pM to 15 pM (see, e.g. Lowman & Wells, 1993; Schier *et al.*, 1996; Yang *et al.*, 1995). Indeed, we cannot exclude the possibility that higher affinity variants of the A4.6.1 antibody could be produced. However, it seems unlikely that further affinity improvement would greatly enhance biological potency or efficacy because for effective inhibition, the antibody must certainly occupy a significant fraction (perhaps >99%) of the available (VEGF) binding sites. Serum VEGF concentrations of about 20 pM in normals, and of >300 pM in patients with metastatic carcinoma, have been observed (Kraft *et al.*, 1999). Local or effective concentrations are likely higher. If we conservatively assume the effective concentration of VEGF *in vivo* to be about 400 pM, then 400 pM of even an infinite-affinity Fab would be required to block all sites.

Other factors may limit the improvement in potency of a full-length IgG resulting from an improvement in intrinsic binding affinity of the Fab for antigen. The full-length IgG form of the antibody may benefit from an avidity effect *in vivo*, especially since VEGF is known to associate with proteoglycans on the cell surface (Gitay-Goren *et al.*, 1992). Even in cell-based assays, the IgG form of Fab-12 is a more effective inhibitor than the Fab form (data not shown). Finally, the half-life for dissociation of the affinity-improved antibody is already significant, even on the time-scale of the half-life of clearance for IgG's (days to weeks). The effect of an improved association rate constant for antibody in this system is unknown.

The fact that point (Ala) mutations in the antibody binding site on VEGF sometimes have lesser effects on the binding of Y0317 than on the binding of Fab-12 may suggest that the optimized binding site is more tolerant than the parental one of variations in the antigen. Indeed, Y0317 showed greatly enhanced affinity for murine VEGF over that of Fab-12 (data not shown), though still >100-fold weaker than its affinity for human VEGF. This could provide an advantage against naturally arising VEGF variants.

Materials and Methods

Construction of phage libraries and mutagenesis

A variant of the Fab-12 antibody (a humanized form of murine antibody A4.6.1) was previously identified from phage-displayed Fab libraries for improved expression on phage particles (Muller *et al.*, 1998a). We made use of the plasmid pY0192, a phagemid construct with ampicillin (or carbenicillin) resistance, as the parental ("wild-type") construct for libraries described here. To prevent contamination by wild-type sequence (Lowman *et al.*, 1991; Lowman, 1998), templates with the TAA stop codon at each residue targeted for randomization were prepared from CJ236 *E. coli* cells (Kunkel *et al.*, 1991). Libraries are designated according to the mutagenic oligonucleotides used for their construction: YC265, TCC TGT GCA GCT TCT GGC NNS NNS TTC NNS NNS NNS GGT ATG AAC TGG GTC CG, randomizing residues 27-28, 30-32 in CDR-H1; YC266, GAA TGG GTT GGA TGG ATT AAC NNS NNS NNS GGT NNS CCG ACC TAT GCT GCG G, randomizing residues 52a-54, 56 in CDR-H2; YC103, GAA TGG GTT GGA TGG ATT NNS NNS NNS NNS GGT GAA CCG ACC TAT G, randomizing residues 52-54 in CDR-H2; YC81, C TGT GCA AAG TAC CCG NNS TAT NNS NNS NNS NNS CAC TGG TAT TTC GAC, randomizing residues 97, 99-100b in CDR-H3; and YC101, CGT TTC ACT TTT TCT NNS GAC NNS TCC AAA NNS ACA GCA TAC CTG CAG , randomizing residues 71, 73, and 76 in the "FR-H3" region. An additional library in CDR-H2 was designed to insert three new residues: YC90, GA TGG ATT AAC ACC TAT NNS NNS NNS ACC GGT GAA CCG ACC.

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

Site-directed mutagenesis for point mutations was carried out as above, using appropriate codons to produce the respective mutations, and sequences were confirmed by single-strand DNA sequencing using Sequenase™ (USB).

Phage binding 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(109) in 50 mM carbonate buffer (pH 9.6) and blocked with 5% (w/v) instant milk in 50 mM carbonate buffer, (pH 9.6). Also included were phage prepared from a non-displaying

control phagemid (pCAT), which confers chloramphenicol resistance, as a means of measuring background and enrichment (Lowman & Wells, 1993). Bound phage were eluted with 0.1 M HCl and immediately neutralized with one-third volume of 1 M Tris (pH 8.0). The eluted phage were propagated by infecting XL1 cells for the next selection cycle as described (Lowman, 1998).

In the first cycle, the VEGF plate was incubated with Fab-phage, then was briefly washed to remove bound phage. In the second cycle, binding and washing were followed by a one hour dissociative incubation at room temperature with binding buffer, after which the plate was again washed prior to acid elution. This process was repeated in rounds 3, 4 and 5, except that 1 μM VEGF was included in the dissociative incubation, and the incubation time was increased to 2, 18, and 37 hours, respectively. During these selections, Y0192 phage showed enrichments ranging from 1.5-fold (at the lowest stringency) to 22,000-fold (using a two hour dissociation incubation). However, further increases in stringency (rounds 4-5) resulted in decreasing enrichments for the control phage, with higher enrichments observed for certain libraries, especially the two CDR-H2 libraries and the CDR-H3 library (Table 1).

In cycle 6, a 17 hour dissociative incubation at room temperature was followed by an additional 30 hour incubation at 37 °C (also including VEGF in the buffer). Under these conditions, Y0192-phage showed only slight binding enrichment (20-fold), whereas the CDR-H3 library phage were enriched by 3500-fold. Cycle 7 was carried out with a 63 hour dissociative incubation, after which only small enrichment factors were observed. However, some libraries were continued through eight cycles (with 120 hours of dissociative incubation in the presence of VEGF), after which Fab-phage were still recoverable by acid elution (data not shown).

Purification of Fab

For small-scale preparations, Y0317 Fab and mutants were prepared from *E. coli* shake-flasks as described (Muller *et al.*, 1998a).

For large-scale preparation, whole cell broth was obtained from a ten liter *E. coli* fermentation. The cells were lysed with a Manton-Gaulin homogenizer (two passes at 6000 psi; lysate temperature maintained at 15-25 °C with a heat exchanger). A 5% (v/v) solution of polyethylene imine (PEI), pH 6.0, was added to the lysate to give a final concentration of 0.25% (v/v). The lysate was mixed for 30 minutes at room temperature. The suspension was centrifuged, and the supernatant (containing the Fab) was processed further. The pH of the supernatant was adjusted to 6.0 with 6 M HCl, followed by dilution to a conductivity of 5 mS/cm with purified water. The conditioned supernatant was loaded onto a BakerBond ABx ion-exchange column. Following a wash with the column equilibration buffer, the Fab was eluted with an increasing sodium chloride gradient in the equilibration buffer. Fractions containing the Fab were identified by SDS-PAGE. The BakerBond ABx column fractions were pooled, pH adjusted to 5.5 with 1 M Mes and diluted to a conductivity of 5 mS/cm with purified water. The conditioned BakerBond ABx pool was loaded onto a SP Sepharose HP cation exchange column (Pharmacia). Once again, the Fab was eluted with a sodium chloride-containing gradient. Fractions containing the Fab were identified by SDS-PAGE. The level of purity of Fab (as determined by SDS-PAGE) after this two column purification was >95%.

BIAcore™ binding 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(109) or VEGF(165) was buffer-exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 μg/ml. Aliquots of VEGF were injected at a flow rate of 2 μl/minute to achieve approximately 700-1400 response units (RU) of coupled protein. A solution of 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, twofold serial dilutions of Fab were injected in PBS/Tween buffer (0.05% Tween-20 in phosphate-buffered saline) at 25 °C or 37 °C at a flow rate of 10 μl/minute. Equilibrium dissociation constants, K_d values from SPR measurements were calculated as k_{off}/k_{on} (Tables 6 and 8).

Radiolabeled VEGF binding assay

Solution binding affinity of Fabs for VEGF was measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled VEGF(109) in the presence of a titration series of unlabeled VEGF, then capturing bound VEGF with an anti-Fab antibody-coated plate.

To establish conditions for the assay, microtiter plates (Dynex) were coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23 °C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [^{125}I]VEGF(109) was mixed with serial dilutions of Fab-12 or Fab Y0317, respectively. Fab-12 was incubated overnight; however, the Fab Y0317 incubation was continued for 65 hours to insure that equilibrium was reached. Thereafter, the mixtures were transferred to the capture plate for incubation at room temperature for one hour. The solution was then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates had dried, 150 μl/well of scintillant (MicroScint-20; Packard) was added, and the plates were counted on a Topcount gamma counter (Packard) for ten minutes. Concentrations of each Fab were chosen to give ≤20% of maximal binding.

For competitive binding assays, Dynex plates were coated and blocked as above, and serial threefold dilutions of unlabeled VEGF(109) were made in PBS/Tween buffer in a Nunc plate. [^{125}I]VEGF(109) was added, followed by addition of a fixed concentration of Fab-12 or Fab Y0317. The final concentrations of Fab-12, and Fab Y0317 were 100 pM and 10 pM, respectively. After incubation (as above), bound VEGF was captured and quantified as described above. The binding data was analyzed using a computer program to perform Scatchard analysis (Munson & Rodbard, 1980) for determination of the dissociation binding constants, K_d, for Fab-12 and Fab Y0317.

ELISA assay of VEGF Ala mutants

The binding affinities of VEGF Ala mutants for full-length Fab-12-IgG (known as rhuMAb VEGF) and Y0317-IgG, a full-length IgG form of the improved antibody expressed in CHO cells (V. Chisholm, unpublished results) were measured as previously described (Muller *et al.*, 1997; Muller *et al.*, 1998a) for the murine antibody A4.6.1, except that the temperature was increased to 37°C, and the incubation time increased to five hours, to insure that equilibrium was reached with the high-affinity antibody.

Cell-based assay of VEGF inhibition

Several versions of the anti-VEGF antibody were tested for their ability to antagonize VEGF(165) 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% (v/v) dia-filtered fetal bovine serum) for 24 hours.

The concentration of VEGF used for inducing the cells was determined by first titrating to identify 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 hours of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [^{3}H]thymidine for 24 hours and harvested for counting, using a TopCount gamma counter.

Crystallization and refinement

The complex between the Fab fragment of affinity-matured, humanized antibody Y0317 Fab and the receptor binding fragment of VEGF (VEGF(109)) was purified and crystallized as described for the analogous complex with the parental humanized Fab-12 fragment (Muller *et al.*, 1998a). The resulting crystals had symmetry consistent with space group $P2_1$ with cell parameters $a = 89.1$ Å, $b = 66.4$ Å, $c = 138.7$ Å, and $\beta = 94.7°$, and were isomorphous with the crystals obtained with the parent complex. A data set was collected from a single frozen crystal at beam line 5.0.2 at the Advanced Light Source, Berkeley, and processed using programs MOSFLM and SCALA (CCP4, 1994). The final data set ($R_{merge} = 7.3\%$) is described in Table 10. Starting with the model of Brookhaven Protein Data Bank entry 1bj1 (Muller *et al.*, 1998a), the structure was refined using the programs X-PLOR (Brünger *et al.*, 1987) and REFMAC (CCP4, 1994). The free *R*-value was monitored using the identical set of reflections sequestered before refinement of parent complex. The differences in the primary structure between Fab-12 and Fab-Y0317 were modeled using the program O (Jones *et al.*, 1991). After correction for anisotropy and application of a bulk solvent correction, the *R*-value reached its final value of 19.9% for all reflections greater than 0.2σ (see Table 10; $R_{free} = 27.4\%$).

Table 10. Crystallographic data and refinement statistics

A. *Data collection*	Overall	Last shell
Resolution range (Å)	30-2.4	2.53-2.40
No. of observations	208,257	22,278
Unique reflections	61,742	8900
Completeness (%)	97.4	96.7
Mean $I/\sigma(I)$	13.6	2.7
R_{sym}	0.073	0.38
B. *Refinement*		
Resolution range (Å)	20-2.4	
No. of reflections	61,689	
No. of atoms	8577	
rmsd bond lengths (Å)	0.013	
rmsd angles (deg.)	1.9	
rmsd improper angles (deg.)	0.92	
rmsd *B*-factors for all bonded atoms, Å^2	3.5	
Number of main-chain torsion angles in disallowed regions of Ramachandran plot[a]	2	

[a] See Laskowski *et al.* (1993).

Protein Data Bank accession number

The coordinates for the VEGF:Y0317 Fab complex have been deposited in the Protein Data Bank, accession number 1cz8.

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Edited by I. A. Wilson

(Received 19 July 1999; received in revised form 7 September 1999; accepted 13 September 1999)

```
        1       10        20        30        40
A4.6.1  EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
         *   *  ** *   *** *  *                *
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                  *  ** * *
humIII  EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA

                50  a      60        70        80
A4.6.1  PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
          * *                             *  *
F(ab)-12 PGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYL
                ** **** ***  *** *   * * *  * *
humIII  PGKGLEWVSVISGDGGSTTYADSVKGRFTISRDNSKNTLYL

          abc    90                            110
A4.6.1  QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS  (SEQ.ID NO:9)
         *** ***   * *                        *  *
F(ab)-12 QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS  (SEQ.ID NO:7)
                        *                   *
humIII  QMNSLRAEDTAVYYCARG----------FDYWGQGTLVTVSS  (SEQ.ID NO:11)
```

FIG._1A

```
        1       10        20        30        40
A4.6.1  DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
           **       *    * *
F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP
                                 *   *       *
humKI   DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP

                50        60        70        80
A4.6.1  DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
        ****                              **   * *
F(ab)-12 GKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
          *   **   *
humKI   GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP

                90       100
A4.6.1  EDIATYYCQQYSTVPWTFGGGTKLEIKR    (SEQ.ID NO:10)
          *                   *   *
F(ab)-12 EDFATYYCQQYSTVPWTFGQGTKVEIKR    (SEQ.ID NO:8)
                  ***
humKI   EDFATYYCQQYNSLPWTFGQGTKVEIKR    (SEQ.ID NO:12)
```

FIG._1B

```
                10          20          30          40
F(ab)-12    DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
Y0243-1     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0238-3     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0313-1     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0317       DIQLTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
                                   CDR-L1
                50          60          70          80
F(ab)-12    GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0243-1     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0238-3     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0313-1     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0317       GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
                       CDR-L2
                90          100         110
F(ab)-12    EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:8)
Y0243-1     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:109)
Y0238-3     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:111)
Y0313-1     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:113)
Y0317       EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:115)
                       CDR-L3
```

FIG._10A

```
                10          20          30          40
F(ab)-12    EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA
Y0243-1     EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
Y0238-3     EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGINWVRQA
Y0313-1     EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
Y0317       EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
                                   CDR-H1
                50          60          70          80
F(ab)-12    PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0243-1     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0238-3     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0313-1     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0317       PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
                       CDR-H2                CDR-7
                90          100         110
F(ab)-12    LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTL   (SEQ.ID NO:7)
Y0243-1     LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTL   (SEQ.ID NO:110)
Y0238-3     LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:112)
Y0313-1     LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:114)
Y0317       LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:116)
                                   CDR-H3
```

FIG._10B

DEPARTMENT OF HEALTH & HUMAN SERVICES

rhuFab VEGF

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

Our Reference: BB-IND 8633

OCT 13 1999

21579

Genentech, Incorporated
Attention: Robert L. Garnick, Ph.D.
Vice President, Regulatory Affairs
1 DNA Way
South San Francisco, CA 94080-4990

Dear Dr. Garnick:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND)**. The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

BB-IND #: 8633

SPONSOR: Genentech, Incorporated

PRODUCT NAME: Humanized Monoclonal Antibody Fragment (rhuFab V2) (E. coli, Genentech) to Vascular Endothelial Growth Factor (VEGF), Intravitreal

DATE OF SUBMISSION: October 6, 1999

DATE OF RECEIPT: October 7, 1999

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file**. Please include three originals of all illustrations which do not reproduce well.

It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in writing of the reasons for placing the IND on hold.

10-18-99 P02:54 IN
10-18-99 P

You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected, fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information, and all serious, unexpected adverse experiences must be reported, in writing, to this Division and to all study centers within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

Sponsors of INDs for products used to treat life-threatening or severely debilitating diseases are encouraged to consider the interim rule outlined in 21 CFR 312.80 through 312.88.

Telephone inquiries concerning this IND should be made directly to me at (301) 827-5101. Correspondence regarding this file should be addressed as follows:

Center for Biologics Evaluation and Research
Attn: Office of Therapeutics Research and Review
HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,

Kay Schneider

Kay Schneider, M.S.
Consumer Safety Officer
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Enclosures (3): 21 CFR Part 312
21 CFR 50.20, 50.25
Information sheet on 21 CFR 25.24

I

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

JAN 27 2006

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality, and Compliance
1 DNA Way
South San Francisco, CA 94080-4990

Dear Dr. Garnick:

We have received your biologics license application (BLA) submitted under section 351 of the Public Health Service Act for the following biological product:

Our Submission Tracking Number (STN): BL #125156/0

Name of Biological Product: Lucentis™ (ranibizumab)

Indication: Treatment for patients with neovascular age-related macular degeneration

Date of Application: December 29, 2005

Date of Receipt: December 30, 2005

User Fee Goal Date: June 30, 2006

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We note that you have not fulfilled the requirement. We are waiving the requirement for pediatric studies for this application.

If you have not already done so, promptly submit the *content of labeling* (21 CFR 601.14(b)) in electronic format as described at the following website: http://www.fda.gov/oc/datacouncil/spl.html.

We will notify you within 60 days of the receipt date if the application is sufficiently complete to permit a substantive review.

We request that you submit all future correspondence, supporting data, or labeling relating to this application in triplicate, citing the above STN number. Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions. Effective August 29, 2005, the new address for all submissions to this application is:

Food and Drug Administration
Center for Drug Evaluation and Research
Therapeutic Biological Products Document Room
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, please contact the Regulatory Project Manager, Lori Gorski, at (301) 796-0722.

Sincerely,

Maureen P. Dillon-Parker
Chief, Project Management Staff
Division of Anti-Infective and
Ophthalmology Products
Office of Antimicrobials
Center for Drug Evaluation and Research

J

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

BLA 125156

MAR 14 2006

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality & Compliance
1 DNA Way
South San Francisco, California 94080-4990

Dear Dr. Garnick:

This letter is in regard to your biologics license application (BLA) submitted under section 351 of the Public Health Service Act.

We have completed an initial review of your application dated December 29, 2005, for Lucentis (ranibizumab injection) to determine its acceptability for filing. Under 21 CFR 601.2(a), your application was filed on February 28, 2006. The user fee goal date is June 30, 2006. This acknowledgment of filing does not mean that we have issued a license nor does it represent any evaluation of the adequacy of the data submitted.

At this time, we have not identified any potential review issues. Our filing review is only a preliminary review, and deficiencies may be identified during substantive review of your application. Following a review of the application, we shall advise you in writing of any action we have taken and request additional information if needed.

Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions.

Please use the following address for any amendments to your application:

Food and Drug Administration
Center for Drug Evaluation and Research
Therapeutic Biological Products Document Room
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Maureen Dillon Parker
Chief, Project Management Staff
Division of Anti-Infective and Ophthalmology Products
Office of Antimicrobial Products
Center for Drug Evaluation and Research

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Paul J. Carter *et al.*-- § 156	Docket No: 22338-80060
Patent No.: 6,407,213	Assignee: Genentech, Inc.
Issued: June 18, 2002	Unit: OPLA
Application No: 08/146,206	

For: METHOD FOR MAKING HUMANIZED ANTIBODIES – Application for § 156 Patent Term Extension

Mail Stop: **Patent Ext.**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

POWER OF ATTORNEY BY ASSIGNEE

The assignee of the entire right, title, and interest in U.S. Patent No. 6,407,213 (granted on application serial no. 08/146,206), Genentech Inc., hereby appoints the practitioners associated with

CUSTOMER NUMBER 33694

as its attorneys and agents to prosecute the captioned patent application, and to transact all business in the U.S. Patent and Trademark Office connected therewith.

Pursuant to 37 C.F.R. § 3.73(b), the undersigned states that Genentech Inc. is the assignee of the entire right, title, and interest in the captioned patent/application by virtue of an assignment by the inventors to Genentech Inc. recorded at Reel 7035/ Frame 0272.

The undersigned, whose title is supplied below, is authorized to act on behalf of the assignee.

Respectfully submitted,

Genentech, Inc.

08/23/06

Jeffrey S. Kubinec
Associate General Counsel – Patent Law

Date

4-19-07

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	Group Art Unit: 1642
Paul J. Carter et al.	Examiner: Minh-Tam Davis
Serial No.: 08/146,206	CONFIRMATION NO:
Filed: November 17, 1993	**CUSTOMER NO: 09157**
For: METHOD FOR MAKING HUMANIZED ANTIBODIES	**EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

RESPONSE TO NOTICE UNDER 37 CFR 1.251 -PATENT

Mail Stop RECONSTUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent , mailed October 17, 2006. The copy of the papers listed in the Notice under 37 CFR 1.251 are a complete and accurate copy of the applicant's record of such papers, except for the following:

1. The PALM INTRANET record states that a Response After Non-Final Action was filed on 07/28/1997. The Response was received by the PTO on 06/27/1997. Please see the enclosed copy.
2. The PALM INTRANET record states that a Notice of Appeal was filed on 08/10/1998. The Notice of Appeal was received by the PTO on 06/26/1998. Please see the enclosed copy.
3. The PALM INTRANET record states that an Extension of Time was filed on 08/10/1998. The Extension of Time was received by the PTO on 06/26/1998. Please see the enclosed copy.
4. The PALM INTRANET record states that an Examiner Interview Summary Record was created on 11/01/2001. Applicants' papers show there was one on 12/11/2001, but not one on 11/01/2001. Please see the enclosed copy.

5. A Request for a Corrected Filing Receipt was mailed on 06/24/1994. Please see the enclosed copy. The PALM INTRANET does not list this.

6. A Request for a Corrected Filing Receipt was mailed on 04/10/1995. Please see the enclosed copy. The PALM INTRANET does not list this.

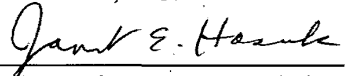
7. A Supplemental Information Disclosure Statement was filed on 10/07/1997. Please see the enclosed copy. The PALM INTRANET does not list this.

8.An Examiner Interview Summary Record was created on 07/16/1999. Please see the enclosed copy. The PALM INTRANET does not list this.

Respectfully submitted,

GENENTECH, INC.

Date: April 17, 2007

By: 

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

Revised (10/18/95)

PTO-2055-B (Rev. 10/03)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

In re Patent No.: 6,407,213 B1

Patentee: Carter et al

Patent Date: June 18 2002

Application No.: 08/146,206

Filing Date: November 17 1993

Direct to:

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

NOTICE UNDER 37 CFR 1.251 - Patent

Statement (check the appropriate box):

☐ The copy submitted with this reply is a complete and accurate copy of applicant's record of all of the correspondence between the Office and the applicant for the above-identified application (except for U.S. patent documents), and applicant is not aware of any correspondence between the Office and applicant for the above-identified application that is not among applicant's records.

☒ The copy of the paper(s) listed in the notice under 37 CFR 1.251 is/are a complete and accurate copy of applicant's record of such paper(s). **Except for the items listed in the Response to Notice under 37 CFR 1.251–Patent**

☐ The papers produced by applicant are applicant's complete record of all of the correspondence between the Office and the applicant for the above-identified application (except for U.S. patent documents), and applicant is not aware of any correspondence between the Office and the applicant for the above-identified application that is not among applicant's records.

☐ Applicant does not possess any record of the correspondence between the Office and the applicant for the above-identified application.

April 17, 2007
Date

WMLee
Signature

Wendy Lee
Typed or printed name

A copy of this notice should be returned with the reply.

Burden Hour Statement: This collection of information is required by 37 CFR 1.251. The information is used by the public to reply to a request for copies of correspondence between the applicant and the USPTO in order to reconstruct an application file. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 60 minutes to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.**

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis Confirmation No: 3992 **Customer No: 09157** **EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

TRANSMITTAL LETTER

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent, mailed October 17, 2006. Transmitted herewith are the following documents:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent
3. Copy of the PALM INTRANET Listing
4. Copies of Correspondence between PTO and Applicant
5. Copies of References Cited in Information Disclosure Statements

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,
GENENTECH, INC.

Date: April 17, 2007

By: Janet E. Hasak

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

4-19-07

COPY

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: November 17, 1993
Mailed On: April 17, 2007

Docket No.: P0709P1
By: Janet E. Hasak - Reg. 28,616
for Wendy M. Lee- Reg. 40,378

The following has been received in the U.S. Patent Office on the date stamped:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent and copy of the PALM INTRANET Listing
3. Copies of Correspondence between PTO and Applicant
4. Copies of References Cited in Information Disclosure Statements

Express Mail No. EV 384 511 097 US
EV 384 511 106 US

EV384511106US 4-19-07

EV384511097US

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis Confirmation No: 3992 **Customer No: 09157** **EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

TRANSMITTAL LETTER

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent, mailed October 17, 2006. Transmitted herewith are the following documents:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent
3. Copy of the PALM INTRANET Listing
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5. Copies of References Cited in Information Disclosure Statements

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,
GENENTECH, INC.

Date: April 17, 2007

By: Janet E. Hasak

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

Day : Tuesday
Date: 10/17/2006
Time: 12:35:09

PALM INTRANET

Patent Number Information

Application Number: **08/146206**
Assignments
Filing or 371(c) Date: **11/17/1993**
Effective Date: **11/17/1993**
Application Received: **11/17/1993**
Patent Number: **6407213**
Issue Date: **06/18/2002**
Date of Abandonment: **00/00/0000**
Attorney Docket Number: **709P1**
Status: **150 / PATENTED CASE**
Confirmation Number: **3992**

Examiner Number: **73622 / DAVIS, MINH TAM**
Group Art Unit: **1642**
Class/Subclass: **530/387.300**
Lost Case: **YES**
Interference Number:
Unmatched Petition: **NO**
L&R Code: Secrecy Code:**1**
Third Level Review: **NO** Secrecy Order: **NO**
Status Date: **05/31/2002**
Oral Hearing: **NO**

Title of Invention: **METHOD FOR MAKING HUMANIZED ANTIBODIES**

Bar Code	PALM Location	Location Date	Charge to Loc	Charge to Name	Employee Name	Location
08146206	16M1	02/23/2006	16X1	DAVIS, MINH TAM	1600,OUTGOING MAIL	REM/00/A 89

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Day : Tuesday
Date: 10/17/2006

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Time: 12:39:56

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Date	Status	Code	Description
10/17/2006		M2512	MAIL RECONSTRUCTION NOTICE - PATENTED APPLIC
10/17/2006		2512	RECONSTRUCTION NOTICE UNDER 37 CFR 1.251 - PATI
08/29/2006		LFLOST	FILE MARKED LOST
10/30/2002		N423	POST ISSUE COMMUNICATION - CERTIFICATE OF COR
06/20/2002		CRFA	SEQUENCE MOVED TO PUBLIC DATABASE
06/18/2002		PGM/	RECORDATION OF PATENT GRANT MAILED
05/31/2002	150	WPIR	ISSUE NOTIFICATION MAILED
06/18/2002		PTAC	PATENT ISSUE DATE USED IN PTA CALCULATION
05/09/2002		R1021	RECEIPT INTO PUBS
05/04/2002		PILS	APPLICATION IS CONSIDERED READY FOR ISSUE
03/18/2002	95	N084	ISSUE FEE PAYMENT VERIFIED
03/18/2002		DRWF	WORKFLOW - DRAWINGS FINISHED
03/18/2002		DRWM	WORKFLOW - DRAWINGS MATCHED WITH FILE AT CO
05/02/2002		R1021	RECEIPT INTO PUBS
03/15/2002		CSRF	WORKFLOW - CUSTOMER SERVICE REQUEST - FINISH
03/26/2002		R1021	RECEIPT INTO PUBS
03/18/2002		DRWI	WORKFLOW - DRAWINGS RECEIVED AT CONTRACTOI
03/18/2002		DRWR	WORKFLOW - DRAWINGS SENT TO CONTRACTOR
03/18/2002		R85B	WORKFLOW -RECEIVED 85B - UNMATCHED
03/18/2002	94	IFEE	ISSUE FEE PAYMENT RECEIVED
03/15/2002		CSRI	WORKFLOW - CUSTOMER SERVICE REQUEST - BEGIN
01/28/2002		SENT	WORKFLOW - FILE SENT TO CONTRACTOR
01/28/2002	93	R1021	RECEIPT INTO PUBS
01/08/2002		D1220	DISPATCH TO PUBLICATIONS
12/18/2001	92	MN/=.	MAIL NOTICE OF ALLOWANCE

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12/18/2001		MN/DR	MAIL FORMAL DRAWINGS REQUIRED
12/14/2001		N/DR	FORMAL DRAWINGS REQUIRED
12/14/2001	90	N/=.	NOTICE OF ALLOWANCE DATA VERIFICATION COMPI
12/14/2001	89	CNTA	NOTICE OF ALLOWABILITY
11/01/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
08/29/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
10/02/2001		AF/D	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEI
10/11/2001		FWDX	DATE FORWARDED TO EXAMINER
10/02/2001		SA..	SUPPLEMENTAL RESPONSE
09/04/2001		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/16/2001		FWDX	DATE FORWARDED TO EXAMINER
07/30/2001		SA..	SUPPLEMENTAL RESPONSE
07/13/2001		FWDX	DATE FORWARDED TO EXAMINER
07/13/2001		SA..	SUPPLEMENTAL RESPONSE
04/26/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
05/02/2001		FWDX	DATE FORWARDED TO EXAMINER
04/26/2001	71	A...	RESPONSE AFTER NON-FINAL ACTION
04/26/2001		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
10/25/2000	41	MCTNF	MAIL NON-FINAL REJECTION
10/23/2000	40	CTNF	NON-FINAL REJECTION
09/06/2000		DOCK	CASE DOCKETED TO EXAMINER IN GAU
11/17/1993	19	IEXX	INITIAL EXAM TEAM NN
02/03/2000		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATAB/
01/11/2000		FWDX	DATE FORWARDED TO EXAMINER
12/28/1999		SA..	SUPPLEMENTAL RESPONSE
01/11/2000		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
11/24/1999	41	MCTMS	MAIL MISCELLANEOUS COMMUNICATION TO APPLIC
11/22/1999	40	CTMS	MISCELLANEOUS ACTION WITH SSP
08/23/1999		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
09/07/1999		FWDX	DATE FORWARDED TO EXAMINER
08/30/1999		SA..	SUPPLEMENTAL RESPONSE
07/19/1999		FWDX	DATE FORWARDED TO EXAMINER
07/16/1999		SA..	SUPPLEMENTAL RESPONSE
05/07/1999		FWDX	DATE FORWARDED TO EXAMINER
04/09/1999	71	ELC.	RESPONSE TO ELECTION / RESTRICTION FILED
03/29/1999	41	MCTRS	MAIL RESTRICTION REQUIREMENT
03/26/1999	40	CTRS	REQUIREMENT FOR RESTRICTION / ELECTION

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03/12/1999		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
02/01/1999		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
02/01/1999		RQPR	REQUEST FOR FOREIGN PRIORITY (PRIORITY PAPERS
01/07/1999		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
01/19/1999		FWDX	DATE FORWARDED TO EXAMINER
01/15/1999		SA..	SUPPLEMENTAL RESPONSE
11/09/1998		FWDX	DATE FORWARDED TO EXAMINER
11/06/1998		SA..	SUPPLEMENTAL RESPONSE
10/16/1998		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
08/26/1998		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/26/1998		AF/D	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEI
09/03/1998		FWDX	DATE FORWARDED TO EXAMINER
08/26/1998	71	R129	REQUEST UNDER RULE 129 TO REOPEN PROSECUTION
08/26/1998		MABN3	MAIL EXPRESS ABANDONMENT (DURING EXAMINATI
08/26/1998	168	ABN3	EXPRESS ABANDONMENT (DURING EXAMINATION)
08/10/1998	120	N/AP	NOTICE OF APPEAL FILED
08/10/1998		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
08/13/1998		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
04/13/1998		C.AD	CORRESPONDENCE ADDRESS CHANGE
12/23/1997	61	MCTFR	MAIL FINAL REJECTION (PTOL - 326)
12/22/1997	60	CTFR	FINAL REJECTION
10/10/1997		FWDX	DATE FORWARDED TO EXAMINER
10/07/1997		SA..	SUPPLEMENTAL RESPONSE
10/10/1997		FWDX	DATE FORWARDED TO EXAMINER
09/01/1997		SA..	SUPPLEMENTAL RESPONSE
10/10/1997		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
09/01/1997		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
10/10/1997		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
10/09/1997		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATAB/
08/01/1997		FWDX	DATE FORWARDED TO EXAMINER
07/28/1997	71	A...	RESPONSE AFTER NON-FINAL ACTION
06/27/1997		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
07/23/1997		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
12/23/1996	41	MCTNF	MAIL NON-FINAL REJECTION
12/23/1996	40	CTNF	NON-FINAL REJECTION
12/03/1996		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
12/10/1996		FWDX	DATE FORWARDED TO EXAMINER

12/03/1996	71	R129	REQUEST UNDER RULE 129 TO REOPEN PROSECUTION
12/03/1996		MABN3	MAIL EXPRESS ABANDONMENT (DURING EXAMINATI
12/03/1996	168	ABN3	EXPRESS ABANDONMENT (DURING EXAMINATION)
08/30/1996		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
08/30/1996		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
04/01/1996	120	N/AP	NOTICE OF APPEAL FILED
04/01/1996		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
04/08/1996		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
12/26/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
10/27/1995	61	MCTFR	MAIL FINAL REJECTION (PTOL - 326)
10/26/1995	60	CTFR	FINAL REJECTION
08/03/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/18/1995		FWDX	DATE FORWARDED TO EXAMINER
06/12/1995	71	A...	RESPONSE AFTER NON-FINAL ACTION
06/12/1995		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
04/17/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
12/09/1994	41	MCTNF	MAIL NON-FINAL REJECTION
12/06/1994	40	CTNF	NON-FINAL REJECTION
10/04/1994		FWDX	DATE FORWARDED TO EXAMINER
09/26/1994	71	ELC.	RESPONSE TO ELECTION / RESTRICTION FILED
08/26/1994	41	MCTRS	MAIL RESTRICTION REQUIREMENT
08/25/1994	40	CTRS	REQUIREMENT FOR RESTRICTION / ELECTION
06/15/1994		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATABA
06/14/1994		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
06/06/1994		A.PE	PRELIMINARY AMENDMENT
11/17/1993		A.PE	PRELIMINARY AMENDMENT
05/24/1994	30	DOCK	CASE DOCKETED TO EXAMINER IN GAU
05/14/1994		FILM	APPLICATION CAPTURED ON MICROFILM
05/03/1994		COMP	APPLICATION IS NOW COMPLETE
05/09/1994		INCD	NOTICE MAILED--APPLICATION INCOMPLETE--FILING
04/15/1994		CRFD	CRF IS FLAWED TECHNICALLY / NOT ENTERED INTO I
04/07/1994		RTAD	RELEASED TO OIPE
04/04/1994		M903	NOTICE OF DO/EO ACCEPTANCE MAILED
03/31/1994		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
03/14/1994		DKTD	371 APPLICATION PREEXAMINATION DOCKETING
02/19/1994		IBPM	IB PAPER MATCH
12/02/1993		DKTD	371 APPLICATION PREEXAMINATION DOCKETING

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12/02/1993		DYWD	APPLICANT DELAY WAIVED
12/02/1993		R331	DEMAND RECEIVED
11/17/1993		R371	RECEIPT OF 371 REQUEST

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UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

JUN 19 2007

Office of Regulatory Policy
HFD-7
5600 Fishers Lane (Rockwall II Rm 1101)
Rockville, MD 20857

Attention: Beverly Friedman

The attached application for patent term extension of U.S. Patent No. 6,407,213, was filed on August 25, 2006, under 35 U.S.C. § 156. It is noted that patent term extension applications for the same regulatory review period for the human biological product, LUCENTIS® (ranibizumab), have been filed in U.S. Patent No. 7,060,269 (as indicated in a letter to FDA mailed on April 3, 2007) and U.S. Patent No. 6,884,879.

The assistance of your Office is requested in confirming that the product identified in the application, LUCENTIS® (ranibizumab), has been subject to a regulatory review period within the meaning of 35 U.S.C. § 156(g) before its first commercial marketing or use and that the application for patent term extension was filed within the sixty-day period after the product was approved. Since a determination has not been made whether the patent in question claims a product which has been subject to the Federal Food, Drug and Cosmetic Act, or a method of manufacturing or use of such a product, this communication is NOT to be considered as notice which may be made in the future pursuant to 35 U.S.C. § 156(d)(2)(A).

Our review of the application to date indicates that the subject patent would be eligible for extension of the patent term under 35 U.S.C. § 156.

Applicant is advised that despite the statement in compliance with 37 C.F.R. § 1.740(a)(14) regarding payment of the applicable fee by check for submission of a patent term extension application, no check was present and no record exists of the Office cashing the check. Therefore, in accordance with the express authorization provided in the same paragraph, the fee of $1,120 as perscribed in 37 C.F.R. § 1.20(j) is being charged to deposit account no. 18-1260.

Inquiries regarding this communication should be directed to the undersigned at (571) 272-7755 (telephone) or (571) 273-7755 (facsimile).

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Jeffrey P. Kushan
Sidley Austin LLP
1501 K Street, N.W.
Washington, DC 20005

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

NOV 2 1 2007

Re: Lucentis
Patent Nos. 6,407,213
6,884,879
Docket Nos. 2007E-0424
2007E-0425

The Honorable Jon Dudas
Under Secretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the application for patent term extension for U.S. Patent Nos. 6,407,213 and 6,884,879 filed by Genentech, Inc. under 35 U.S.C. § 156. The human biological product claimed by these patents is Lucentis (ranibizumab), which was assigned biologic license application (BLA) No. 125156/0.

A review of the Food and Drug Administration's official records indicates that this product was subject to a regulatory review period before its commercial marketing or use, as required under 35 U.S.C. § 156(a)(4). Our records also indicate that it represents the first permitted commercial marketing or use of the product, as defined under 35 U.S.C. § 156(f)(1), and interpreted by the courts in *Glaxo Operations UK Ltd. v. Quigg*, 706 F. Supp. 1224 (E.D. Va. 1989), *aff'd*, 894 F. 2d 392 (Fed. Cir. 1990).

The BLA was approved on June 30, 2006, which makes the submission of the patent term extension applications on August 25, 2006, timely within the meaning of 35 U.S.C. § 156(d)(1).

Should you conclude that the subject patents are eligible for patent term extension, please advise us accordingly. As required by 35 U.S.C. § 156(d)(2)(A) we will then determine the applicable regulatory review period, publish the determination in the *Federal Register*, and notify you of our determination.

Please let me know if we can be of further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

JAN - 8 2008

Office of Regulatory Policy
HFD - 7
5600 Fishers Lane (Rockwall II Rm. 1101)
Rockville, MD 20857

Attention: Beverly Friedman

Dear Ms. Axelrad:

Transmitted herewith is a copy of the application for patent term extension of U.S. Patent No. 6,407,213. The application was filed on August 25, 2006, under 35 U.S.C. § 156. It is noted that patent term extension applications for the same regulatory review period for the human biological product, LUCENTIS® (ranibizumab), have been filed in U.S. Patent Nos. 6,884,879 and 7,060,269.

The patent claims a product that was subject to regulatory review under the Federal Food, Drug and Cosmetic Act. Subject to final review, the subject patent is considered to be eligible for patent term extension. Thus, a determination by your office of the applicable regulatory review period is necessary. Accordingly, notice and a copy of the application are provided pursuant to 35 U.S.C. § 156(d)(2)(A).

Inquiries regarding this communication should be directed to the undersigned at (571)272-7755 (telephone) or (571) 273-7755 (facsimile).

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

RE: LUCENTIS® (ranibizumab)
FDA Docket No. 2007E-0424

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
Rockville MD 20857

APR 28 2008

Re: LUCENTIS - 6,407,213
Docket No.: 2007E-0424
LUCENTIS - 6,884,879
Docket No.: 2007E-0425
LUCENTIS - 7,060,269
Docket No.: 2007E-0146

The Honorable Jon Dudas
Undersecretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the applications for patent term extension for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269, filed by Genentech, Inc., under 35 U.S.C. section 156 et seq. We have reviewed the dates contained in the application and have determined the regulatory review period for LUCENTIS (ranibizumab), the human biological product claimed by the patents.

The total length of the regulatory review period for LUCENTIS is 2,430 days. Of this time, 2,247 days occurred during the testing phase and 183 days occurred during the approval phase. These periods of time were derived from the following dates:

1. The date an exemption under subsection 505(i) of the Federal Food, Drug, and Cosmetic Act involving this biologic product became effective: November 6, 1999.

   The applicant claims October 7, 1999, as the date the investigational new drug application (IND) became effective. However, FDA records indicate that the IND effective date was November 6, 1999, which was thirty days after FDA receipt of the IND.

2. The date the application was initially submitted with respect to the human biological product under section 351 of the Public Health Service Act: December 30, 2005.

   The applicant claims December 29, 2005, as the date the biologics license application (BLA) for LUCENTIS (BLA 125156/0) was initially submitted. However, FDA records indicate that BLA 125156/0 was submitted on December 30, 2005.

3. The date the application was approved: June 30, 2006.

   FDA has verified the applicant's claim that BLA 125156/0 was approved on June 30, 2006.

This determination of the regulatory review period by FDA does not take into account the effective date of the patents, nor does it exclude one-half of the testing phase as required by 35 U.S.C. section 156(c)(2).

Please let me know if we can be of further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

a person with Medicare could be identified because the sample is small enough to identify participants. CMS would make exceptions if the information is needed for one of the routine uses or if it's required by law.

POLICIES AND PRACTICES FOR STORING, RETRIEVING, ACCESSING, RETAINING, AND DISPOSING OF RECORDS IN THE SYSTEM:

STORAGE:

Records are stored on both tape cartridges (magnetic storage media) and in a DB2 relational database management environment (DASD data storage media).

RETRIEVABILITY:

Information is most frequently retrieved by HICN, provider number (facility, physician, IDs), service dates, and beneficiary state code.

SAFEGUARDS AND PROTECTIONS:

CMS has protections in place for authorized users to make sure they are properly using the data and there is no unauthorized use. Personnel having access to the system have been trained in the Privacy Act and information security requirements. Employees who maintain records in this system cannot use or disclose data until the recipient agrees to implement appropriate management, operational and technical safeguards that will protect the confidentiality, integrity, and availability of the information and information systems.

This system would follow all applicable Federal laws and regulations, and Federal, HHS, and CMS security and data privacy policies and standards. These laws and regulations include but are not limited to: the Privacy Act of 1974; the Federal Information Security Management Act of 2002 (when applicable); the Computer Fraud and Abuse Act of 1986; the Health Insurance Portability and Accountability Act of 1996; the E-Government Act of 2002, the Clinger-Cohen Act of 1996; the Medicare Modernization Act of 2003, and the corresponding implementing regulations. OMB Circular A–130, Management of Federal Resources, Appendix III, Security of Federal Automated Information Resources also applies. Federal, HHS, and CMS policies and standards include but are not limited to all pertinent National Institute of Standards and Technology publications, the HHS Information Systems Program Handbook, and the CMS Information Security Handbook.

RETENTION AND DISPOSAL:

Records are maintained with identifiers for all transactions after they are entered into the system for a period of 20 years. Records are housed in both active and archival files. All claims-related records are encompassed by the document preservation order and will be retained until notification is received from the Department of Justice.

SYSTEM MANAGER AND ADDRESS:

Director, Centers for Beneficiary Choices, CMS, Mail stop C5–19–07, 7500 Security Boulevard, Baltimore, Maryland 21244–1850.

NOTIFICATION PROCEDURE:

For purpose of notification, the subject individual should write to the system manager who will require the system name, and the retrieval selection criteria (e.g., HICN, facility/pharmacy number, service dates, etc.).

RECORD ACCESS PROCEDURE:

For purpose of access, use the same procedures outlined in Notification Procedures above. Requestors should also reasonably specify the record contents being sought. (These procedures are in accordance with Department regulation 45 CFR 5b.5 (a)(2).)

CONTESTING RECORD PROCEDURES:

The subject individual should contact the system manager named above, and reasonably identify the record and specify the information to be contested. State the corrective action sought and the reasons for the correction with supporting justification. (These procedures are in accordance with Department regulation 45 CFR 5b.7.)

RECORD SOURCE CATEGORIES:

Summary prescription drug claim information contained in this system is obtained from the Part D Sponsor daily and monthly drug event transaction reports, Medicare Beneficiary Database (09–70–0530), and other payer information to be provided by the TROOP Facilitator.

SYSTEMS EXEMPTED FROM CERTAIN PROVISIONS OF THE ACT:

None.

[FR Doc. E8–11949 Filed 5–28–08; 8:45 am]
BILLING CODE 4120–03–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket Nos. FDA–2007–E–0461 (formerly Docket No. 2007E–0424), FDA–2007–E–0165 (formerly Docket No. 2007E–0425), FDA–2007–E–0459 (formerly Docket No. 2007E–0146)]

Determination of Regulatory Review Period for Purposes of Patent Extension; LUCENTIS

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) has determined the regulatory review period for LUCENTIS and is publishing this notice of that determination as required by law. FDA has made the determination because of the submission of applications to the Director of Patents and Trademarks, Department of Commerce, for the extension of patents which claim that human biological product.

ADDRESSES: Submit written or electronic comments and petitions to the Division of Dockets Management (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Submit electronic comments to *http://www.regulations.gov.*

FOR FURTHER INFORMATION CONTACT: Beverly Friedman, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Ave., Bldg. 51, rm. 6222, Silver Spring, MD, 20993–0002, 301–796–3602.

SUPPLEMENTARY INFORMATION: The Drug Price Competition and Patent Term Restoration Act of 1984 (Public Law 98–417) and the Generic Animal Drug and Patent Term Restoration Act (Public Law 100–670) generally provide that a patent may be extended for a period of up to 5 years so long as the patented item (human drug product, animal drug product, medical device, food additive, or color additive) was subject to regulatory review by FDA before the item was marketed. Under these acts, a product's regulatory review period forms the basis for determining the amount of extension an applicant may receive.

A regulatory review period consists of two periods of time: A testing phase and an approval phase. For human biological products, the testing phase begins when the exemption to permit the clinical investigations of the biological product becomes effective

and runs until the approval phase begins. The approval phase starts with the initial submission of an application to market the human biological product and continues until FDA grants permission to market the biological product. Although only a portion of a regulatory review period may count toward the actual amount of extension that the Director of Patents and Trademarks may award (for example, half the testing phase must be subtracted as well as any time that may have occurred before the patent was issued), FDA's determination of the length of a regulatory review period for a human biological product will include all of the testing phase and approval phase as specified in 35 U.S.C. 156(g)(1)(B).

FDA recently approved for marketing the human biologic product LUCENTIS (ranibizumab). LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration. Subsequent to this approval, the Patent and Trademark Office received patent term restoration applications for LUCENTIS (U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269) from Genentech, Inc., and the Patent and Trademark Office requested FDA's assistance in determining this patent's eligibility for patent term restoration. In letters dated July 24, 2007, and November 21, 2007, FDA advised the Patent and Trademark Office that this human biological product had undergone a regulatory review period and that the approval of LUCENTIS represented the first permitted commercial marketing or use of the product. Shortly thereafter, the Patent and Trademark Office requested that FDA determine the product's regulatory review period.

FDA has determined that the applicable regulatory review period for LUCENTIS is 2,430 days. Of this time, 2,247 days occurred during the testing phase of the regulatory review period, while 183 days occurred during the approval phase. These periods of time were derived from the following dates:

1. *The date an exemption under section 505(i) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 355(i)) became effective*: November 6, 1999. The applicant claims October 7, 1999, as the date the investigational new drug application (IND) became effective. However, FDA records indicate that the IND effective date was November 6, 1999, which was 30 days after FDA receipt of the IND.

2. *The date the application was initially submitted with respect to the human biological product under section 351 of the Public Health Service Act (42 U.S.C. 262)*: December 30, 2005. The applicant claims December 29, 2005, as the date the biologics license application (BLA) for LUCENTIS (BLA 125156/0) was initially submitted. However, FDA records indicate that BLA 125156/0 was submitted on December 30, 2005.

3. *The date the application was approved*: June 30, 2006. FDA has verified the applicant's claim that BLA 125156/0 was approved on June 30, 2006.

This determination of the regulatory review period establishes the maximum potential length of a patent extension. However, the U.S. Patent and Trademark Office applies several statutory limitations in its calculations of the actual period for patent extension. In its applications for patent extension for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269, this applicant seeks 378 days; 307 days or 17 days, respectively, of patent term extension.

Anyone with knowledge that any of the dates as published are incorrect may submit to the Division of Dockets Management (see **ADDRESSES**) written or electronic comments and ask for a redetermination by July 28, 2008. Furthermore, any interested person may petition FDA for a determination regarding whether the applicant for extension acted with due diligence during the regulatory review period by November 25, 2008. To meet its burden, the petition must contain sufficient facts to merit an FDA investigation. (See H. Rept. 857, part 1, 98th Cong., 2d sess., pp. 41–42, 1984.) Petitions should be in the format specified in 21 CFR 10.30.

Comments and petitions should be submitted to the Division of Dockets Management. Three copies of any mailed information are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. Comments and petitions may be seen in the Division of Dockets Management between 9 a.m. and 4 p.m., Monday through Friday.

Please note that on January 15, 2008, the FDA Division of Dockets Management Web site transitioned to the Federal Dockets Management System (FDMS). FDMS is a Government-wide, electronic docket management system. Electronic comments or submissions will be accepted by FDA only through FDMS at *http://www.regulations.gov.*

Dated: May 8, 2008.

Jane A. Axelrad,

Associate Director for Policy, Center for Drug Evaluation and Research.

[FR Doc. E8–12007 Filed 5–28–08; 8:45 am]

BILLING CODE 4160–01–S

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket No. FDA–2007–M–0467] (formerly Docket No. 2007M–0408), [Docket No. FDA–2007–M–0481] (formerly Docket No. 2007M–0467), [Docket No. FDA–2007–M–0480] (formerly Docket No. 2007M–0409), [Docket No. FDA–2007–M–0472] (formerly Docket No. 2007M–0413), [Docket No. FDA–2007–M–0468] (formerly Docket No. 2007M–0446), [Docket No. FDA–2007–M–0494] (formerly Docket No. 2007M–0380), [Docket No. FDA–2007–M–0493] (formerly Docket No. 2007M–0411), [Docket No. FDA–2007–M–0492] (formerly Docket No. 2007M–0410), [Docket No. FDA–2007–M–0490] (formerly Docket No. 2007M–0415), [Docket No. FDA–2007–M–0491] (formerly Docket No. 2007M–0447]

Medical Devices; Availability of Safety and Effectiveness Summaries for Premarket Approval Applications

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is publishing a list of premarket approval applications (PMAs) that have been approved. This list is intended to inform the public of the availability of safety and effectiveness summaries of approved PMAs through the Internet and the agency's Division of Dockets Management.

ADDRESSES: Submit written requests for copies of summaries of safety and effectiveness data to the Division of Dockets Management (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Please cite the appropriate docket number as listed in Table 1 of this document when submitting a written request. See the **SUPPLEMENTARY INFORMATION** section for electronic access to the summaries of safety and effectiveness.

FOR FURTHER INFORMATION CONTACT: Samie Allen, Center for Devices and Radiological Health (HFZ–402), Food and Drug Administration, 9200 Corporate Blvd., Rockville, MD 20850, 240–276–4013.

SUPPLEMENTARY INFORMATION:

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
Rockville MD 20857

JAN 8 2009

Re: Lucentis
Docket Nos.: FDA-2007-E-0461
FDA-2007-E-0165
FDA-2007-E-0459

The Honorable Jon Dudas
Under Secretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the patent term extension applications for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269 filed by Genentech, Inc., under 35 U.S.C. § 156. The patent claims Lucentis (ranibizumab), biologic license application (BLA) 125156/0.

In the May 29, 2008, issue of the Federal Register (73 Fed. Reg. 30949), the Food and Drug Administration published its determination of this product's regulatory review period, as required under 35 U.S.C. § 156(d)(2)(A). The notice provided that on or before November 25, 2008, 180 days after the publication of the determination, any interested person could file a petition with FDA under 35 U.S.C. § 156(d)(2)(B)(i) for a determination of whether the patent term extension applicant acted with due diligence during the regulatory review period.

The 180-day period for filing a due diligence petition pursuant to this notice has expired and FDA has received no such petition. Therefore, FDA considers the regulatory review period determination to be final.

Please let me know if we can provide further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

MAR 26 2009

Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

In Re: Patent Term Extension
Application for
U.S. Patent No. 6,407,213

NOTICE OF FINAL DETERMINATION AND REQUIREMENT FOR ELECTION

A determination has been made that U.S. Patent No. 6,407,213, claims of which cover the human biologic drug product LUCENTIS® (ranibizumab), is eligible for patent term extension under 35 U.S.C. § 156. The period of extension has been determined to be 378 days.

A single request for reconsideration of this final determination as to the length of extension of the term of the patent may be made if filed within one month of the date of this notice. Extensions of time under 37 CFR § 1.136(a) are not applicable to this time period.

Applicant also has applied for patent term extension of U.S. Patent No. 6,884,879 and U.S. Patent No. 7,060,269 based on the regulatory review period for the human biologic drug product LUCENTIS® (ranibizumab).

When patent term extension applications are filed for extension of the terms of different patents based upon the same regulatory review period for a product, the certificate of extension is issued to the patent having the earliest date of issuance, unless applicant elects a different patent. In the absence of an election by applicant within ONE MONTH of the date of this notice, and in accordance with 37 CFR 1.785(b), the applications for patent term extension of U.S. Patent No. 6,884,879 and U.S. Patent No. 7,060,269 will be denied. Accordingly, the application for patent term extension of the patent having the earlier date of issuance will be granted, i.e., a certificate of extension will be issued to U.S. Patent No 6,407,213 for a period of 378 days.

In the absence of a request for reconsideration, and if U.S. Patent No. 6,407,213 is elected, the Director will issue to the applicant a certificate of extension, under seal, for a period of 378 days in U.S. Patent No. 6,407,213.

The period of extension, if calculated using the Food and Drug Administration determination of the length of the regulatory review period published in the Federal Register of May 29, 2008 (73 Fed. Reg. 30949), would be 828 days. Under 35 U.S.C. § 156(c):

Period of Extension	=	½ (Testing Phase) + Approval Phase
	=	½ (2,247 days - 956 days) + 183 days
	=	828 days (2.3 years)

Since the regulatory review period began November 6, 1999, before the patent issued (June 18, 2002), only that portion of the regulatory review period occurring after the date the patent issued has been considered in the above determination of the length of the extension period 35 U.S.C. § 156(c). (From November 6, 1999, to and including, June 18, 2002, is 956 days; this period is subtracted for the number of days occurring in the testing phase according to the FDA's determination of the length of the regulatory review period.) No determination of a lack of due diligence under 35 U.S.C. § 156(c)(1) was made.

However, the 14 year exception of 35 U.S.C. § 156(c)(3) operates to limit the term of the extension in the present situation because it provides that the period remaining in the term of the patent measured from the date of approval of the approved product plus any patent term extension cannot exceed fourteen years. The period of extension calculated above, 828 days, would extend the patent from June 18, 2019, to September 23, 2021, which is beyond the 14-year limit (the approval date is June 30, 2006, thus, the 14 year limit is June 30, 2020). The period of extension is thus limited to 378 days, by operation of 35 U.S.C. § 156(c)(3). Accordingly, the period of extension is the number of days to extend the term of the patent from its original expiration date, June 18, 2019, to and including, June 30, 2020, or 378 days.

The limitations of 35 U.S.C. 156(g)(6) do not operate to further reduce the period of extension determined above.

Upon issuance of the certificate of extension, the following information will be published in the Official Gazette:

U.S. Patent No.:	6,407,213
Granted:	June 18, 2002
Original Expiration Date[1]:	June 18, 2019
Applicant:	Paul J. Carter et al.
Owner of Record:	Genentech, Inc.
Title:	Method for Making Humanized Antibodies
Product Trade Name:	LUCENTIS® (ranibizumab)
Term Extended:	378 days
Expiration Date of Extension:	June 30, 2020

[1]Subject to the provisions of 35 U.S.C. § 41(b).

Any correspondence with respect to this matter should be addressed as follows:

By mail: Mail Stop Hatch-Waxman PTE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450.

By FAX: (571) 273-7755

Telephone inquiries related to this determination should be directed to the undersigned at (571) 272-7755.

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Office of Regulatory Policy
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 51, Rm. 6222
Silver Spring, MD 20993-0002

Attention: Beverly Friedman

RE: LUCENTIS® (ranibizumab)
Docket No.: FDA-2007-E-0461

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. Patent No.	**6,407,213 – § 156**	Unit:	OPLA
Serial No.:	**08/ 146,206**		
Confirmation No.:	3992		
Filed:	25 August 2006		
First Inventor:	P.J. CARTER		
Patent Owner:	Genentech, Inc.		
For:	Method for making humanized antibodies Application for patent term extension under 35 U.S.C. § 156		

Mail Stop **Hatch-Waxman PTE**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ELECTION UNDER 37 C.F.R. § 1.785(b)

Sir:

This letter responds to the Notice of Final Determination and Requirement for Election mailed in the captioned application for patent term extension on 26 March 2009. The Notice observes that applicant filed applications to extend the terms of U.S. Patent Nos. 6,407,213, 6,884,879, and 7,060,269 based on the regulatory review period for LUCENTIS®. The Notice further states a requirement that applicant elect one of the patents to receive a term extension certificate within a period of one month of the date of the Notice. This election is filed within the stated period and is therefore timely.

Pursuant to § 1.785(b), **applicant elects U.S. Patent No. 6,407,213** to receive a certificate of extension under § 1.780 and 35 U.S.C. § 156(e)(1). Applicant requests that the Director proceed to issue a certificate of extension of U.S. Patent No. 6,407,213 based on the regulatory review period for LUCENTIS® for a period of 378 days, as indicated in the Notice of Final Determination and Requirement for Election issued in this application for patent term extension.

We believe that no fee is due in respect of this election. However, the Director is requested to debit any fee required for entry or consideration of this paper from our Deposit Account No. 18-1260.

Respectfully submitted,

/David L. Fitzgerald/

David L. Fitzgerald, Reg. No. 47,347
Attorney for Genentech, Inc.

24 April 2009

SIDLEY AUSTIN LLP
1501 K Street, NW
Washington, DC 20005

tel. (202) 736-8818
fax (202) 736-8711

Electronic Acknowledgement Receipt

EFS ID:	5212426
Application Number:	08146206
International Application Number:	
Confirmation Number:	3992
Title of Invention:	METHOD FOR MAKING HUMANIZED ANTIBODIES
First Named Inventor/Applicant Name:	PAUL J. CARTER
Customer Number:	33694
Filer:	David Laurence Fitzgerald
Filer Authorized By:	
Attorney Docket Number:	709P1
Receipt Date:	24-APR-2009
Filing Date:	17-NOV-1993
Time Stamp:	10:46:21
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	Lucentis_213_PTE_election.pdf	78045 b6baae5477e1a50f05bedfe33594cb57572ff209	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	78045

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

NOV 18 2009

Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

In Re: Patent Term Extension
Application for
U.S. Patent No. 6,407,213

Dear Mr. Kushan :

A certificate under 35 U.S.C. § 156 is enclosed extending the term of U.S. Patent No. 6,407,213 for a period of 378 days. While a courtesy copy of this letter is being forwarded to the Food and Drug Administration (FDA), you should directly correspond with the FDA regarding any required changes to the patent expiration dates.

Inquiries regarding this communication should be directed to the undersigned by telephone at (571) 272-7755, or by e-mail at mary.till@uspto.gov.

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Office of Regulatory Policy
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 51, Rm. 6222
Silver Spring, MD 20993-0002

Attention: Beverly Friedman

RE: LUCENTIS® (ranibizumab)
Docket No.: FDA-2007-E-0461

UNITED STATES PATENT AND TRADEMARK OFFICE

(12) CERTIFICATE EXTENDING PATENT TERM
UNDER 35 U.S.C. § 156

(68)	PATENT NO.	:	6,407,213
(45)	ISSUED	:	June 18, 2002
(75)	INVENTOR	:	Paul J. Carter et al.
(73)	PATENT OWNER	:	Genentech, Inc.
(95)	PRODUCT	:	LUCENTIS® (ranibizumab)

This is to certify that an application under 35 U.S.C. § 156 has been filed in the United States Patent and Trademark Office, requesting extension of the term of U.S. Patent No. 6,407,213 based upon the regulatory review of the product LUCENTIS® (ranibizumab) by the Food and Drug Administration. Since it appears that the requirements of the law have been met, this certificate extends the term of the patent for the period of

(94) 378 days

from June 18, 2019, the original expiration date of the patent, subject to the payment of maintenance fees as provided by law, with all rights pertaining thereto as provided by 35 U.S.C. § 156(b).

I have caused the seal of the United States Patent and Trademark Office to be affixed this 18th day of November 2009.

David J. Kappos
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

Mail Stop Interference
P.O. Box 1450
Alexandria Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 1

Filed 2 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. CARTER AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

DECLARATION - Bd.R. 203(b)[1]

Part A. Declaration of interference

An interference is declared (35 U.S.C. § 135(a)) between the above-identified parties. Details of the application(s), patent (if any), reissue application (if any), count(s) and claims designated as corresponding or as not corresponding to the count(s) appear in Parts E and F of this DECLARATION.

[1] "Bd.R. x" may be used as shorthand for "37 C.F.R. § 41.x". 69 Fed. Reg. 49960, 49961 (12 Aug. 2004).

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for 2:00 p.m. on 16 March 2010 (the Board will initiate the call).

No later than four business days prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors:	Paul J. Carter San Francisco, CA
	Leonard G. Presta San Francisco, CA

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part F. Count and claims of the parties

Count 1

A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter:	PCT/US92/05126, filed 15 June 1992; and 07/715,272, filed 14 June 1991, now abandoned.
Adair:	08/846,658, filed 01 May 1997; 08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999; 07/743,329, filed on 17 September 1991; PCT/GB90/02017, filed 21 December 1990; and GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. CARTER AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Part H. Order form for requesting file copies

When requesting copies of files, use of SO Form 4 will greatly expedite processing of the request. Please attach a copy of Parts E and F of this DECLARATION with a hand-drawn circle around the patents and applications for which a copy of a file wrapper is requested.

/Sally Gardner Lane/
Administrative Patent Judge

Enc:
Copy of STANDING ORDER
Form PTO-850
Copy U.S. Patent 6,407,213
Copy of claims of 11/284,261

cc (via overnight delivery):

Attorney for Carter:

Sidley Austin, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Attorney for Adair:

Cozen O'Connor, P.C.
1900 Market Street
Philadelphia, PA 19103-3508

Mail Stop Interference
P.O. Box 1450
Alexandria Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 1

Filed 2 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

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San Francisco, CA

Leonard G. Presta
San Francisco, CA

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Assignee:	Genentech, Inc.

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Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

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Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
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(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

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/Sally Gardner Lane/
Administrative Patent Judge

Enc:
Copy of STANDING ORDER
Form PTO-850
Copy U.S. Patent 6,407,213
Copy of claims of 11/284,261

cc (via overnight delivery):

Attorney for Carter:

Sidley Austin, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Attorney for Adair:

Cozen O'Connor, P.C.
1900 Market Street
Philadelphia, PA 19103-3508

Mail Stop Interference
P.O. Box 1450
Alexandria, Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 81

Filed 2 September 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL,
and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Before SALLY GARDNER LANE, RICHARD TORCZON, and SALLY C. MEDLEY, *Administrative Patent Judges.*

LANE, *Administrative Patent Judge.*

Judgment– Merits – Bd. R. 127

The Carter motion for judgment on the basis that the single involved Adair claim is barred under 35 U.S.C. § 135(b) was granted. (Paper 80). Because Adair no longer has an interfering claim that is not barred under 35 U.S.C. §135(b) it is appropriate to

enter judgment against Adair. *Berman v. Housey*, 291 F.3d 1345, 1351 (Fed. Cir. 2002).

It is

ORDERED that judgment on priority as to Count 1 (Paper 1 at 4), the sole count of the interference, is entered against senior party Adair;

FURTHER ORDERED that claim 24 of Adair application 11/284,261, which claim corresponds to Count 1 (Paper 1 at 4), is FINALLY REFUSED, 35 U.S.C. §135(a):

FURTHER ORDERED that if there is a settlement agreement, the parties are directed to 35 U.S.C. 135(c) and Bd. R. 205; and

FURTHER ORDERED that a copy of this judgment shall be entered into the administrative record of the Carter involved patent and application and the Adair involved application.

cc (via electronic filing):

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