Antibody-based therapy

Humanized antibodies

Greg Winter and William J. Harris

Hybridoma technology enabled rodent monoclonal antibodies to be created against human pathogens and cells, but these had limited clinical utility. Protein engineering, reviewed here by Greg Winter and William Harris, *is now generating antibodies for treatment of infectious disease, autoimmune disease and cancer by 'humanizing' rodent antibodies. Humanized antibodies have improved pharmacokinetics, reduced immunogenicity and have been used to clinical advantage.*

The antibody is an adaptor molecule containing binding sites for antigen at one end and for effector molecules at the other, and has evolved to bind to a vast range of antigens. Binding alone may be sufficient to neutralize some toxins and viruses, however, more commonly, the antibody triggers the complement system and cell-mediated killing. Although antibodies are natural therapeutic agents, it has proved difficult to make human monoclonal antibodies (mAbs) by hybridoma technology.

Rodent mAbs unfortunately have serious disadvantages: a short half-life in serum; only some of the different classes can trigger human effector functions; and the mAbs can also elicit an unwanted immune response in patients (human anti-mouse antibodies or HAMA). HAMA can result in enhanced clearance of the antibody from the serum, blocking of its therapeutic effect and hypersensitivity reactions. These problems have prompted the use of protein engineering technologies to 'humanize' rodent mAbs by transplanting antigen-binding sites from rodent to human antibodies. In principle, humanizing allows access to a large pool of well-characterized rodent mAbs for therapy, including those with specificities against human antigens that are difficult to elicit from a human immune response[1].

The engineering of antibodies is facilitated by the modular arrangement of protein domains: the heavy- and light-chain variable (V) domains are responsible for binding to antigen, and the constant domains to effector functions. As both complement and cell-mediated killing require fully glycosylated antibody, the engineered mAbs are expressed in mammalian hosts. Each antibody domain is encoded by a different genetic exon and, to build recombinant antibodies, the exons are pasted together. The exons encoding the variable domains (*V* genes) can be cloned from the genomic DNA of a B-cell hybridoma: more conveniently, the *V* genes of hybridomas are isolated

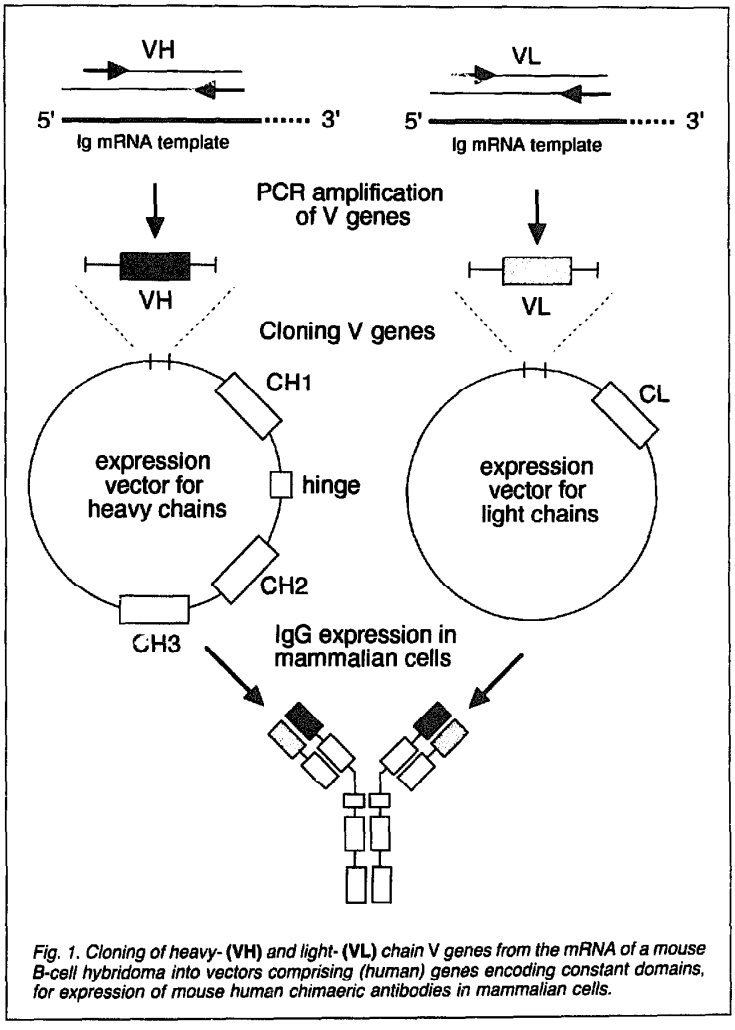

Fig. 1. Cloning of heavy- **(VH)** *and light-* **(VL)** *chain V genes from the mRNA of a mouse B-cell hybridoma into vectors comprising (human) genes encoding constant domains, for expression of mouse human chimaeric antibodies in mammalian cells.*

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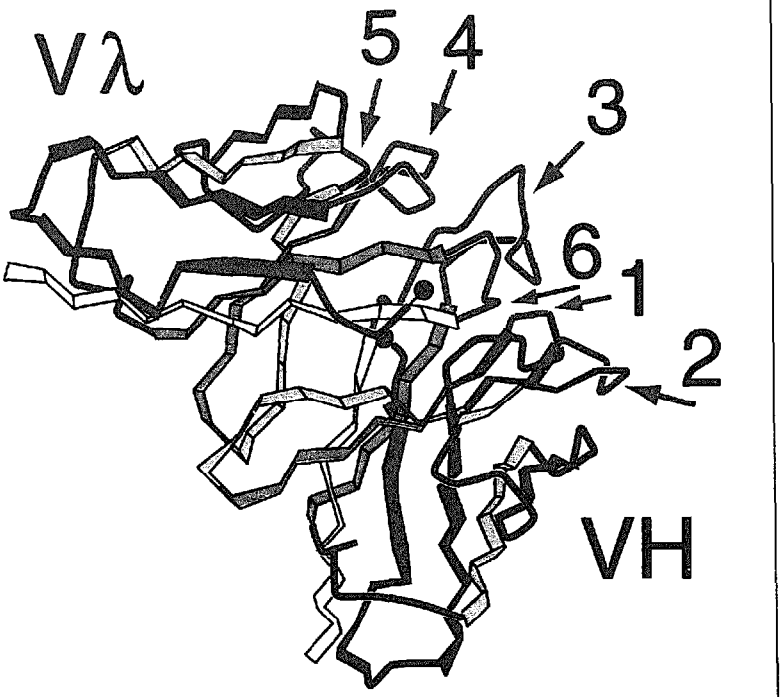

Fig. 2. The β-sheet framework structure of heavy- **(VH)** *and light-* **(Vλ)** *chain variable domains with hypervariable loops 1–6.* Reproduced, with permission, from Winter, G. and Milstein, C. (1991) *Nature* 349, 239–299.

from the mRNA by use of the polymerase chain reaction (PCR). The *V* genes are readily linked to those exons encoding constant domains for expression of mAbs[2] (Fig. 1). Expression vectors have been built with both antibody and viral promoters and enhancers, with *V* and *C* genes as different exons ('genomic') or linked together ('cDNA'). Different markers for selection of transformed cells are available and in both myeloma and CHO hosts[3,4], mAb expression is greatly enhanced by amplifying the number of integrated copies, resulting in yields of up to 0.7 gl^{-1} in fermenters[5].

Building humanized antibodies

The first generation of humanized antibodies were simple chimaeric mAbs, in which the variable domains of a rodent mAb are transplanted to the constant domains of human antibodies (Fig. 1). This reduces the immunogenicity of the rodent mAb (see below) and allows the effector functions to be selected for the therapeutic application. Thus, the human γ1 isotype appears to be the most effective for complement and cell-mediated killing, while the human γg4 isotype appears more suitable for imaging and blocking[6].

The second generation of humanized antibodies were the so-called CDR-grafted antibodies, in which the antigen-binding loops of the rodent mAb were built into a human antibody. The architecture of each antibody V-domain consists of a β-sheet 'sandwich' surmounted by antigen-binding loops (complementarity determining regions or CDRs): in different antibodies, these CDR loops are hypervariable in sequence (Fig. 2). It is this hypervariability that allows the antibody repertoire to bind a potentially vast array of antigens. By transplanting (or grafting) the CDRs from rodent mAb to human antibody, the antigen-binding site can also be transferred[7]; indeed the same human framework can be used for mounting different antigen-binding sites[7–9]. However, to recreate the antigen-binding site it is also necessary to consider other possible interactions between the β-sheet framework and the loops. With the help of molecular modelling it is possible to design framework substitutions that maintain key contacts with the CDR loops.

For example, with the rat antibody CAMPATH-1 directed against the CDw52 antigen of human lymphocytes, the simple grafting of the CDRs failed to transplant the binding activity to a human antibody. When the three-dimensional folding of the VH-CDR1 loop of the rat antibody and its contacts with the rat framework were modelled by computer graphics, the framework amino acid residue Phe27 was predicted to pack against the loop. However, in the human framework of the CDR-grafted antibody, Phe27 was replaced by Ser27; indeed when Ser27 was mutated to Phe in the CDR-grafted antibody, the binding activity was restored[8]. In other examples, enhancement of antigen affinity was achieved stepwise by combining several framework substitutions[10,11]. Indeed analysis of antibody structures is leading towards the identification of sets of framework residues that may exert an influence on CDR structure[11] and also on the packing of the strands of the β-sheet[12].

The first CDR-grafted antibodies were based on the known crystallographic structures of the human myeloma proteins[7–9]. CDR-grafted antibodies have also been built with consensus human frameworks based on several human heavy chains[13]. The use of a single or a limited number of human frameworks offers the prospect of a range of therapeutic antibodies that are almost identical, apart from the CDR sequences. Conversely a range of framework structures should be capable of supporting the CDR loops, and 'hyperchimaeric' CDR-grafted antibodies have used mouse–human frameworks. For example, to humanize a mouse antibody directed against the human IL-2 receptor (anti-Tac), a human framework sequence was selected by homology. A molecular model was then used to identify those framework residues of the rodent antibody that might interact with the antigen-binding loops, and these were built into the selected human framework[14] (Fig. 3). Chimaeric frameworks have also been proposed in which the

internal residues that pack between the domains and with the antigen-binding loops are derived from the rodent sequence and the solvent-accessible residues are taken from a human sequence[15].

Most generally, all of the rodent CDRs are transplanted from mouse to human antibody. However, some CDRs are more important than others for binding of antigen, as evident from the crystallographic structures of antibody–antigen complexes. The interaction of antibody loops with antigen involves both main-chain and sidechain contacts: as the CDR loops of mouse and human antibodies fold in a limited number of ways[16], it is possible to maintain some main-chain contacts while varying some of the sidechains (and sequence) of the CDRs[17].

There is some loss in binding affinity on CDR grafting but, in combination with some framework alterations, it is usually possible to obtain a reshaped antibody with an affinity within three-fold of the parent monoclonal antibody. High binding affinities may be critical for neutralization of a cytokine or toxin in the serum; they appear to be less important where multiple interactions can occur with high avidities, as with multimeric (cell surface) antibody binding to repeated epitopes on a viral coat[9]. However, small improvements in affinity have been seen for some CDR-grafted antibodies[18], and binding affinities can also be improved *in vitro*, by chain shuffling[19], or random mutation[20].

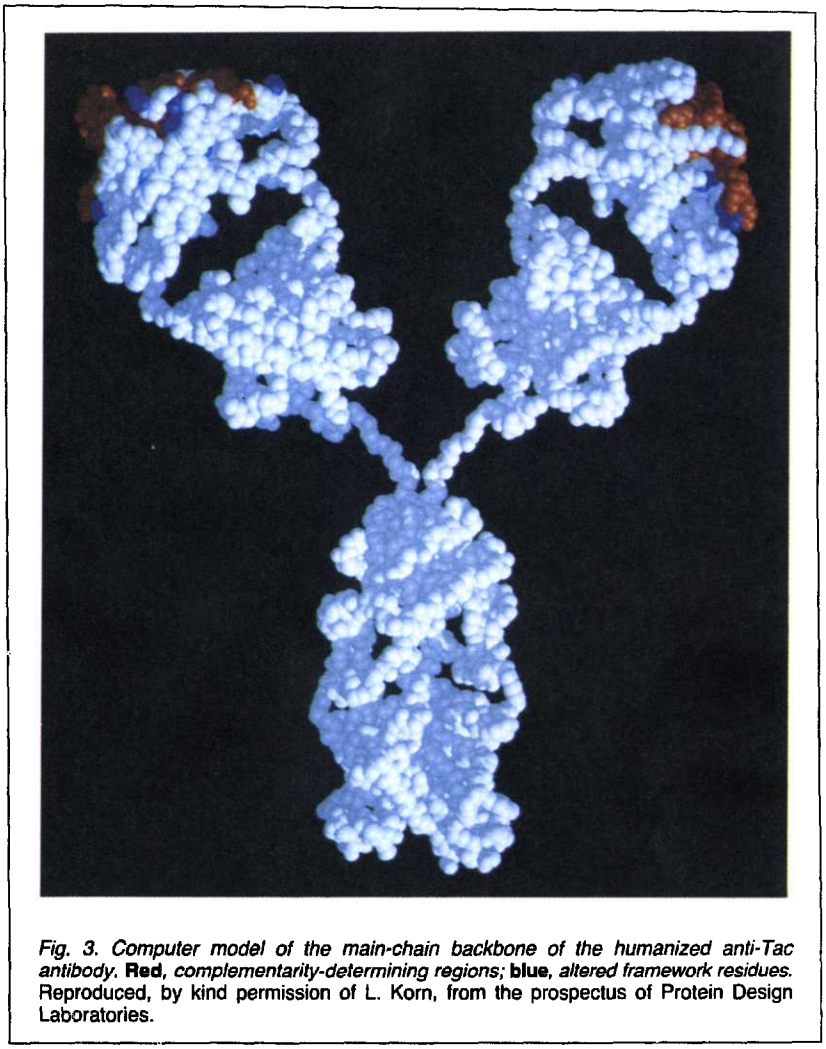

Fig. 3. Computer model of the main-chain backbone of the humanized anti-Tac antibody. **Red,** *complementarity-determining regions;* **blue,** *altered framework residues.* Reproduced, by kind permission of L. Korn, from the prospectus of Protein Design Laboratories.

Using humanized antibodies

Both chimaeric and CDR-grafted antibodies appear to have better pharmacokinetics than rodent mAbs, with extended serum half-life (>75 hours) in humans and cynomolgus monkeys. Likewise the immunogenicity is reduced. Much of the HAMA response to mouse antibodies in patients is directed against the constant region: chimaeric antibodies and CDR-grafted antibodies appear to elicit much less response with the immunogenic epitopes being located in the variable regions. Indeed much of this response is directed against the antigen-binding site (for review see Ref. 4).

No antibody response was detected against the CDR-grafted anti-CDw52 antibody during the treatment of two patients with non-Hodgkin's lymphoma for up to 43 days with escalating doses of antibody ranging from 1 to 20 mg per day[21]. Also, no response was reported when the antibody was used in a single course of therapy for rheumatoid arthritis patients[22] or in conjunction with an anti-CD4 antibody in treatment of a patient with intractable systemic vasculitis[23]. However, an antibody response was detected on further treatment of the rheumatoid patients[22]. Antibody responses were not detected against other CDR-grafted antibodies used for radio-imaging in tumour patients[24] or treating acute graft-versus-host disease[25].

Chimaeric and CDR-grafted antibodies have been constructed against a wide range of viral and bacterial pathogens, and against human cell-surface markers including tumour cell antigens. Some of the targets are summarized in Table I. CDR-grafted antibodies against lymphocyte markers have already been used to clinical advantage. The anti-CDw52 antibody was used to deplete a large tumour mass in two lymphoma patients, and to achieve clinical remission[21]. Likewise, this antibody resulted in significant clinical benefit for a patient with systemic vasculitis[23], and for rheumatoid arthritis patients[22]. The anti-Tac antibody was used for immunosuppression following allogeneic marrow transplantation, and resulted in improvement in several cases of acute graft-versus-host disease[25].

Future prospects

As shown above, humanized antibodies can be engineered from rodent mAbs. Their use has been demonstrated in the clinic, and they have a longer serum half-life and reduced immunogenicity

TABLE I. CDR-grafted antibodies for therapy (see also Ref. 3)

Target	Clinical potential
CDw52	lymphomas, systemic vasculitis, rheumatoid arthritis
CD3	organ transplantation
CD4	organ transplants, rheumatoid arthritis, Crohn's disease
IL-2 receptor	leukaemias and lymphomas, organ transplants, graft-versus-host disease
Tumour necrosis factor β	septic shock
Human immunodeficiency virus	AIDS
Rous sarcoma virus	respiratory syncytial virus infection
Herpes simplex virus	neonatal, ocular and genital herpes infection
Lewis-Y	cancer
$p185^{HER-2}$	cancer
Placental alkaline phosphatase	cancer
Carcinoembryonic antigen	cancer

compared to rodent mAbs. They therefore appear to be promising as therapeutics, especially for a single course of treatment. It is not yet clear whether humanized (or even human) antibodies will elicit a blocking immune response over longer or several courses of treatment.

The immunogenicity of humanized antibodies is likely to depend on several factors, including the immune state of the patients, and the dose and regimen of antibody administration. The target is also likely to be important; antibodies directed against cell-bound antigens might be expected to be more immunogenic than those binding to soluble antigen. Furthermore, since foreign framework regions can elicit an immune response[26], we might also expect that the differing strategies to select and mutate human frameworks (as described above) could lead to reshaped antibodies with differing immunogenicity. For example, human antibody frameworks that are mutated (*in vivo* or *in vitro*) with respect to the germ-line segments could prove immunogenic: even buried residues could form the critical element of a T-cell epitope if presented as a denatured peptide by a class II MHC molecule[27]. Indeed it may be desirable to design CDR-grafted antibodies by using framework regions based on human germ-line *V*-gene segments.

The design of antibodies for therapy would certainly be revolutionized if *in vitro* assays were available to test the immunogenicity of different constructs. However the key practical issues are not whether the immune response can be avoided entirely, but how it can be bypassed, for example by changing the idiotype of the engineered antibody, and whether the antibodies can be used for long enough to achieve clinical benefit.

So far we have focussed almost entirely on the construction and use of glycosylated antibodies expressed in mammalian cells. However, the use of antibody fragments may be advantageous in some applications, since they penetrate tissues more readily, and are cleared more rapidly from the serum. This may help in neutralizing and clearing drugs from the serum, or in imaging tumours with radioactive entities coupled to the fragments[1]. Although antibody fragments, lacking the glycosylated Fc portion, cannot trigger effector functions, they could in principle be equipped to do so, for example by chemically linking Fab fragments together[28] as bispecific antibody fragments (with one arm binding a tumour cell antigen and the other binding and triggering effector cells such as cytotoxic T cells or monocytes).

Furthermore, antibody fragments can be expressed by secretion from bacteria[29,30], and can be readily derived from the *V* genes of hybridomas, or from *V* gene repertoires. The repertoires are cloned for display on the surface of filamentous bacteriophage by fusion of the encoded antibody fragment to a coat protein of the phage, and phage with the desired activities selected by binding to antigen. Indeed this technology mimics the strategy of immune selection, and human antibody fragments with specificities against many different foreign and human self-antigens have been isolated from the same 'single pot' of phages (see Refs 31, 32 for review).

Over the past century we have seen three generations of antibody therapeutics: polyclonal animal antibodies, rodent monoclonal antibodies and now humanized antibodies. We anticipate that the use of 'repertoire selection' technologies to make human antibodies and fragments will provide the next generation. However, in the meantime it seems likely that humanized antibodies will prove clinically useful for treating several diseases, and the experience should prove valuable for designing and formulating the next generation of antibody therapeutics.

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The use of monoclonal antibodies to achieve immunological tolerance

Herman Waldmann and Stephen Cobbold

Monoclonal antibodies are potentially useful immunosuppressive agents. Short courses of CD4/CD8 monoclonal antibody can be used to guide the immune system of experimental animals to accept organ grafts and to arrest autoimmunity. This reprogramming, reviewed by Herman Waldmann and Stephen Cobbold, *is accompanied by potent T-cell dependent, 'infectious' regulatory mechanisms. A goal for therapeutic immunosuppression should be to understand and harness these innate immunoregulatory mechanisms.*

The ideal form of therapeutic immunosuppression would be one that could be given over a short-term period to achieve long-term unresponsiveness to the desired antigen, without impairing the response to infectious agents. Current immunosuppressive regimens are relatively non-antigen-specific, require long-term administration and incur a sustained risk of infection and undesirable side-effects.

If we are to achieve tolerance as a therapeutic goal in autoimmunity or in transplantation, then it is essential that we understand how the body normally establishes 'self tolerance'. From this basis we can determine which of these natural mechanisms might be exploited for pharmaceutical control. As thymus-derived cells (T cells) are required for driving most forms of immune response it is appropriate that we focus our discussion on how to control their functions.

T-cell recognition of nonself

T cells recognize foreign or 'nonself' antigen as peptide fra-ments displayed to them in the clefts of major histocompatibility complex (MHC) class I and II molecules expressed on the surfaces of many body cells. In addition, T cells have unique clonally distributed receptors that respond to these antigens displayed to them on specialized cells in the lymphoid tissues (antigen-presenting cells or APC). They then proliferate and differentiate to effector mode (Fig. 1). It is now clear that the cells with the greatest ability to present antigen and activate T cells are the dendritic cells[1,2]. Two features of dendritic cells that endow them with this property are their possession of a particular array of cell surface ligands complementary to an array of adhesion molecules on T cells, and their abundant MHC class II expression. A further requirement for T-cell activation is one of collaboration or help from other T cells responsive to the same antigen[3–5] (Fig. 2).

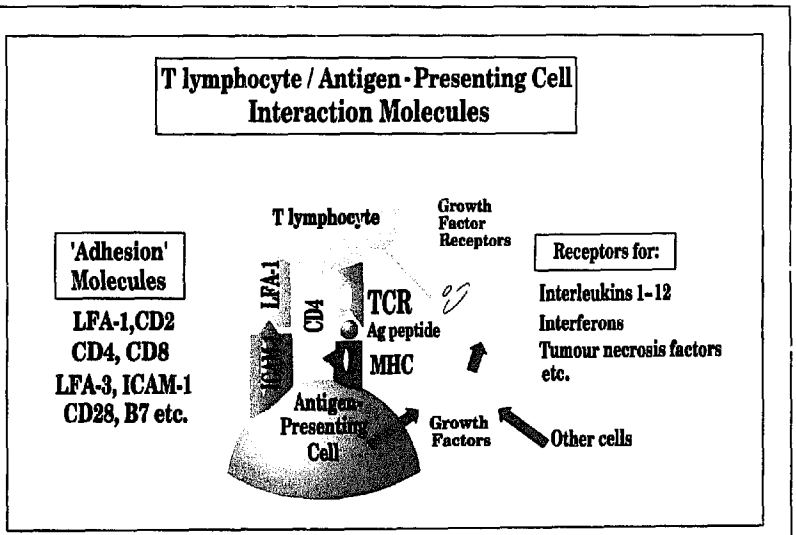

Fig. 1. There are three types of molecular interactions between a T cell and an antigen-presenting cell (APC). First, the specific T-cell receptor (TCR) recognizes the foreign antigen, in the form of a processed peptide [the antigen (Ag) peptide], bound in the cleft of the major histocompatibility complex (MHC) molecules of the APC. Secondly, a series of adhesion molecules on the T cell bind to their ligands on the APC. These include CD4 and CD8, which are co-receptors for the MHC molecules, and CD28, which interacts with the APC ligand B7 to provide 'costimulation'. Thirdly, the T cell expresses receptors for various factors that regulate growth and differentiation (cytokines), such as interleukin 2, some of which are produced by other activated T cells and form the basis of 'help' and collaboration. Although the T-cell receptor binding provides the primary signal to trigger the T cell, the outcome, which can be either a proliferative response or the induction of a non-responsive state, depends on further signals from these adhesion molecules and growth factor receptors in order to put the antigen recognition 'in context'.

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