

Human antibodies by design

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Received 3 March 1998; accepted 1 May 1998

Monoclonal antibodies (Mabs) have long been considered a good class of natural drugs, both because they mimic their natural role in the body and because they have no inherent toxicity. Although rodent Mabs are readily generated, their widespread use as therapeutic agents has been hampered because they are recognized as foreign by the patient. Evidently, clinical Mabs should be as human as possible and results with some of the more recently developed chimerized and humanized Mabs are testimony to this. Mabs that are entirely human are now being produced from phage display and transgenic mice. The first fully human Mabs generated by phage display have already entered clinical trials, and together with recent advances in these technologies, may finally realize the full potential of antibodies.

Keywords: applied immunology, protein engineering, phage display, transgenic mice

It is almost a quarter of a century since the first mouse monoclonal antibodies (Mabs) were created¹, yet the full benefit of Mabs in the clinic is only now being realized². Historically, the first Mabs were murine and as such were often recognized as foreign by the patients leading to the development of human anti-mouse antibody (HAMA) responses. Technical difficulties have impeded the production of fully human Mabs by cell fusion (although it may be possible to clone variable regions from unstable cells³), and it is ethically unacceptable to immunize humans to order. Together, these issues have driven the development of chimerizing and complementary-determining region grafting (humanizing) so that the Mabs appear more human. This has resulted in some of the more recent humanized Mabs eliciting little or no HAMA response⁴, although more data are required on repeated administration to non-immunocompromised patients. Mabs that are entirely human should further reduce the risk of being recognized as foreign, and over the last few years there have been notable advances in the generation of human Mabs using phage display and transgenic mice. This review describes the current status and relative merits of the different approaches to producing human antibody therapeutics.

Structure and function of antibodies

Immunoglobulins (Ig) are made up of distinct structural and functional domains as exemplified by the γ -immunoglobulin (IgG) class of antibodies. These are Y-shaped molecules comprising two identical 50 kDa heavy chains and two identical 25 kDa light chains. Disulfide bonds link together the heavy and light chain pairs as well as the two heavy chains (Fig. 1). Each chain consists of one domain that is variable (V domain) in sequence and responsible for binding its target antigen, and one or more domains that are relatively constant (C domains) in sequence and bind effector molecules. Light chains consist of one V domain (V_L) and a single constant domain (C_L) whereas heavy chains comprise one V domain (V_H) and three constant domains (C_{H1} , C_{H2} , and C_{H3}). The C_{H2} domain contains an N-linked glycosylation site and the C_{H2} and C_{H3} domains contain sequences that mediate recruitment of cytotoxic effector cells and fixation of complement. Within each V domain are three regions that are hypervariable in sequence and crystallographic studies have shown that these residues form loops at the ends of rigid β -sheets. The hypervariable loops are primarily responsible for antigen recognition and are referred to as "complementarity-determining regions" (CDRs). The remaining V region

amino acids act as a scaffold to support the loops and are known as "framework residues".

This domain structure of immunoglobulins is conserved across species and has facilitated Ig manipulation by protein engineering. Thus, fragments containing effector functions (Fc) can be exchanged between antibodies, or CDRs can be transplanted from one antibody to another in order to transfer antigen-binding capability. Furthermore, molecules carrying only antigen-binding capabilities have been produced, and these include $F(ab')_2$, Fab, and scFvs (Fig. 1). Together these developments have enabled a plethora of Mabs to be designed for specific therapeutic purposes.

Chimerized and humanized antibodies

The major drawback of rodent Mabs is that they are frequently recognized as foreign by the patient. To improve their therapeutic potential, chimeric antibodies have been constructed in which rodent constant regions are replaced by their human counterparts^{5,6} (Fig. 2). Initial comparisons of murine and chimeric Mabs in clinical settings showed much promise. For example, use of the chimeric Mab 17-1A in patients with colorectal adenocarcinoma showed improved pharmacokinetics together with a considerable reduction in immunogenicity⁷. Lack of immunogenicity of chimeric Mabs is not a general feature, however, with >50% of patients developing an antiglobulin response against the rodent V-domains after repeated dosing^{8,9}. Despite such difficulties, a limited number of chimeric Mabs have successfully progressed through clinical trials².

The realization that the conserved structure of IgGs across species also extended to the antigen-binding site facilitated the introduction of humanized, CDR-grafted or reshaped antibodies where only the CDRs are of foreign origin^{10,11} (Fig. 2). Because the transfer of murine CDR residues alone can result in significant loss of antigen binding, it was often necessary to reintroduce a number of foreign framework residues to restore affinity. The first examples of humanization were therefore based on human V-domains of known crystal structure¹² enabling the identification of framework residues that may contribute to antigen binding. This, however, can be a time-consuming process as the identity and number of foreign residues required has to be determined empirically and may vary from one to six residues^{13,14}. Another approach is to identify human V-domains with closest homology to the foreign V-region and then to construct computer-generated models to determine which

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framework residues to reintroduce¹⁵. The number of potential residues that might impact on antigen binding is large¹², and introducing all of these changes, up to nine in some cases¹⁵, would make the antibody less human. A compromise approach might be to choose the most homologous human germ line or consensus sequence and, using known structural data, make an iterative series of Mabs to identify the optimal humanized Mab that retains affinity but with the minimal number of foreign residues. This balance between retention of antigen binding and increasing risk of immunogenicity has led to the notion that only a small number of surface-exposed residues might need to be replaced in order to make the surface of a murine Mab appear human^{16,17}. The internal packing and interface residues could remain unchanged with the expectation that affinity would be preserved. Several murine antibodies have now been veneered or resurfaced in this way¹⁷, but, as yet, none have been used in man. While humanization can be highly successful, it does have a number of practical limitations: (1) there are a limited number of options of routes for efficient construction of humanized Mabs; (2) a detailed knowledge of antibody structure or modeling is usually necessary; (3) there often has to be a compromise between affinity retention and introduced foreign amino acids leading to unpredictable immunogenicity; (4) the antibody repertoire is limited to the animal in which the progenitor Mab originated; and (5) the rodent Mab of the required specificity has to have already been generated.

The first humanized Mab to enter the clinic was CAMPATH-1H (ref. 11) for the treatment of non-Hodgkin's lymphoma¹⁸ and rheumatoid arthritis (RA)^{19,20}. Although evidence of clinical benefit was observed, with repeated therapy over half of RA patients developed HAMA responses. Studies with other humanized Mabs, however, have shown negligible antiglobulin responses in patients. For example, multiple dosing with an anti-CD33 Mab in myeloid leukemia patients²¹, or with an anti-Tac antibody^{15,22} in patients with graft-versus-host disease or those undergoing renal transplanta-

tion, resulted in clinical efficacy with only one out of a total of 38 patients developing an immune response. More than 20 humanized Mabs have now progressed into clinical trials⁴ for a variety of indications. The general consensus of these studies is that these agents are proving more efficacious and are well-tolerated with little or no intrinsic immunogenicity or toxicity.

Phage display of human antibodies

Display of antibody fragments as Fabs or scFvs on filamentous phage²³ (Fig. 2) was first described in 1990²⁴, the first human antibodies were generated soon after²⁵. Apart from the completely human nature of the antibodies, at the time the major advantage of phage display was viewed as the speed to Mab isolation, with antibody selection and ELISA screening taking just 1–2 weeks. The major drawback was that the isolated Mabs were only of moderate affinity²⁵. More recently, however, high-affinity antibodies have been isolated from very large antibody repertoires within the same time frame^{26,27}, thereby facilitating the rapid generation of lead Mabs for therapy. Although any given selection protocol will generate a panel of antibodies with different properties, a variety of selection and screening strategies can be used to enrich for Mabs with the desired characteristics. This ability to tailor the selections as required and the number and diversity of different human Mabs generated is now viewed as one of the major benefits of using phage display. Strategies include selecting for high affinity^{27–29} and/or neutralization potency^{28,30–32}, for receptor agonists²⁹, the ability to recognize specific epitopes^{33,34} or specific cell types^{35,36}, for generating human equivalents of existing rodent Mabs³⁷ akin to humanization (above), or for use as Western blot or immuno-cytochemical reagents³⁸. Because the constraints of the immune response and immunogenicity of the epitope are not relevant in phage display, Mabs to traditionally difficult antigens are also readily isolated. These include Mabs to carbohydrate-containing epitopes³⁹, toxic moieties such as doxorubicin²⁷, and, importantly from a therapeutic viewpoint, anti-self antibodies⁴¹.

The phage system also permits selection of complex antigens such as protein complexes or whole cells, where the cell-surface components are presented in their native configuration. Red blood cells³⁵, peripheral blood lymphocytes³⁶, and melanoma cells⁴¹ have all been successfully used, and the possibility now exists to apply cell surface selection to generate antibodies with therapeutic potential. A selection procedure has been developed that facilitates the selection of antibodies that recognize specific, discrete regions of cell surfaces. This technique³⁴ utilizes the binding specificity of an existing ligand to identify antibodies that bind in close proximity to the ligand binding site. It is possible to take whole cell selections one stage further and perform selections on the whole organism. Recently, such methodology has been developed for the *in vivo* panning of phage libraries to select for binding members that recognize a given target organ. In this application, a phage display library was used to identify peptides that selectively bind to target organs such as kidney blood vessels and brain⁴². Selection procedures are extremely flexible and are continually evolving to ensure that Mabs are isolated that satisfy the researcher's predetermined requirements.

By selecting from very large non-immunized repertoires of antibody fragments (Fabs or scFvs), a good diversity of high-affinity Mabs can be directly isolated^{26,27}, the best of which have sub-nanomolar affinities against both hapten²⁷ and protein²⁹ antigens. Once an antibody with the desired epitope specificity has been isolated, the system provides the format for the directed evolution of antibody affinity in order to improve its neutralization potency and/or binding kinetics. There are a number of approaches to the affinity maturation of antibodies⁴³ including mutation of the CDRs of the antibody gene segments³², chain shuffling⁴⁴, use of mutator

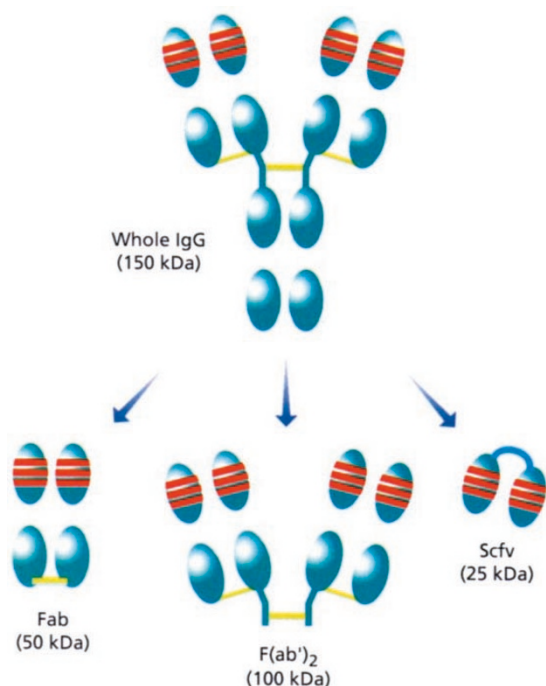


Figure 1. Structure of an antibody and derived antibody fragments. The variable (V) domains encompass the CDRs, which are highlighted in red. Disulfide bonds are indicated in yellow, scFv linker is indicated in blue. Fab: fragment antigen binding; F(ab')₂: bivalent Fab; scFv: single-chain variable fragment.

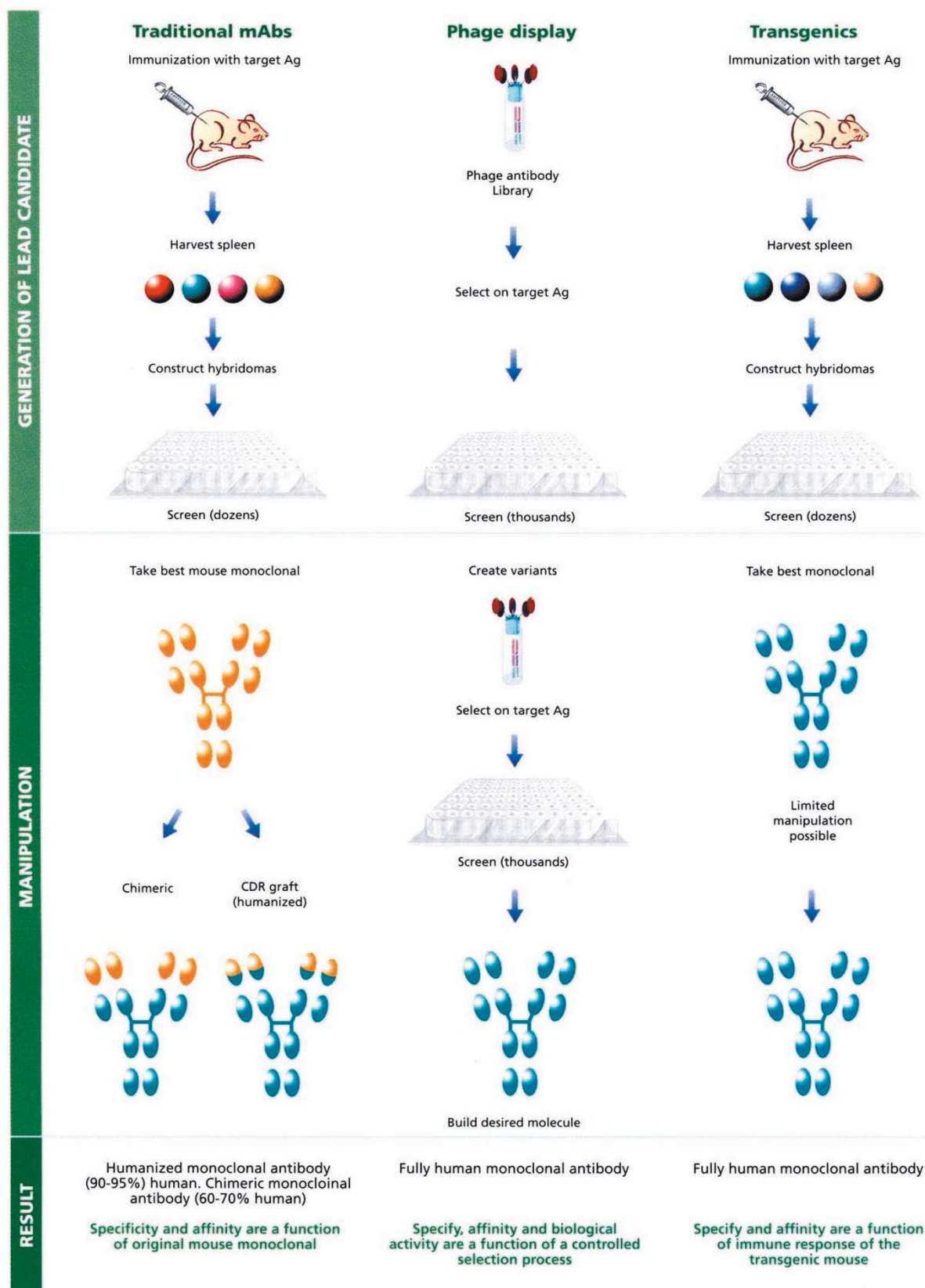


Figure 2. Comparison of the different approaches for producing human monoclonal antibodies. With traditional or transgenic technology, the final mAb is a whole IgG. In phage display, the panel of lead Mabs are either in scFv or Fab format. This facilitates further engineering for the desired characteristic(s) and this may take from zero (none necessary) to several months. In all cases, the clinical candidate Mabs currently require recloning into mammalian expression vectors and the establishment of high antibody-producing cell lines. Ag: antigen.

strains of *Escherichia coli*⁴⁵ and DNA shuffling⁴⁶, all combined with stringent selection protocols^{30,47}. Such approaches can be combined and/or reiterated to give stepwise improvements in the affinity culminating in a final Mab that may have >100-fold improved affinity. For example, an anti-C-erb-B-2 scFv antibody, originally isolated from a naive phage library, with a K_d of 16 nM has been affinity matured through iterative CDR mutagenesis to a K_d of 13 pM²⁸ as a scFv. Such changes, particularly in CDR3 of the heavy chain, are unlikely to compromise the human nature of these antibodies. The extent of randomness, degeneracy, and redundancy within CDR3 are so great that, in the vast majority of cases, it would be impossible to assign any given change as “non-human”.

Compared with antibody fragments, whole IgGs have an extended serum half-life and are therefore the preferred format for therapeutic Mabs. This necessitates recloning of the phage display-derived scFvs or Fabs into mammalian expression vectors containing the appropriate constant domains and establishing stable expressing cell lines. Typically, this may take 6–8 weeks after which time around 10–50 mg of IgG could be purified for preclinical studies. The specificity and affinity of the antibody fragments are generally very well retained by the whole IgG, and, in some instances, the affinity may significantly improve²⁹ due to the bivalent nature of the IgG.

In the past year the first human Mabs derived from phage display have entered phase I/IIa clinical trials for treatment of rheumatoid arthritis (anti-tumor necrosis factor [TNF- α]) and ocular fibrosis (anti-transforming growth factor β)⁴⁸. Although the results of the trials are awaited, these Mabs provide an early indication of the powerful potential of antibody phage display libraries.

Human antibodies from transgenic mice.

An alternative strategy for producing fully human Mabs is offered by transgenic mice (Fig. 2)^{49–51}. This technology, which ultimately remains dependent on immunization, involves two prior *in vivo* genetic manipulations: inactivation of the endogenous mouse Ig genes and the subsequent introduction of unrearranged human Ig gene segments. Because the human Ig loci are extremely large and complex structures comprising hundreds of genes spanning several Mb of DNA⁵², large fragments of these loci must be introduced into the mouse germ line⁵² if functional human Mabs are to be produced. Essentially, this has been achieved using either minigene constructs where up to 80 kb of a limited number of cloned human Ig genes are artificially juxtaposed^{53–55} or through yeast artificial chromosomes (YACs) that enable the cloning of very large contiguous Ig gene fragments^{56,57} that can be over 1 Mb in size⁵⁸. To achieve maximal expression of the human antibody transgenes, it is also essential to silence the endogenous Ig expression prior to introduction of the human loci⁴⁹. This has been achieved by knockout of critical mouse V-genes in embryonic stem cells, thereby blocking B-cell development and endogenous antibody production⁵⁰. Consequently, the levels of human Ig in the serum of transgenic mice are typically around 100 μ g/ml^{53,56}, but levels up to 800 μ g/ml have also been reported⁵⁸.

The first fully human antigen-specific Mabs derived from transgenic mice were of moderate affinity (100 nM–1 μ M) with only a limited number of these Mabs being raised against each antigen^{55,56,59}. Although this likely reflected the makeup of the plasmid-based minigene constructs that contained only a fraction of the human Ig-gene segments, the principle and feasibility of using a transgenic approach was established. With the recent advent of larger loci in the form of megabase-sized YACs comprising large contiguous regions of 66 V_H and 32 V_K genes, has come a significant improvement in both the number and affinity of specific Mabs that can be recovered⁵⁸. This observation parallels that of phage display in that the larger phage display repertoires ($\geq 10^{10}$ Mabs²⁷) yield a

greater diversity of higher-affinity antigen-specific Mabs than their smaller counterparts (10⁷–10⁸ Mabs⁶⁰). In both cases, it is now evident that increasing complexity of the initial V-gene repertoire, whether as a transgene or as a phage library, is accompanied by an improvement in the diversity and affinity of the isolated Mabs. To date, only three different protein antigens—interleukin 8, epidermal growth factor receptor and TNF- α —have been used to immunize the “megabase mice”⁵⁸. The affinities for the isolated IgGs are good, generally being in the 0.1–1.0 nM range. Although only the best of the antibody fragments derived from phage display^{27,29} have affinities that can rank alongside this, IgGs possess an inherent avidity component that may often result in improved affinity compared with its equivalent monomeric scFv. Accordingly, the affinities of two anti-MuSK scFvs were significantly improved from 5.1 nM and 4.8 nM to 12.5 pM and 29.5 pM, respectively, upon conversion into IgGs²⁹.

The transgenic system is still developing and we await the establishment of mice harboring the full complement of human Ig genes, notably the Ig λ locus. This should further increase the diversity of the isolated Mabs as around 40% of natural human Mabs contain λ light chains⁶¹. The main advantage of the transgenic system is that whole Mabs, not antibody fragments, are generated by the mice. The isolated hybridomas could also potentially be used as production systems if the current levels of IgG (2–19 μ g/ml⁵⁸ in static culture) are improved. It should also be possible to construct a set of transgenic mice, each expressing a different human isotype, thereby enabling the generation of Mabs with appropriate effector functions. Nevertheless, no Mabs derived from transgenic mice have yet entered clinical trials, but this will, no doubt, change with time.

Future perspectives

Humanization has proven to be a powerful method for transferring the specificity and affinity of an existing appropriate rodent Mab into human format. It is, however, wholly dependent upon the existence of an rodent Mab. Although in the absence of such, the generation and identification of suitable rodent Mabs could be undertaken, this would significantly add to the time taken to develop an appropriate therapeutic candidate. With the isolation of more and more fully human Mabs from phage display and transgenic mice, it seems likely that the frequency with which rodent Mabs are humanized will slowly decline. Furthermore, an analogous phage display selection technology, epitope imprinted selection³⁶, has already been developed to generate human equivalents of existing rodent specificities. Together, these factors will likely effect a shorter “shelf-life” for humanization compared with its counterpart technologies. The most recently reported affinities for Mabs derived from transgenic mice and phage display are equally good, matching the best that the immune response can offer. This makes other factors, such as Mab diversity and speed of isolation, more important considerations when deciding upon which approach to take.

Over the last year, there has been significant progress in the development of antibody therapeutics with the first two fully human Mabs derived from phage display entering phase I/IIa clinical trials for RA and ocular fibrosis⁴⁸. The generation of large human phage display repertoires and “megabase mice” is still evolving, and further improvements to both systems are to be expected. Together, these technologies are now beginning to procure a “production line” of efficacious human Mabs suitable for therapy.

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