Man-made antibodies

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Monoclonal antibodies can now be genetically engineered and endowed with new properties. In the future, gene technology could enable antigen-binding fragments to be made by exploiting repertoires of variable domain genes derived from immunized animals and expressed in bacteria. How readily can this approach be extended to production of *'in vitro'* repertoires of variable domain genes, and obviate the immunization of animals?

IN 1975 a method was described for making cell lines that secrete a single species of antibody (monoclonal antibody) with the desired specificity to antigen[1]. The technique—'hybridoma technology'—proved to be general, and a wide range of monoclonal antibodies have been made which bind to protein, carbohydrate, nucleic acids and hapten antigens, and which even have catalytic activities[2,3], leading to many practical applications for monoclonal antibodies in research and human health-care[4-7] and to patent disputes[8]. The technology has been improved over the years, particularly by the preselection of antigen-binding B cells[9] and by screening with antigen-coated filters[10].

Hybridoma technology was first extended by somatic cell genetics, which allowed antibody mutants to be selected[11,12], their functional properties to be changed by switching heavy chain constant regions[13] and antibodies to be made with dual specificity[14]. Gene technology later revolutionized this potential as antibody genes could now be altered to order. New vistas appeared, reviving the forgotten excitement of the old discipline of immunochemistry of antibodies. Initially antibody genes were taken from hybridomas, cloned into plasmid vectors and expressed as complete antibodies in mammalian cells[15,16] or as fragments in bacteria[17-20]. The ready manipulation of the genes by cutting and pasting of restriction fragments, or by site-directed mutagenesis, has allowed the construction of new antibody reagents and fine mapping of antibody structure-function relationships.

More recently a new approach has been proposed with the potential to bypass hybridomas[21,22]. Antibody genes are cloned directly from lymphocytes of immunized animals and expressed in bacteria, and the antibody products are screened for binding to antigen[23,24]. As does hybridoma technology, the process relies on animal immunization to give rise to many antigen-specific cells. In the animal, antibodies of low affinity are first produced by antigen-induced proliferation of cells, and then higher affinity variants are generated by point mutation and selection. Hybridoma technology can immortalize these cells; gene technology can immortalize their genes. In both cases, however, it is animals that 'invent' the new molecules.

Looking ahead, can we even bypass animals and make new antibodies in vitro[25]? Two current strategies recapitulate the great immunological controversy of the 1950s—instruction versus selection[26]. The selectionists are making naive repertoires of antibody genes and selecting those with antigen-binding activity, so mimicking nature. The modern instructionists are using computer graphic techniques to build specific antigen-binding sites. Here we shall describe the manipulations that have already produced some designer antibodies of practical value, and discuss other possibilities for improving on nature.

Starting with hybridomas

The antibody (IgG) is a Y-shaped molecule, in which the domains forming the tips of the arms bind to antigen and those forming the stem are responsible for triggering effector functions that eliminate the antigen. The domain structure of the molecule makes it particularly accessible to protein engineering, as functional domains carrying antigen-binding activities (Fv, Fab fragments) or effector functions (Fc fragments) can be used separately as fragments, or swapped between antibodies[27,28] (see Fig. 1 and legend). Furthermore, the rigid β-sheet framework structure of the variable (V) domains, surmounted with antigen-binding loops, allows the transplanting of binding sites from one antibody to another[29]. These structural features have spawned a range of designer antibodies, from complete antibodies, capable of destorying pathogens or tumour cells through their natural effector mechanisms, to antigen-binding fragments that can be used to target attached toxins, or for diagnosis though bound radioisotopes (Fig. 2).

Antibodies kill cells by triggering the complement cascade at the cell surface, with consequent lysis, or by binding to receptors on the surface of specialized effector cells, such as phagocytes or killer cells and triggering phagocytosis or antibody-dependent cell-mediated cytolysis. The potential of an antibody in lysis is determined mainly by the class of the constant (C) domains (isotype). This has been dissected by making chimaeric antibodies, in which the variable domains of a rodent antibody are attached to the constant domains of human γ isotypes. The human γ1 isotype emerges as being highly effective in both complement and cell-mediated killing, and therefore the most suitable for therapeutic use against pathogens or tumour cells[30,31]. Conversely, the inactive human γ4 isotype may be more suitable for diagnostic imaging, or for blocking a damaging immune or allergic response by binding to the antigen and thus competing with destructive antibodies[32]. Analysis of the roles of individual amino-acid residues should permit the engineering of variants of a single antibody isotype with differing effector mechanisms. Thus the binding sites for the high-affinity receptor (FcRI) include the lower hinge region of the antibody[33], and the 'core' binding site for the first component of complement is a strand of β sheet in the second heavy chain constant (CH2) domain[34] (Fig. 1).

Although some rodent antibodies (particularly the mouse γ2a and rat γ2b isotypes), can trigger human effector mechanisms, they are immunogenic in human therapy. In view of the difficulties of making human monoclonal antibodies directly (see later), rodent antibodies have been 'humanized' by linking rodent variable regions and human constant regions[28] (chimaeric antibodies, Fig. 2). This reduces the immunogenicity of the antibody as shown in clinical trials[35]. But residual immunogenicity is retained (at least in part) by virtue of the foreign V-region framework[36].

A more complete way of humanizing rodent antibodies includes the replacement of the V-region framework ('reshaped' antibodies, Fig. 2). It relies on the architecture of V domains as a framework of β sheets topped with antigen-binding loops (see ref. 37 and Fig. 1). By grafting the loops, the antigen-binding site can be transferred from rodent to human antibody[29,31,38,39]. The technique was used to humanize a rat therapeutic antibody directed against mature human leukocytes[31] which proved clinically effective in destroying a large mass of tumour in two patients[40]. But reshaping requires that the different framework

regions are structurally conserved, both in the orientations of the two β sheets of each domain and in the packing of heavy chain variable (VH) and light chain variable (VL) domains together; that the hypervariable loops make the majority of contacts with antigen; and that the loops are supported in a similar way by the underlying β-sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved necessary in others. Simple molecular modelling can help identify contacts and design small changes to optimize affinities[31,41].

Although natural effector functions are powerful, antibodies can also be engineered to recruit other effector functions. For example, by generating antibodies whose antigen-binding sites have dual specificity for a target cell and an effector such as a toxin. Such 'hybrid hybridomas' are made by fusion of hybridomas of two-different specificities[14,42], each of the two

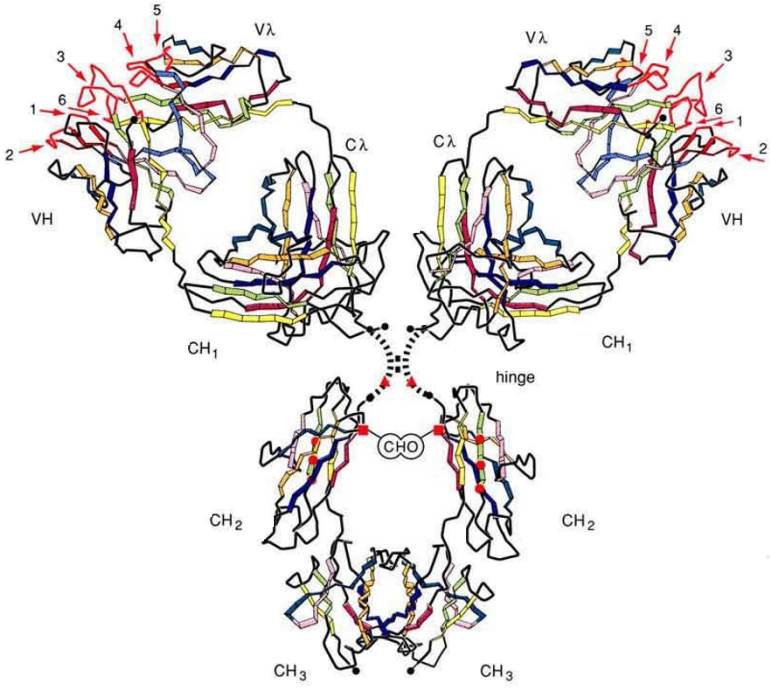
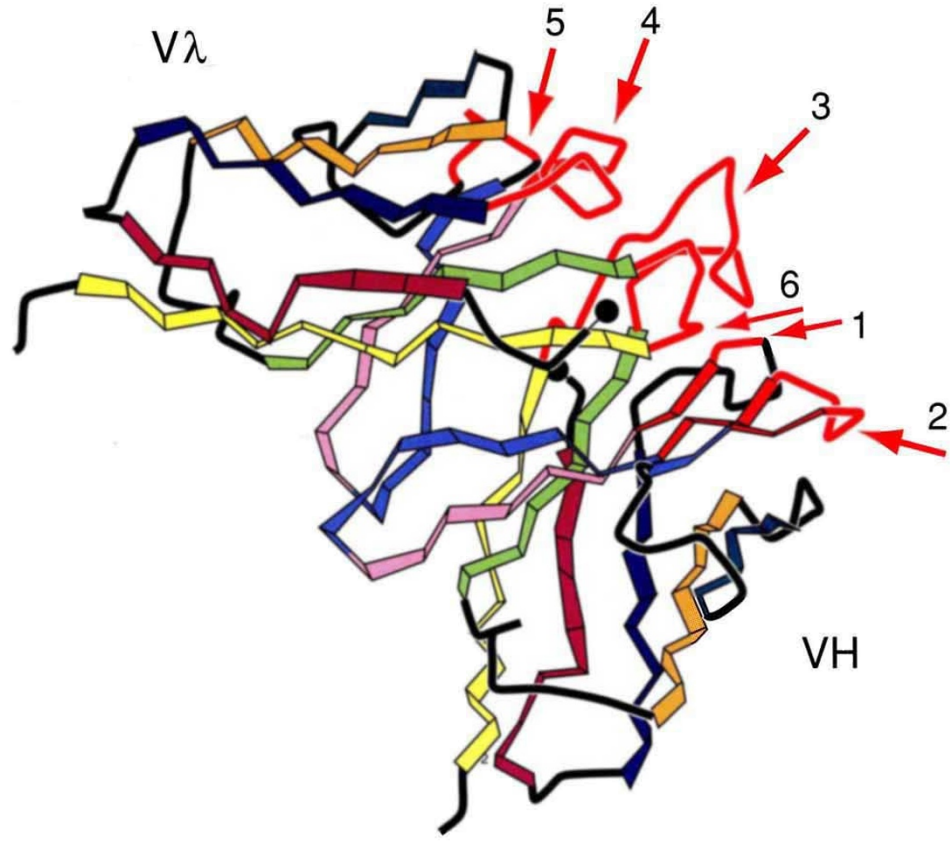


FIG. 1 Antibody structure. The antibody (IgG) consists of four chains, two heavy (H) and two light (L), which in turn are built by stringing together domains of similar architecture. Each chain is paired with another chain by lateral packing of the domains and also by at least one disulphide bond. Each domain consists of two β sheets which pack together to form a sandwich, with exposed loops at the ends of the strands. Thus the C domains have three β strands in one sheet (strands C, F, G) and four strands (strands A, B, D, E) in the other. The V domains have an extra two strands in one sheet (C, C′, C″, F, G). This framework is highly conserved in different antibodies. The three loops at the top of the V domain are hypervariable in sequence and fashion the antigen-binding site[37]. Despite the sequence hypervariability, most of the antigen-binding loops have a small repertoire of main-chain conformations. The antibody can be proteolytically cleaved at the flexible hinge region, yielding Fab fragments which bind antigen (comprising heavy chain VH and CH1 domains, top of the hinge and the entire light chain), and Fc fragments which bind to effector functions (comprising lower hinge and heavy chain CH2 and CH3 domains). The model is taken from the solved X-ray crystallographic structures of Fab and Fc domains of myeloma protein KOL[85], represented according to Lesk[86] and redrawn by an artist. Each strand of the β sheets has been colour-coded. The β sheet defined by the A, B, D, E strands is more intensely coloured. The hypervariable regions (VH, 1–3; VL, 4–6) are in red; the binding sites for high-affinity receptor[33], Clq[34] and carbohydrate attachment site are also marked in red, as triangles, circles or squares respectively.



halves of a single antibody molecule is from one of the two parental antibodies, and the molecule therefore carries two different antigen-binding sites. Such bispecific antibodies can bind both to a cell target (for example in a tumour) and to a toxin or cytotoxic T cell[43,44]. Novel effector functions can also be recruited by fusing gene segments encoding antigen-binding sites (as Fc or Fab fragments) to genes encoding toxins[45] and enzymes[27]. This can, for example, allow agents such as tissue plasminogen activator to be targeted to blood clots through Fab binding to fibrin, leading to the local activation of plasminogen[46]. Fusion of antibodies with enzymes may also prove invaluable for local activation of prodrugs[7,47]. Conversely antibody Fc fragments can equip other proteins with antibody effector functions. For example, CD4 linked to Fc fragments binds to viral glycoprotein gp120 on the surface of cells infected with human immunodeficiency virus (HIV), and kills the cells by antibody-dependent cell-mediated cytolysis (ref. 48) (CD4 immunoadhesin, Fig. 2).

Complete antibodies (relative molecular mass 150,000 (M_r 150K)) are large molecules but much smaller fragments can be prepared that retain antigen-binding activity. Small fragments (M_r 12K–50K) equipped with radioisotopes could be used for imaging or therapy and are in some ways particularly attractive for use *in vivo* as they penetrate tissue boundaries more effectively[49]. Fragments are also cleared faster from the serum and tissues[50] and although this may compromise their use as targeting agents, it can aid the clearance of toxic drugs, such as digoxin, from the circulation[51]. Small fragments also have advantages in fundamental research, for high-resolution X-ray crystallographic studies of antigen-binding sites. The size of antibodies and flexibility of the hinge connecting Fab arms and the Fc domain have prompted crystallographers to turn to Fab fragments[52] and now Fv fragments[53] (Fig. 2). In future we expect that fragments will be used extensively as they are readily expressed in an active form from genes introduced into mammalian[27,54] or bacterial cells[19,20,23,55]. But antibody fragments may require further engineering to eliminate undesirable properties.

For example, Fv fragments are noncovalently associated heterodimers of VH and VL domains which may thus dissociate. Although some Fv fragments are less prone to dissociation than others[56], stable Fv fragments can be engineered either by linking the domains with a hydrophilic and flexible peptide[57,58] to create single-chain Fv fragments (scFv, Fig. 2), or by introducing disulphide bonds between the domains[56]. Single VH domains (dAb, Fig. 2) with antigen-binding activities[23] are likely to require more extensive engineering. The domains have an exposed hydrophobic surface (where they normally interact with light chain), rendering them 'sticky'. If their properties can be improved, for example by introducing hydrophilic residues to the interface, these single domains may prove to be the forerunners of a new generation of small recognition molecules.

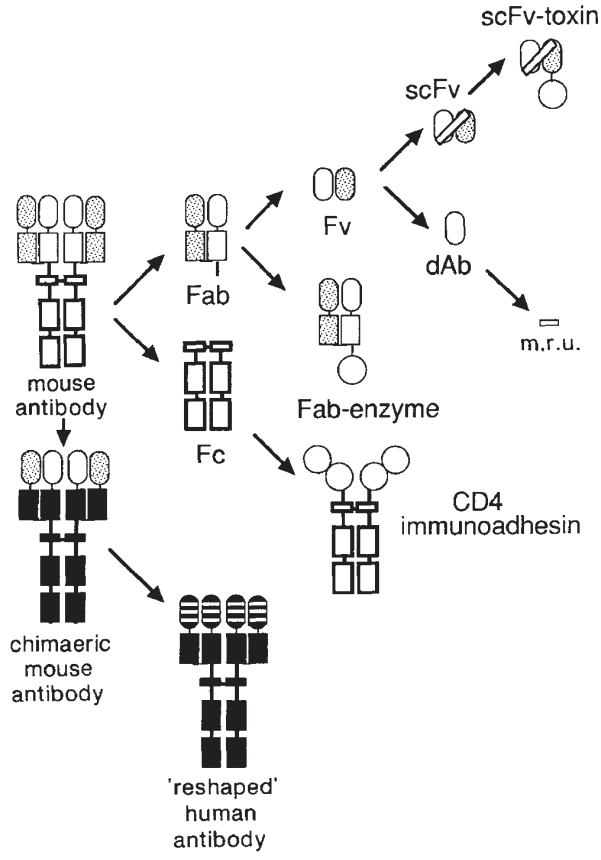


FIG. 2 Engineered antibodies and fragments. A range of engineered antibodies and fragments is depicted. Each box represents a domain. Single-chain Fv fragments (scFv), in which VH and VL domains are linked by a peptide (see text), and Fab fragments have been used to target enzymes and toxins. In immunoadehesins, a ligand specific for receptor (here CD4) is attached to an Fc fragment. Single VH domains (dAbs)[23] and even single CRRs (minimal recognition units or m.r.u.)[87,88] have been identified with antigen-binding activities. Site-directed mutagenesis of antibodies and fragments has also been used to alter effector functions and improve affinities[41] (see text).

Bypassing hybridomas

Immunization is essential to derive hybridomas secreting high-affinity antibodies. In the animal these antibodies are produced in two stages[59,60]. The first stage is fast and leads to the production of antibodies of low affinity (10^5–$10^7 M^{-1}$). It involves the

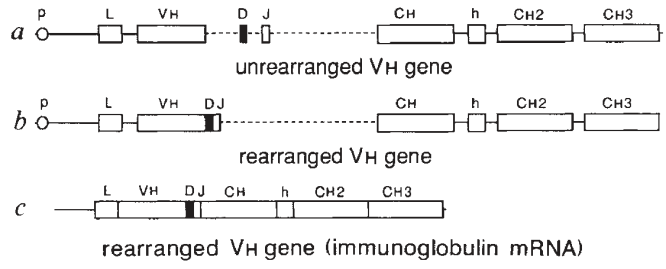


FIG. 3 Organization of V genes. The domain structure of the antibodies is mirrored at the level of the gene, as the individual domains are encoded by separate exons. In turn, the VH and VL domains are built from separate genetic elements, and assembled by DNA rearrangements during lymphocyte differentiation. Thus the first and second hypervariable loops are encoded by the germ-line V genes, but the third hypervariable loop by the combination of V, D and J elements (for the VH domain) and V and J elements (for the VL domain). The third hypervariable loop of VH is accordingly the most diverse in sequence and backbone conformation and is often the longest of the loops. Exon structure is illustrated for the unrearranged and rearranged VH genes and is similar (not shown) for VL genes (Vκ or Vλ). VH, unrearranged or rearranged heavy chain variable region as appropriate; D, D segment; J, J segment; CH1, CH2, CH3, first, second and third heavy chain constant exons, respectively; h, hinge exon; L, signal sequence; p, immunoglobulin promoter (octanucleotide motif). The potential diversity of the mouse primary repertoire is huge as a consequence of the combinatorial arrangements of the genetic elements. It can be estimated as follows. (1) Diversity due to combinatorial integration: $300\,V\kappa \times 4J\kappa = 1.2 \times 10^3$ and $200\,VH \times 15\,D \times 4J = 12 \times 10^3$. (2) Diversity due to junctional alternatives (alternative readings at junction due to differences in reading frame, and lengths of J and D segments): Vκ to Jκ ~ 3 and VH to D to J ~ 40. (3) Diversity due to N segment for heavy chain >10. (4) Hence combined diversity for each chain: light chains $= 3 \times 10^3$ and heavy chains $> 5 \times 10^6$. Conclusion: diversity due to combinatorial association of heavy and light chains $> 10^{10}$. Additional factor due to somatic mutation $> 10^{30}$ (see text).

TABLE 1 Immortalization of antibodies using unselected cells from hyperimmunized animals

	Hybridomas	EBV transformation	Random combinatorial gene cloning (bacteria)
Species restrictions	Yes	Only human	No
Chain pairs	H and L	H and L	H and L separate
Positive/negative clones	1/50	1/500(?)	$<1/10^4$ ('artificial')* $<1/10^8$ ('original')
Assay on plates (number of clones as replicas or overlays per plate)	~300	ND	$\sim10^{3-5}$(?)
Assays on supernatants	Well established, very sensitive	Well established, less sensitive	Possible
Doubling time of cells	18 h	~40 h	30 min
Stability	Good	Poor	Very good
Product	Full molecules	Full molecules	Fragments
Yield of product	20–100 μg ml^{-1}	0.5–5 μg ml^{-1}	0.5–10 μg ml^{-1}

* These values are calculated as follows. If P is probability of finding a particular combination of light (L) and heavy (H) chains in an N-size combinatorial library, and P_L and P_H are the probabilities of random occurrence of each component, $P=1-e^{-q}$ where q is NP_LP_H. If we assume that 1% of the mRNA molecules encode antigen-specific immunoglobulin ($P_L=P_H=10^{-2}$), there is ~60% probability that $1/10^4$ recombinants will contain one H and one L chain encoding any antigen-specific immunoglobulin: most are comprised of artificial combinations, which may or may not specify antigen-binding activity. If there is a dominant lymphocyte clone which represents 1% of the antigen-specific immunoglobulin lymphocytes, the frequency of each chain is 10^{-4} and testing 10^8 clones will give us a 60% probability of finding the 'original' combination. But regardless of whether they originate from antigen-binding lymphocytes, L chains may be capable of complementing the H chains from antigen-specific immunoglobulin. This frequency may be high particularly in primary responses and early secondary responses, before extensive diversification through hypermutation, but will depend on the cut-off affinity used in the assay. Taking a value of 1:100 (ref. 69), there is a 60% chance that $\sim10^4$ of the recombinants will give an 'artificial' positive signal. EBV, Epstein–Barr virus.

proliferation of cells drawn from the repertoire already available at the time of immunization. The potential repertoire is huge ($>10^{10}$) (Fig. 3 and legend), but at any given time, only a fraction of the potential repertoire of a mouse is available through the limited number of clones (10^7–10^8) expressing antibodies.

The second stage produces high-affinity antibodies, starting with the genes used in the first stage. Its main tool is the hypermutation of these genes followed by selection of those cells which produce antibodies of increased affinity. This is a darwinian process, involving variation resulting from point mutations followed by selection driven by the requirement for antigen for cell survival. It is an inheritable process, but only at the somatic level among the lymphoid cells of the individual. The resulting memory cells seem to be able to undergo new rounds of hypermutation and selection following antigenic challenge[61].

The rate of mutation approaches 10^{-3} or 3×10^{-4} mutations per base pair per cell division in a more recent estimate[60,61,62]. Mutations are localized to the segment coding for the variable portion of the antibody genes. The potential diversity generated by hypermutation is astronomical. Even if only the hypervariable region (~30 residues) of a single antibody variable domain could mutate into 10 out of the 20 amino acids, this would produce about 10^{30} variants for each chain. So the problem at this stage is not the generation of diversity, but the continuous selection of improvements against a background of degeneracy[63]. As immunization proceeds, additional high-affinity antibodies gradually emerge with V-D-J combinations which are rarely found in the primary repertoire (repertoire shift)[64].

In 'classical' antibody engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged antibody VH and VL genes. However, using 'universal' primers and the polymerase chain reaction (PCR) it is possible to rescue V genes[21,23,65,66] and by building restriction sites into these primers the amplified DNA can be cloned directly for expression in mammalian cells[21] or bacteria[23,24].

This offers new routes for the derivation of monoclonal antibody-producing cell lines (Fig. 5). At its simplest, it allows V genes to be rescued from hybridomas[21], unstable hybridoma fusions (for example mouse-human hybridomas), single hybridoma cells[67,68] or even single B lymphocytes. Hybridomas have advantages because fusion enriches for antigen-stimulated cells. However, rescue from single B lymphocytes bypasses cell fusion, allowing access to terminally differentiated B cells which are rich in messenger RNA but are not able to fuse. Single hybridomas or B cells might also be isolated by binding to antigen, immobilized on solid supports (for example on plates or magnetic beads), by fluorescence-activated cell sorting (FACS) or by rosetting with antigen-coated red cells. The single set of V genes from individual cells could then be co-expressed in bacteria. But in this case each cell must be processed separately.

Cloning from heterogeneous cell populations has the advantage that all the cells can be processed together. For example,

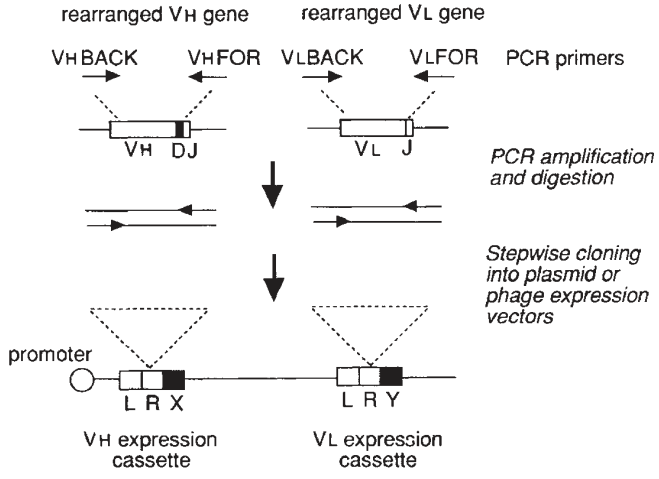


FIG. 4 PCR cloning of rearranged VH and VL genes into expression vectors. Primer mixtures can be designed for PCR amplification of most families of V genes, as the nucleotide sequences at the 5′ and 3′ ends of the rearranged V genes are relatively conserved[21]. At the 5′ end, mixed PCR primers have been based within the signal sequence[65,67] or at the N-terminal end of the mature variable domain[21,66]. At the 3′ end, PCR primers have been located within the J region or the C region. One set of PCR primers, located entirely within the rearranged V-gene exon, amplifies both mRNA and chromosomal DNA[21,23]. The PCR primers incorporate restriction sites and after amplification and digestion with restriction enzymes can be cloned into specialized plasmid or λ phage vectors directly for expression of the V genes. The cloning of VH and VL genes into a plasmid expression vector is illustrated here. (L, leader sequence for secretion into bacterial periplasm and/or beyond outer membrane; R, polylinker cloning site; X and Y, extra polypeptide sequence (for example, heavy chain CH1 domain, light chain CL domain or peptide tag for recognition by monoclonal antibody).)

VH and VL genes, taken from total unfractionated cells from an immunized mouse have been combined at random and Fab fragments expressed from λ phage screened for antigen-binding activities[24]. The disadvantage is that the original VH and VL pairing, selected for high affinity by immunization, is lost. A combinatorial library with only 1,000 different VH and 1,000 different VL gene elements equally represented, would necessitate the screening of between 10^6 and 5×10^6 clones to recover most of the original pairings. Thus the chances of recovering original pairs of V genes from a large random combinatorial library from an immunized mouse are low, and even more remote for the highest affinity antibodies of hyperimmune animals. Those pairs of VH and VL domains that are found to be capable of binding antigen[69,70] are likely to do so at lower affinity or with lower specificity than those selected by antigen. Although isolation of an anti-hapten Fab fragment with a good affinity has been reported[24] using such an approach, if the aim of this technology is to bypass hybridomas, it will need to make the high-affinity antibodies required for diagnosis and therapy, antibodies which are difficult to derive even with hybridomas.

To improve the chances of recovering original pairs, the complexity of the combinatorial libraries could be reduced by using small populations of antigen-selected B lymphocytes (Fig. 5). But all combinatorial approaches will rely heavily on powerful screening methods. Here the use of membrane filters for screening large numbers of clones is promising. A variety of formats have been deployed; for example, capture of antibodies on filters coated with antigen, and detection with anti-globulin reagents[10]. Alternatively, antibodies or Fab fragments have been immobilized directly on membrane filters[24] or indirectly through antiglobulin reagents (A. Skerra, M. Dreher, E. Gheradi, G.W. and C.M., unpublished results) and probed with labelled antigen.

Bypassing animals?

Looking ahead, it may become possible to build antibodies from first principles, taking advantage of the structural framework on which the antigen-binding loops fold. In general these loops have a limited number of conformations which generate an endless variety of binding sites by alternative combinations and by diverse side chains[71,72,64]. Recent successes in modelling antigen-binding sites[41,72] augurs well for *de novo* design. This approach might become attractive for making catalytic antibodies, particularly for small substrates. Here side chains or binding sites for prosthetic groups[73] might be introduced, not only to bind selectively to the transition state of the substrate, but also to participate directly in bond making and breaking. The only question is whether the antibody architecture, specialized for binding, is the best starting point for building catalysts. Genuine enzyme architectures, such as the TIM barrel, might be more suitable. Like antibodies, TIM enzymes also have a framework structure (a barrel of β strands and α helices) and loops to bind substrate. Many enzymes with a diversity of catalytic properties are based on this architecture[74] and the loops might be manipulated independently of the frameworks[75] for design of new catalytic and binding properties.

Instead of the 'design and build' approach, could we build an artificial selection system, for example harnessing bacteria or phage, to select for antigen-binding activities? Here no structural information about the antigen is needed. But we see no future in trying to select high-affinity binding activities in a single step. The strategy of the immune system, in which low affinity evolves to high affinity seems more realistic[25]. Can we imitate this strategy and indeed improve on it?

Our first task is to prepare a naive repertoire of antibody genes (Fig. 6). At its simplest we could use the polymerase chain reaction (PCR) and universal primers to reproduce *in vitro* the repertoire of rearranged V genes expressed by naive animals. However 'naive' animals are not really naive and the available repertoire is limited in size and shaped, for example by tolerance to self-epitopes. In principle we could adopt the mammalian strategy of assembling a much larger repertoire by random combination between restriction fragments encoding the germline V, D and J elements. To match the potential repertoire of the animal, we would also have to reproduce the junctional diversity created by recombination. Large repertoires might also be made by trying to immitate the process of gene conversion adopted by birds[76].

But such 'natural' repertoires are not ideal. The sequences of germ-line V genes and the multiplicity of highly related V genes in the genome are presumably themselves the result of chance and evolutionary pressures, for example towards recognition of pathogens and against recognition of self-components. Such repertoires are both biased and highly redundant. More efficient repertoires might be constructed by using V genes from a variety of animal sources, excluding highly related V genes and even designing entirely new V genes or D segments. For example, the structures of individual antibody loops are often very similar

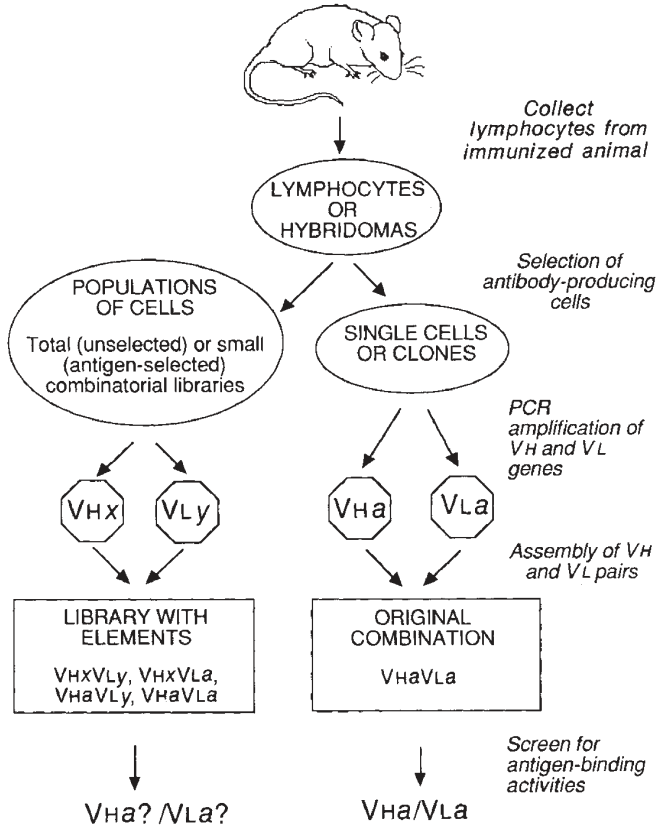


FIG. 5 Strategies for cloning paired VH and VL genes from lymphocytes of an immunized animal. VH and VL genes (expressed as pairs VHx/VLy) from *n* lymphocytes can be amplified by PCR from the mRNA or genomic DNA of single cells or populations. The lymphocytes can also be selected by antigen-binding, or immortalized as hybridomas by fusion with myeloma cells or by infection with Epstein–Barr virus. From single lymphocytes, or clones, the original VHa/VLa gene combination is readily rescued. From populations of lymphocytes, repertoires of VH–VL genes (VL*n*) could be combined at random, and antigen-binding combinations selected. But for large populations of lymphocytes, original combinations of VH and VL genes will be a minor proportion of heterologous combinations, some of which may display residual antigen-binding activity. The library will contain $(n-1)^2$ VHxVLy elements, $(n-1)$ each of VHxVLa and VHaVLy elements and a single VHaVLa element. VHa and VLa (or close derivatives), however, may occur repeatedly. Thus, at earlier stages or immunization, there are likely to be some artificial pairs which originate from different but closely related clones which can bind antigen, particularly with responses dominated by 'unique' VH and VL gene combinations (restricted or idiotypic responses[59,60]). Ref. 90 is in agreement with this prediction. As maturation for high affinity proceeds, the number of mutations increase and the likelihood of effective heterologous complementation diminishes.

in their overall fold[71,72], and the loops could be fleshed with diverse side chains.

The next stage is to express the library and screen for antigen-binding activities by random combination of VH and VL domains. Ideally the library should be 'complete', containing antibodies of a minimum binding affinity for any conceivable epitope. The tighter the binding of a primary antibody, the larger the library required. For example, it has been estimated that a primary repertoire of 10^7 different antibodies is likely to recognize more than 99 per cent of epitopes with an affinity constant of 10^5 M^{-1} or better, and rarely contributes high-affinity antibodies to an epitope taken at random ($>10^9$ M^{-1}) (ref. 77). If millions of VH and VL combinations from an artificial library could be screened, for example on membrane filters with a cut-off affinity of at least 10^5 M^{-1}, then the library would be as complete as the primary repertoire of a single mouse (10^7 antibody species). In the future, the screening of large libraries may well be replaced by methods of selection. For example, by expressing functional antibody fragments at the surface of phage, desired V-gene combinations can be enriched by binding to antigen[78]. If these screening and selection techniques worked as well as those in the animal, we could imitate the primary response and generally obtain low-affinity antibodies.

The enhancement of affinity *in vivo* can be contributed by a single point mutation, or several mutations[64]. Hypermutation of the genes corresponding to antibodies of low affinity should be easy to imitate *in vitro*. Point mutations could be introduced into the V genes by many techniques, for example, by using error-prone polymerases, PCR amplification through a large number of cycles, biased ratios of nucleotide triphosphates or 'spiked' oligonucleotide primers. Multiple mutations could be targeted throughout the body of the gene simultaneously, or to each of the hypervariable loops[79].

The screening or selection of the mutants with improved affinity is likely to be more difficult, as it needs to discriminate between mutants differing slightly in affinity. Such discrimination is possible for hybridoma clones in agarose[10], by binding secreted antibody on coated membranes with different antigen density. The differential binding to antigen-coated membranes might also be achieved by competiton with low-affinity antibody. The system we are proposing, however, is primitive compared with the animal. Ideally we would like to hypermutate specific segments of DNA within cells rather than in isolated DNA, and at the same time express the products on the cell surface, and select in a darwinian fashion variants of steadily increasing affinity. Animals are superb at this job, and it will not be easy to compete with their efficiency. We may learn to imitate the animal strategy, but in the meantime for the production of high-affinity antibodies we will normally do better by stealing hypermutated V genes from immunized animals.

Human antibodies: today and tomorrow

Making human monoclonal antibodies has posed difficulties for hybridoma technology. For example, the use of the mouse myeloma as a fusion partner for human cells leads to preferential loss of human chromosomes, and intolerable instability of the hybrids. Hybridomas are derived from spleen or lymph nodes, whereas the primary source from humans, the peripheral blood lymphocytes, contains few blast cells actively involved in the immune response. As an alternative to fusion, immortalization of human cells by Epstein-Barr virus does not lead to preferential immortalization of blasts engaged in antibody responses, and also leads to lines which are low producers of antibody and unstable[80]. Further, humans can rarely be hyperimmunized to order, especially with noxious chemicals, pathogenic viruses or cancer cells. In any case, the isolation of human antibodies to human cell-surface antigens would have to overcome tolerance mechanisms which eliminate lymphocytes with self-reactivity. Human lymphocytes have recently been used to populate severe combined immunodeficient (SCID) mice and these animals can be immunized[81,82] to make human antibodies.

Gene technology offers alternatives. The 'humanizing' of rodent monoclonal antibodies is currently the most practical approach. It allows access to a vast pool of rodent antibodies with good affinities and specificities. This is a major advantage, particularly when dealing with antibodies for tumour therapy, or for the manipulation *in vivo* of the human immune system. Thousands of antibodies have already been made against human

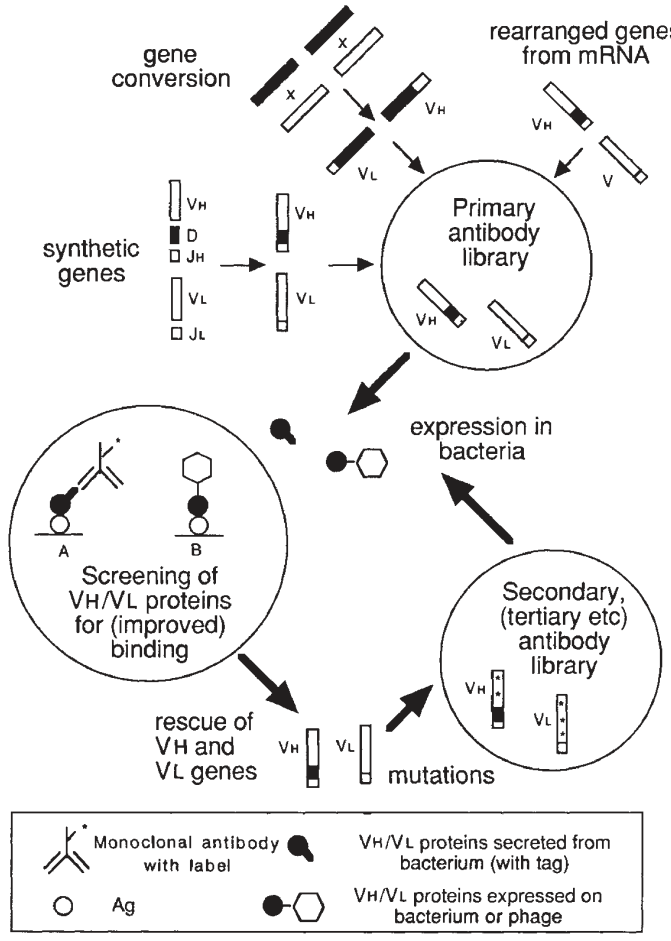


FIG. 6 Mimicking the immune system. VH and VL gene repertoires derived by a range of methods, such as reproduction of the available library from lymphoid cells, assembly of V, D and J elements, or gene cross over (to mimic gene conversion). The genes are cloned and expressed, and VH–VL pairs binding to antigen can be selected or screened, for example on membrane filters. Here the VH–VL pair, binding to antigen (Ag), is tagged with a C-terminal peptide recognized by a monoclonal antibody (mAb), or is expressed on the surface of a bacterium or phage. For detection, the mAb or Ag can, for example, be coupled to radioisotope, or to an enzyme for production of a coloured dye. A, Capture of VH–VL with antigen and detection with mAb. Other schemes could involve capture by binding to membrane and detection with Ag or capture by mAb and detection with Ag. B, Capture with Ag and detection of phage plaques. The genes encoding VH–VL pairs, identified as antigen-binding, can be rescued and hypermutated to form the secondary antibody libarary. These genes can then be expressed and screened for improved binding to antigen. This process will need to be repeated several times to achieve the efficiency of animal immune systems. Selection systems for catalytic antibodies might differ from those above. Such antibodies have been made through hybridoma technology by immunization with transition-state analogues, or by chance[2,3]. But animals select antibodies on the basis of binding, not catalysis, and the transfer of V-gene repertoires to bacteria gives a new twist to the field[24]. Vast libraries might be screened directly, not for binding, but for catalysis for example with substrates yielding fluorescent products, or by complementation of bacterial mutants deficient in a catalytic step of interest. In this case, antibodies will need to be expressed intracellularly[89].

cell-surface antigens, and particularly against human leukocytes[4]. Reshaping these antibodies, by transplanting only the antigen-binding loops to human antibodies[29,31], yields humanized antibodies which may have similar immunogenicity to truly human antibodies. In future, several other approaches may become available through gene technology as discussed in earlier sections. Furthermore, transgenic mice have been made which carry immunoglobulin heavy V, D, J and human C regions[83] and this should allow human antibodies to be produced directly from hyperimmunized mice. It remains to be seen whether the size of the repertoires, limited by the amount of new DNA that can be carried by the transgenic animal, will be a drawback.

But all these methods will have to compete with immortalization by Epstein–Barr virus and cell fusion, which themselves are constantly improving, particularly as they start to incorporate ideas and techniques involving DNA manipulations. There are immune responses which are unique to humans, or which need to be characterized in humans, including those to antibodies in certain auto-immune, parasitic or infectious diseases, or cancer. Here the rescue by gene technology of human hybridomas or cell lines immortalized by Epstein–Barr virus may provide a powerful way of defining the properties of such antibodies and immortalizing them[68].

As Ehrlich wrote 90 years ago[84], '... we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics'. We see a jungle of technologies, old and new, stimulating each other: in the immediate future, most of them start with immunized animals. □

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