Monoclonal Fab Fragments from Combinatorial Libraries Displayed on the Surface of Phage

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The combinatorial antibody approach captures the immune repertoire within a library of bacteria or their viruses (phage). Current molecular biology techniques allow the construction of repertoires of a size that at least matches if not surpasses the size of the primary animal repertoire, about $10^6$. Escherichia coli are competent producers of antibody Fab and scFv fragments. To survey repertoires of this size for antibody fragments of the proper specificity and highest affinity, a phage display system that allows an affinity-based selection of clones was developed. These developments have at least three important consequences: (i) they allow direct cloning and expression of human Fab fragments; (ii) they allow the creation of synthetic antibodies; and (iii) they allow the in vitro evolution of antibody specificity and affinity. For the crystallographer, these developments will provide unique opportunities for the study of molecular recognition. © 1993

Crystallography of antibody Fab fragments requires access to monoclonal antibodies. Initially, these were derived from humans with multiple myeloma. The limitation with myeloma proteins is that the corresponding eliciting antigen is unknown, so that study of antibody–antigen complexes is confined to antigens selected on the basis of an exhaustive screening process. The arrival of hybridoma technology (1) meant that rodent monoclonal antibodies against defined antigens could be readily generated, allowing a flurry of activity in crystallographic studies on antibody–antigen complexes. A primary limitation of hybridoma technology for the structural investigator is that it has not been readily extended to human antibodies. Indeed, Epstein-Barr virus immortalization of B cells has probably been of greater value, although it too has practical limitations (2). Although many of the features of antibody–antigen interactions have been and continue to be elucidated using rodent antibodies, there are cases, e.g., autoimmunity, where one would like to study human antibodies. Another limitation of hybridoma technology is that the expression of the antibody in the hybrid cell does not permit ready genetic manipulation for detailed structure–function studies. In comparison to eukaryotic cells, bacteria have many advantages as the basis for much of modern molecular biology. The idea therefore developed that if one could express antibody repertoires in bacteria, then one could derive antibodies from any animal, including human, or furthermore could manipulate such antibodies with relative ease.

ESTABLISHING ANTIBODY REPERTOIRES IN BACTERIA: COMBINATORIAL LIBRARIES (SCFV PHAGE)

An important development in efforts to establish antibody repertoires in bacteria was the demonstration that single antigen-binding fragments of antibodies (Fab or scFv) could be expressed in bacteria (3, 4). Similarly, it became possible to obtain enough heavy- and light-chain
DNA for cloning, even though beginning with a mixture of cells with many different antibody genes, using the polymerase chain reaction (PCR) (6–7). The next stage was to choose a cloning vehicle or vector to get the antibody genes into bacteria and expressed. Phage, viruses infecting bacteria, were favored because of their high efficiency of infection, which allows the cloning of a large number of antibody genes. The ensemble of phage, each containing one heavy-chain and one light-chain gene, is described as a combinatorial antibody library. More correctly, the ensemble is a random combinatorial library since heavy-chain DNA and light-chain DNA are prepared and cloned into the vector separately and no knowledge of the in vivo combination remains. The first combinatorial antibody library was constructed from an immunized mouse using lambda phage as the cloning vector (8). Antibody Fab fragments binding the immunizing antigen were successfully isolated from the library by probing with the antigen.

The methodology was then advanced to the expression of antibody fragment libraries on the surface of filamentous phage (9–12), building on the pioneering work of Smith on the expression of peptides at the phage surface (13). Antibody display phage have antibody genes contained inside the phage particle and antibody expressed on the outside. The advantage is that specific antibody phage can now be selected from the library by binding to immobilized antigen, a technique referred to as panning. Once selected, individual antibody phage can be used to infect bacteria, multiply, and produce large amounts of antibody. Thus, recognition and replication become linked as for the B cell in vivo. We now describe the combinatorial library system in use at the Scripps Research Institute (11, 14–24) in overview and then in some detail. An alternative but similar system has been developed at the Laboratory of Molecular Biology in Cambridge (12, 25–28) and elsewhere (29). We have covered much of this material in other reviews (Refs. 30–32). Here we provide an update and emphasize points of interest to the crystallographic community.

CONSTRUCTION AND SELECTION OF BINDERS FROM COMBINATORIAL PHAGE DISPLAY LIBRARIES

Figure 1 shows an outline of the strategy. The antibody genes are derived from either an animal or a human, immunized or nonimmunized, or semisynthetically. In the former case, antibody-producing cells are isolated, and RNA is prepared and reverse transcribed into DNA in a reaction catalyzed by the enzyme reverse transcriptase. Heavy (Fd part) and light chains are then amplified using the PCR reaction as described in Fig. 2. This provides the genetic information necessary to produce the Fab antigen-binding fragment of the antibody molecule. The Cambridge group has focused more on single-chain Fv’s. The heavy- and light-chain DNAs are then cut with restriction enzymes and cloned sequentially into a phagemid vector designated pComb3 (11), which is described in more detail in Fig. 3. This vector is then electroporated into bacterial cells. One phagemid, carrying one set of antibody genes, enters each cell. There it is capable of producing antibody Fab fragments that assemble in the periplasm of the cell anchored in the inner membrane as shown in Fig. 3. The efficiency of the transformation process with a ligation mixture of this vector is on the order of $10^7$ to $10^8$ µg DNA. It is this step that limits the library size to the order of $10^8$–$10^9$. A larger size would require a vast number of electroporation steps. Bacterial cells carrying plasmid are now selected by growth in medium containing ampicillin: only the phagemid and not the bacterial chromosome carries a gene for ampicillin resistance (β-lactamase).

The next stage is the rescue of the phagemid DNA by adding infective helper phage to the cells. In a complex process, considered further in Fig. 4, helper phage enters the cell and packages the DNA from the phagemid, carrying the antibody genes, in preference to its own. As it leaves the cell, the phage is capped at one end by 3–5 molecules of a protein known as coat protein III (cpIII). Some of the cpIII molecules are linked to Fab molecules, so a typical phage will carry one Fab molecule on its surface and the corresponding antibody genes inside. This rescue process applied to a library of phagemids generates a library of phage–Fabs. The process also serves to amplify the initial library so that typically $10^8$ copies of each phage (and therefore each set of antibody genes) are generated.

This library is now “panned” against antigen, as shown in Fig. 5. Typically, the library will contain $10^{12}$ phage–Fabs (about $10^8$ copies of each member of the initial library as above) in about 50 µl of solution. This solution is layered onto antigen immobilized on plastic and incubated, and then nonspecifically bound phage are removed by repeated washings. The remaining phage, greatly enriched for specific phage–Fabs, are
, e.g., by acid or excess soluble antigen. This round of panning, generally produces an enactor for specific phage on the order of 1000. This procedure is then repeated, usually three or four times, which should be sufficient to reveal specific phages carrying only singly in the initial library, i.e., the percentage on the order of 1 in 10^3. Finally, the phage–Fabs are converted to plasmids expressed as soluble Fabs by excision of the cplIII gene, otherwise directs the Fab to the phage surface, otherwise.

The plasmids are used to transform bacteria and in renal clones, carrying one plasmid and one set of genes, grown up. The supernatants or pericellular extracts from each clone contain Fab which is screened for reactivity with antigen, usually in conventional ELISA.

**ANTIBODIES DERIVED FROM IMMUNE DONORS**

Antigen-specific Fab fragments have been derived from libraries prepared from immunized mice and humans. In principle, any animal can be used provided that enough sequence information is available to design PCR primers capable of amplifying a substantial portion of the antibody repertoire. Specific Fab fragments have also been prepared from humans who have not been actively immunized but who have had contact with antigen, most usually an infectious disease, as described by Y. (21). This could be many years prior to making a library. Probably the most useful prognostic indicator for the library approach is the titer of antibody against antigen in the serum of the donor animal.

**FIG. 1.** Strategy for cloning monoclonal Fab fragments from combinatorial libraries on the surface of phage. RNA from tissue source containing antibody-producing cells, e.g., peripheral blood, bone marrow, or spleen from a human, is transcribed to cDNA and then the Fd part of the heavy chain and the light chain are amplified using the PCR reaction. Heavy- and light-chain DNA is amplified from semisynthetic genes. The PCR primers incorporate restriction sites to clone the PCR products into a phagemid vector (Fig. 3). The ensemble of heavy-chain inserts in the vector is chain library and similarly for the light chain. When both chains are present, the ensemble is referred to as a combinatorial phagemid library. The light-chain library is now “rescued” to a phage display library (Fig. 4) in which each phage Fab is selected by its ability to bind to antigen. These specific phage–Fabs can now be converted to two Fab expressed Fab, and the fragments into bacterial cells, produces soluble Fab. Adapted from Ref. 30.
words, if a reasonable level of specific polyclonal antibodies to a given antigen can be identified in the serum of the donor animal, then it is likely that a library prepared from the appropriate donor tissue will yield monoclonal antibodies to the antigen. It should also be noted that Fab fragments specific for many different antigens can be isolated from the same library. Thus, for example, if mouse antibodies were required against a set of different antigens, it would not be necessary to immunize individual mice. All the antigens could be introduced into a single mouse, one library prepared, and selection introduced during the panning process.

The affinities for the relevant antigen of Fab fragments isolated from immune libraries appear to be in the range $10^7$ to $10^9$ M$^{-1}$ (11–15). Many of the affinities for binding to proteins are estimates from inhibition ELISA studies, although surface plasmon resonance (33) studies confirm these values. These affinities are similar to those found for monoclonal antibodies derived from application of the hybridoma approach to immunized mice (34, 35).

The number of different antibodies generated against the immunizing antigen can be readily assessed by sequencing of the antibody genes on the appropriate plasmid DNA. Typically, one finds a limited number of heavy chains combined with a greater number of light chains (12, 15–18, 21–22, 36). In other words, a given heavy chain is often found in combination with several different light chains with retention of antigen affinity. This phenomenon is referred to as chain promiscuity and has been described for antibodies derived by conventional means (37). Frequently, families of closely related sequences are seen. For example, 33 Fabs reacting with high affinity with the HIV-1 surface glycoprotein gp120 were obtained from a library derived from an HIV-1-seropositive individual (23). Seven groups of heavy chains in which the CDR3 region was identical or nearly so were described, implying that the chains arose from a common precursor (same VDJ gene rearrangement). In some groups the heavy chains were paired with closely similar light chains. In others, they were paired with very different light chains. Simply by panning the library, therefore, one derives families of related antibodies. These can be further manipulated as discussed below.

**GENETIC MANIPULATION OF FABS**

The cloning of Fabs in bacteria means that, following the initial identification of antigen-specific Fabs, the

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![Diagram of antibody structure](image)

**FIG. 2.** The antibody molecule. The human IgG1 molecule is shown schematically. PCR primers are chosen to amplify DNA corresponding to the Fd part ($V_H$ C$H_1$) of the heavy chain and the whole of the light chain. Equivalent positions on the protein are represented by arrows. The 3 primer for the heavy chain hybridizes to a hinge region sequence to include the Cys involved in the heavy–light chain disulfide bridge in IgG1. Other antibody isotypes have been amplified by a similar strategy (e.g., Ref. 51).

![Diagram of pComb vector](image)

**FIG. 3.** The composition of the pComb vector and the proposed pathway for Fab assembly. Expression of Fab (Hc, heavy chain)/cpIII fusion and light chain (Lc) is controlled by lac promoter/operator sequences. The chains are directed to the periplasmic space by pelB signal sequences, which are subsequently cleaved. The heavy chain is anchored to the membrane by the cpIII fusion, whereas the light chain is secreted into the periplasm. The two chains then assemble on the membrane. Adapted from Ref. 30. Filamentous phage biology is reviewed in Ref. 55 and phage display first described in Ref. 13. Comparable vector systems have been described in other laboratories, e.g., Refs. 29 and 48.
clones may be manipulated to improve their affinity or alter their specificity. One strategy that could prove to be of general use in altering the properties of antibodies isolated from combinatorial libraries is “chain shuffling.” We summarize here our own experience from two approaches to chain shuffling using a set of HIV-1 gp120-binding Fabs.

In the first, a given chain was recombined with the complete original library of the complementary chain (23). We have found that this approach, with either chain fixed, yields a spectrum of related clones, all of an affinity for antigen comparable to that of the original pairing.

In the second approach to shuffling, heavy and light chains were cloned into separate plasmid vectors with differing antibiotic resistance (24). Cells were then transformed and double transformants expressing both chains selected. In a set of 21 Fabs binding to gp120, this system revealed a surprising degree of chain promiscuity, particularly in the heavy chain. Thus, some heavy chains that would productively pair with all light chains in the set were found. Other heavy chains were more selective in their choice of partner. The system was further used to look at recombination of chains from Fabs binding to gp120 with those binding to tetanus toxoid. The results clearly supported the notion that specificity for antigen is dominated by the heavy chain. Heavy chains from some gp120 binders could retain affinity for gp120 with a light chain from a tetanus toxoid binder. A heavy chain from a tetanus toxoid binder could retain affinity for toxoid with light chains from gp120 binders. However, none of the light chains in the experiment could dictate a new specificity to heavy-chain partners.

Alternative strategies for the refinement of clones involve mutagenesis and reselection. Random muta-

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**FIG. 4.** Helper phage rescue of phagemid to give a phage display library. Helper phage, a single-stranded DNA virus in a protein coat, infects *E. coli*, and the single-stranded DNA is converted to double-stranded DNA. This codes for a number of phage proteins. Some of these are coat proteins which accumulate in the inner membrane of the cell. Others act on the pComb3 phagemid DNA, because of the FI sequence (Fig. 3), causing the packaging of a single-stranded copy of the phagemid DNA. This is packaged in preference to the helper phage DNA which has been mutated to decrease packaging efficiency. As the assembling phage is extruded from the cell, it is “capped” by coat protein III, which includes native cplII and the cplII-Fab complex. Thus, a phage results with an Fab displayed on the surface and the phagemid DNA, containing the corresponding antibody genes, inside. The advantages of phagemid rescue over direct cloning of antibody genes into phage are twofold. Direct cloning would mean that every cplII molecule would carry Fab. This would greatly reduce infectivity since cplII is involved in entry to *E. coli*. Further multivalent display is expected to hinder selection of binders on the basis of affinity in the panning process because of chelation effects. Phagemid rescue probably leads to primarily monovalent Fab display.
genesis of the variable regions is possible by a number of different approaches such as chemical mutagenesis (38), polymerase-induced mutagenesis (39), and in vivo mutagenesis using mutator strains of Escherichia coli (40). Focused mutagenesis, in which several residues are targeted, does not mimic the supposed random mutation and selection of the immune system but allows all possible mutations in a defined region to be explored. This strategy has been successful in the generation of high-affinity variants of human growth hormone (41, 42) and, when applied to the CDRs of antibody, in improving affinity for antigen (29, 28).

Although positive selection for variants of increased affinity is an obvious aim, mutagenesis can also be employed to increase or decrease cross-reactivity (43). The panning process can assist here. For instance, specificity can be increased by including the antigen giving rise to unwanted cross-reactivity in the wash solution during the selection or by preselection of the phage with the antigen. Conversely, cross-reactivity can be encouraged by panning alternately against the antigens in question.

Another genetic manipulation of particular importance for any therapeutic application of library-derived antibodies is to splice the Fabs to Fc (Fig. 2) to generate whole antibody molecules. This is relatively straightforward. Expression of the whole antibody molecules, at least in part because of the glycosylation requirement of the Fc part of the molecule, is then carried out in eukaryotic cell lines. We have shown how a whole IgG1 molecule can be expressed in Chinese hamster ovary (CHO) cells using the Fd and light chains derived from phage (44). As expected, the molecule binds antigen with retained antigen affinity.

**ANTIBODIES DERIVED WITHOUT IMMUNIZATION**

There are two principal types of libraries for derivation of antibodies without immunization. The first is termed a "naive" library, which is derived from an animal but typically from the IgD or IgM class of antibody rather than the more usual IgG class. The former should be less influenced by the immunization history of the animal. The antibodies selected by this route (19, 26, 27) will tend to be of lower affinity than those

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**FIG. 5.** Panning for selection of specific Fabs from the combinatorial phage display library. Antigen immobilized, e.g., on a plastic surface is exposed to the phage display library (A) when specific phage-Fabs bind and most of the rest of the phage are removed by washing (B). Specific phage-Fabs are then eluted, e.g., by low pH or excess soluble antigen (C) and isolated (D). In practice, after one round of panning many irrelevant phage-Fabs are present at (D) and the process (A–D) is repeated as several rounds of panning. After each round of panning the eluted phage-Fabs are amplified to minimize the chances of losing important phage. Amplification is carried out by infecting bacterial cells with the phage (which are then converted from single-stranded phage to double-stranded phagemid DNA), growing up the cells, and then rescuing phage-Fabs using helper phage as in Fig. 4. Finally the phage-Fabs from (D) are converted to the phagemid form, the DNA is prepared, and the gene for eptIII is excised. Religation then gives a reconstructed phagemid which can be used to transform bacterial cells for the production of soluble Fab fragment. After Ref. 53.
that have been subjected to rounds of mutation and selection \textit{in vivo}. Nevertheless, by subjecting the first selected antibodies to some of the procedures described above, high-affinity antibodies can be generated. For instance, an antibody against a small hapten could be selected from a nonimmunized donor with an affinity of $3 \times 10^6 \text{ M}^{-1}$. Light-chain shuffling increased this value by 20-fold and shuffling of CDR1 and 2 of the heavy chain by a further 15-fold to $1 \times 10^9 \text{ M}^{-1}$.

The second type of library is referred to as semisynthetic in that the antibody frameworks are derived from natural antibodies but some or all of the CDRs are derived from synthetic gene segments. Initial explorations of semisynthetic antibodies utilized a single clone selected from a library from an immune donor (20). A 16-amino-acid random sequence was then introduced over the CDR3 region of the heavy chain to generate a vast library of antibodies. Selection of the library against a variety of antigens allowed the cloning of new specificities. The complete randomization of 16 amino acids would require the generation of a library of greater than $10^{10}$ clones, far in excess of the number that is obtainable by the transformation of \textit{E. coli}. However, libraries can be constructed to match or exceed the diversity of clones examined by an animal at a given moment, approximately $10^7$. Selection of this library derived from a human anti-tetanus clone against a new antigen, fluorescein, resulted in the isolation of clones that bound fluorescein with affinities approaching that obtained by the secondary boost of a mouse. This strategy is proving useful for the generation of antibodies against a variety of antigens, including otherhaptons, metal ions, metal oxides, DNA, and self-proteins (44-46), and has also recently been demonstrated in the Cambridge laboratory (28). Extension of this strategy to the synthesis of all the CDRs or the use of natural libraries of FR1-FR3 fragments in combination with synthetic CDR3s should yield libraries from which almost any given specificity is re-trievable. This approach has one distinct advantage over the use of naive libraries. Diversity of these libraries is controlled at the level of nucleic acid synthesis, whereas the diversity of a naive library is limited by the source of RNA, which is susceptible to bias by RNA derived from plasma cells or activated B cells.

Synthetic antibodies can also be selected to mimic a receptor or ligand (46). This can be achieved with a limited knowledge of critical residues involved in binding. For example, many members of the integrin family of cell adhesion receptors bind Arg-Gly-Asp (RGD)-containing ligands. Although this simple tripeptide se-

sequence binds with only millimolar affinity, it is sufficient to guide the synthesis and selection of antibodies with affinities of $10^{10} \text{ M}^{-1}$. This was achieved by placement of the RGD sequence within HCDR3 and construction and selection from a library in which sequence surrounding the tripeptide sequence was randomized. This procedure served to optimize the conformational display of the sequence. Furthermore, the resulting antibodies exhibit specificity for the receptor on which they were selected. This approach may find application in drug design since the optimized CDR can be viewed as a constrained peptide lead compound. Ligand-mimicking antibodies may also prove to be useful surrogates when structural investigation of the natural ligand is intractable.

The generation of antibodies without immunization (47, 48) clearly has many advantages over the use of immunized animals. There are two principal disadvantages at present. One is that a greater diversity of specific antibodies is typically obtained from an immunized library, although this is likely to change as more semisynthetic libraries are produced. The second is that, for human therapy, the extent to which semisynthetic antibodies will be perceived as foreign by the human is unknown. Nevertheless semisynthetic libraries in particular offer a myriad of new possibilities as discussed below.

\section*{FUTURE PERSPECTIVES}

We see the availability of library-derived antibodies impacting on structural studies on antibodies and antibody-antigen complexes in a number of ways. We anticipate a movement toward the study of human antibodies and away from rodent antibodies. Human antibody responses to infectious agents, for instance, can often be clearly distinguished from murine responses, and human responses are of much greater interest. For instance, the neutralizing murine response to immunization with HIV-1 gp120 is very much focused on anti-V3 loop antibodies. In contrast, the human neutralizing response to long-term HIV-1 infection shows a strong component of anti-CD4 site antibodies, which is difficult to elicit in mice (49). Furthermore, the heavy-chain CDR3, which is generally accepted to be the most important component in antigen recognition, shows important differences between mice and humans. In humans, it is often much longer and its generation by genetic recombination a more complex process,
probably allowing a greater diversity of sequences (50). Autoimmunity is another area in which human antibodies are clearly of considerably more relevance to understanding disease.

For many aspects of molecular recognition, the most exciting developments are likely to come from structural studies on antibodies derived by randomization of the CDRs and selection by phage display. Such antibodies might be variants of antibodies derived from natural libraries or wholly from semisynthetic libraries. The approach has advantages over site-directed mutagenesis in that it makes fewer assumptions about our understanding of protein structure-function. One could envisage, for example, detailed structural analyses of closely related antibodies of ascending affinity and graduated specificity. This could bring us closer to a molecular understanding of those important features of antibody binding to antigen: affinity and specificity.

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The combinatorial phagemid library is now "rescued" to a phage display library (Fig. 4) in which each phage expresses an Fab on the surface and has the corresponding genes inside the phage. This library is now "panned" against immobilized antigen (Fig. 5) and specific Fabs are selected by their ability to bind to antigen. These specific phage–Fabs can now be converted to a phagemid form which, when injected into bacterial cells, produces soluble Fabs. Adapted from Ref. 30.