Human Antibodies from Combinatorial Libraries

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I. Introduction

The survival of animals in a hostile environment teeming with pathogens has necessitated the evolution of complex systems of immune defense. These systems contain molecules, primarily antibodies, and T-cell receptors, with exquisite specificities which have naturally attracted attention as potential therapeutic agents. Antibodies are clearly more straightforward to use in this role since they are soluble protein molecules whereas T-cell receptors are of course cell associated and require MHC compatibility for recognition. Indeed over 100 years ago, Emil von Behring demonstrated the efficacy of hyperimmune horse serum containing high titers of antibody to diptheria in combating an outbreak of disease in humans (Behring, 1893). However, despite this early success, the use of passive immunization through the 20th century has been very limited. This probably reflects the great success of antibiotics in combating bacterial disease, of vaccines in the prevention of viral disease, and the difficulties of obtaining antibodies useful for passive immunization. The emergence of AIDS in the 1980s and of a spectrum of infections in a large pool of immunocompromised people has once more focused attention on infectious disease. This in turn has renewed interest in passive immunization at a time, we will argue, of rapid progress in the generation and design of antibodies for medicine.

Antibodies are generally thought to have evolved to recognize and eliminate foreign pathogens. However, the ability to target antibodies to self-molecules provides new therapeutic opportunities. These include immunomodulation, e.g., immunosuppression, anti-inflammation, and cancer. Further human antibodies could be used to explore the function of certain molecules in humans by neutralizing such molecules in vivo.

Immunotherapy prefers a ready supply of specific human antibodies. Hyperimmune human serum preparations have proved useful in a number of instances, notably to achieve a great decrease in the developed countries in incidence of hemolytic disease of the newborn through anti-rhesus D prophylaxis. The disadvantages of hyperim-
mune serum are that it is only practically available in limited instances and that it is inherently extremely inefficient since only a small proportion of the preparation constitutes specific functional antibody. Hybridoma technology offers unlimited supplies of specific rodent monoclonal antibodies but has been much less successful for human monoclonal antibodies (James and Bell, 1987). Epstein-Barr virus transformation of human B cells (Casali and Notkins, 1989) has met with some success but again has major limitations. "Humanization" of existing mouse monoclonal antibodies by variable domain replacement (Boulianne et al., 1984; Morrison et al., 1984; Neuberger et al., 1985) or by CDR grafting (Jones et al., 1986) has been achieved and this allows for the generation of anti-self-antibodies. The chimeric antibodies are easily produced but retain the mouse variable domains with possible consequences for immunogenicity. The CDR-grafted antibodies are more nearly human but take longer to produce and may require adjustments to the human frameworks.

A new technology for the generation of human monoclonal antibodies has been developed over the past 5 years based on cloning antibody fragments in phage libraries. The vectors used were initially λ phage (Huse et al., 1989) but these have been superseded by filamentous phage display vectors (McCafferty et al., 1990; Kang et al., 1991a; Barbas et al., 1991; Clackson et al., 1991).

II. Principles of Combinatorial Libraries

A. The Combinatorial Approach

The "combinatorial approach" of cloning antibody repertoires was initially developed for application in the field of catalytic antibodies. Traditional hybridoma methodologies allow for a limited sampling of the immune repertoire. Cloning the repertoire in *Escherichia coli* would allow a much more extensive survey of the immune response and might be essential for screening or selecting for rare catalytic activities. This approach became possible due to two developments. First, it was demonstrated in 1988 that the antigen binding fragments Fv (variable heavy, \( V_H \), variable light, \( V_L \), Fig. 6) and Fab could be expressed and functionally assembled in *E. coli* (Skerra and Plückthun, 1988; Better et al., 1988). These experiments were successful because the antibody fragments were secreted from the cytoplasm to the oxidizing environment of the periplasm in *E. coli* under the guidance of bacterial leader sequences. The oxidizing environment and possibly the secretory event were necessary for disulfide bond forma-
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tion and proper folding of antibody domains. This is directly analogous
to the production of antibodies in eukaryotic cells where the two chains
are transported from the cytoplasm to the lumen of the endoplasmic
reticulum. Second, the development of the polymerase chain reaction
(PCR) allowed for the rapid cloning of antibody genes from hybridomas
(Larrick et al., 1989; Orlandi et al., 1989) and mixed populations of
antibody producing cells (Sastry et al., 1989). Within the year, these
two developments were utilized to produce the first combinatorial
antibody library (Huse et al., 1989). At the same time an alternative
approach consisting of single heavy-chain variable domains (dAbs)
was reported (Ward et al., 1989). This dAb approach makes the assump-
tion that the role of the light chain in antigen binding is negligible.
Crystallographic studies indicate that light chains often make consid-
orable contact with antigen and can play an important role both in de-
termining affinity and in specificity. Furthermore and equally impor-
tant, single V H domains are not physicochemically well-behaved
due to exposure of a large hydrophobic patch on the domain usually
involved in light-chain association. Indeed, the dAb approach has not
proven to be generally applicable.

The first combinatorial library experiment reported in 1989 involved
screening antibody Fab fragments for binding of labeled antigen. In
this case the libraries were expressed in λ phage, a lytic phage which
upon lysis releases the periplasmically sequestered Fab (Huse et al.,
1989). In these experiments a mouse was immunized with the hapten
designed to elicit catalytic antibodies and RNA was prepared from the
spleen. After reverse transcription, the cDNA of antibody heavy chains
(Fd part of IgG1) and light chains were amplified by the PCR reaction
and ligated into modified λ phage vectors to give libraries of heavy
and light chains. The two libraries were then combined by digestion
of opposite arms of the vectors and religation to generate a random
combinatorial library containing the genetic information for the produc-
tion of Fab fragments. The library was screened by transfer of Fabs
produced by λ phage-lysed E. coli onto nitrocellulose filters. The filters
were then probed with 125I-labeled hapten conjugate (Fig. 1) and re-
vealed a high frequency of positives in the library (about 1 in 5000)
which allowed 200 monoclonal Fab fragments to be identified follow-
ing an examination of 10 6 Fabs. The examination of this library of a
million clones, which as we see later is a relatively modest library
size, required 20 filter lifts. Analysis of 22 of the positive Fabs showed
sequence diversity and apparent binding affinities of the order of
10 5 M -1.

With the demonstration of the applicability of the system for the
Fig. 1. Screening the first combinatorial library for antigen binding (Huse et al., 1989). A mouse was immunized with the hapten NPN on the carrier KLH and a spleen library prepared in λ phage. The Fab library (A and B), heavy-chain alone library (E and F), and light-chain alone library (G and H) were screened in duplicate on nitrocellulose filters at approximately 30,000 plaques per filter using a radiolabeled
cloning of mouse antibodies, the next step required a model system for cloning human antibodies. Peripheral blood lymphocytes from an individual recently boosted with tetanus toxoid (Persson et al., 1991) were utilized to construct an IgGlκ library. The antibodies generated in this case showed considerable sequence diversity and apparent affinities in the range 10⁷–10⁹ M⁻¹. Similar studies have also been reported elsewhere (Mullinax et al., 1990). Recent boosting was very important in that it was not possible to isolate antigen binding Fabs from an individual with a high anti-tetanus toxoid titer who had not been boosted. This probably reflects the presence of antigen-specific plasma cells with their high concentration of specific mRNA in the peripheral blood of boosted subjects as compared to the low resting level of plasma cells in PBLs. The method has also been applied to the generation of human autoantibodies (e.g., Portolano et al., 1991; Hexham et al., 1991).

B. PHAGE DISPLAY OF ANTIBODY FRAGMENTS

The screening procedure limits the size of the library which may be examined in the λ phage system. For example, the screening of a diverse library of 5 x 10⁸ antibodies would require an examination of a minimum of 10,000 filter lifts (at 50,000 plaques per plate). Furthermore, the screening procedure places restrictions on the antigens which are being examined in that the antigen must be available in significant quantities in purified form, be amenable to labeling with ¹²⁵I or enzymes, and should not stick significantly to filters in the absence of antibody. This is very restrictive if the interest is in isolating antibodies against proteins which have yet to be identified or characterized, found for example on the surface of a cell or in a crude protein extract such as a viral lysate.

Selection is inherently a more efficient method for deriving positive clones from a library than screening since virtually no effort is spent examining the vast majority of negative clones. Selection is practiced very effectively by the immune system through the expression of immunoglobulin on the surface of B cells. The selected clones are then amplified by the linkage of this recognition device to the replication of the genetic information within the B cell and clonal expansion. It

library for antigen binding (Huse et al., en NPN on the carrier KLH and a spleen ry (A and B), heavy-chain alone library and H) were screened in duplicate on plaques per filter using a radiolabeled BSA–NPN conjugate. Filters C and D illustrate the duplicate secondary screening of a positive from a primary screening (e.g., arrows on filters A and B). As expected for a bona fide positive, the frequency of positives in the secondary screening was much higher than that in the primary screening. No positives were identified in either the heavy- or light-chain alone libraries.
was therefore logical to seek a selection system in which recognition and replication could be linked. George Smith (1985) had shown that peptides could be expressed on the surface of filamentous phage indicating how this linkage might be achieved. The concept of selectable phage display libraries had been established in 1990 with peptide libraries (Scott and Smith, 1990; Devlin et al., 1990; Cwiria et al., 1990). Fragments of $\beta$-galactosidase had previously been displayed on phage (Farnley and Smith, 1988). The concept needed only be extended to libraries of proteins. This would allow selection of specific antibodies based on their ability to bind to immobilized antigen largely circumventing the problems associated with screening. An outline of the strategy is presented in Fig. 2. For a general review of phage display see Barbas, 1993.

A brief introduction to filamentous (Ff) phage biology makes their use in cloning or creating antibodies more obvious (reviewed in Model and Russel, 1988). Ff phage, most notably f1, fd, and M13 (the three are

\textbf{Fig. 2.} Strategy for cloning human monoclonal Fab fragments from combinatorial libraries on the surface of phage. The strategy has also been applied to single-chain Fv fragments (scFv). The antibody fragment is shown as a fusion with the capping protein $\delta_5$ (c$\delta_5p$ or c$\delta$H) in monovalent display. Multivalent display using c$\delta_5p$ has also been described. Fusion with phage coat protein 8 (c$\delta_8p$ or c$\delta$VIII) displaying the antibody fragment multivalently along the phage surface is another format which has been adopted. After Burton and Barbas (1993a).
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otably fl, fd, and M13 (the three are
almost identical) infect gram-negative bacteria by virtue of a specific
interaction between one end of the phage and the bacterial pilus. Ff
phage are long and thin (ca. 900 x 6–10 nm, Fig. 3); essentially they
are tubes of protein encapsulating a single-stranded closed circular
DNA genome.

The mature Ff phage consists of five proteins and a covalently closed
gene of approximately 6.4 kb. Approximately 2700 copies of the
 gene 8 protein (g8p or cpVIII) form all but the end structures. Minor
coat proteins (g3p or cpIII, g6p, g7p, g9p) are present in about 5 copies
each. The proteins are arranged so that g3p and g6p are expressed at
the tail of the phage, g7p and g9p at the head. g3p is the phage protein
that makes specific contact with the bacterial receptor. Rough genetic
mapping demonstrated that gp3 has two domains, an amino-terminal
domain important for phage infectivity and a carboxy-terminal domain
important for closing or capping the phage tube. In addition to the coat
proteins is an endonuclease/topoisomerase (g2p), a single-stranded
DNA binding protein (g5p), and several less-studied proteins: g1p,
gXp, g4p. An important regulatory element, the intergenic region (IR),
is also present. The IR contains a DNA origin of replication and a
DNA packaging signal. A plasmid with a Ff IR, most commonly fl, is
known as a phagemid. The IR is sufficient to allow helper phage-
mediated replication and packaging of the phagemid single-stranded
DNA. Packaged phagemids can infect bacteria and are known as trans-
ducing particles.

Unlike λ phage in which the first repertoire cloning experiments
were performed, the Ff phage are not assembled in the cytoplasm and
are not released by cell lysis. They are instead extruded through the
outer membrane leaving the cell unharmed. The Ff phage are extruded
as they are assembled in the bacterial membrane. Assembly begins
with the coating of genomic (or phagemid) single-stranded DNA by
g5p. This nucleoprotein complex then migrates to the inner membrane.
Coat proteins then replace g5p in a vectorial fashion—g7p and g9p
are first, followed by polymerization of g5p onto the DNA rod, and
finally g6p and g3p cap the particle. Since g3p and g8p are secreted
and anchored in the periplasmic space of E. coli and are assembled
to form the surface of the phage, they are ideal targets for fusion with
antibody domains. Note that secretion into the periplasmic space is a
prerequisite for proper folding and assembly of antibodies in all but
specifically engineered E. coli (C.F.B., unpublished data). Phage dis-
playing antibody fragments can then be sorted by a process known as
panning to isolate those which encode the desired specificity and
highest affinity as described below.
C. Phage Display Vector Systems

The phage genome itself may serve as a cloning vehicle, i.e., genes can be directly cloned into the phage genome. Genomic g3p fusion systems allow the polyvalent display of foreign peptides. In these systems every copy of the three to five copies of g3p is fused with the displayed protein or peptide. With multivalent display of antibodies on the phage surface, avidity effects minimize the selection efficiencies between clones which bear antibodies even if they differ by two orders of magnitude in affinity. The standard genomic fusion phage vector is that first described by Smith, fd-tet. With this system it is difficult to prepare the double-stranded DNA necessary for cloning. Genomic g3p fusions have been highly successful in the area of peptide libraries. The display of proteins has been described using this system and includes the display of fragments of β-galactosidase and murine single-chain antibodies. Genomic cloning need not be limited to fusions with the native coat protein. As an alternative, a second coat protein gene may be introduced as the carrier of the fusion. This type of system would have a decreased valency of display but has yet to be utilized for antibody expression.

Phagemid systems offer an attractive alternative to cloning directly into the phage genome. Phagemids are simply plasmids which bear the IR region of an Ff phage. The genomes of phagemids, when propagated in cells superinfected with a helper phage, will be packaged as phage particles in a fashion identical to the phage itself. Phagemid systems have two distinct advantages over cloning into the phage genome. First, double-stranded DNA is easily obtained making library construction less tedious and, second, the valency of the displayed protein may be controlled. Phagemid systems have been described using both g8p and g3p fusions (Fig. 4). For display purposes the phagemid also carries either g3 or g8 on which the gene of the protein to be displayed is fused. Subsequently the use of helper phage superinfection leads to the expression of two forms of the coat protein, one

Fig. 3. (a) Electron microscopy of hepatitis B surface antigen particles and filamentous phage. Fresh phage were prepared from the third pan stage of a library from a donor recently boosted with the hepatitis antigen. The phage were incubated with antigen particles, added to grids coated with antibody against the hepatitis antigen, and negatively stained. Magnification 25,000×. From Zebedee et al., 1992; figure kindly provided by Suzanne Zebedee. (b) Transmission electron microscopic images of anti-herpes D antigen (Rh(D)) expressing phage bound to the surface of an Rh(D)-positive red cell. Magnification 44,000×. From Siegel and Silberstein (1994); figure kindly provided by Don Siegel.
native and one fusion protein. On phage assembly there is a competition between these proteins for incorporation into the virion. The g8p system like the genomic g3p system is multivalent. The display of Fab fragments as g8p fusions was described initially by Kang et al. (1991a) and later by Chang et al. (1991). Sorting of antibodies of different specificities was demonstrated in one case with a 1000-fold enrichment in a single panning step. Examination of packaged phagemid displaying antibodies revealed the presence of a variable number of Fab fragments ranging from 1 to 24. Further examination of this system demonstrated that the avidity effect of multiple copies of Fab on the phage surface does not allow for the selection of antibodies of the highest affinity. In panning experiments with human anti-tetanus toxoid antibodies—which differed by 100-fold in affinity only a 5-fold enrichment could be demonstrated in a single selection (Barbas et al., 1991).

In contrast to g8p fusions, g3p fusions can be made monovalent. In the virion there are normally three to five copies of g3p and phage morphogenesis leads to incorporation of the Fab–g3p fusion and the native helper phage produced g3p into the virion (Fig. 5). Native g3p is necessary for infection as the infectivity domain should not be present in the Fab–g3p fusion (vida infra). The fusion, generally one

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**Fig. 4.** (a) The composition of a phage display vector, pComb3, and the proposed pathway for Fab assembly. Expression of Fd (Hc, heavy chain)/cpIII (g3p) 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. (b) 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 f1 sequence (a), 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 cpIII and the cpIII–Fab complex. Thus a phage results with an Fab displayed on the surface and the phagemid DNA, containing the corresponding antibody genes, inside. The advantage of phagemid rescue as opposed to directly cloning antibody genes into phage are twofold. Direct cloning would mean that every cpIII molecule would carry Fab. This would greatly reduce infectivity since cpIII 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.
copy per phage particle, is displayed in functional form on the surface of the phage and is therefore available for antigen selection. Fusions with g3p should respect the function of this 406-residue protein. Studies of g3p have revealed the two functional properties, infectivity and normal (nonpolyphage) morphogenesis, which map to roughly the first and second half of the gene, respectively. The N-terminal domain of g3p binds to the F pilus, allowing for infection of E. coli, whereas the membrane-bound C-terminal domain, P198-S406, serves the morphogenic role of capping the trailing end of the filament according to the vectorial polymerization model. Another important biological feature of the N-terminal domain of g3p is its role in providing immunity to an Ft-infected cell to superinfection by other Ft phage. This fact has important consequences in the design of phagemid vectors which utilize g3p fusions. Since superinfection with helper phage is required in this approach, the N-terminal domain should be deleted in the fusion provided by the phagemid. Otherwise, superinfection will be prevented. This feature was incorporated in the first vector designed for Fab display, pComb3 (Barbas et al., 1991), and is also found in pDH188 (Garrard et al., 1991) and pEXmide3 (Söderlind et al., 1992), which are also designed for Fab display. Previously this same design was utilized in the display of growth hormone (Bass et al., 1990). Several g3p phagemid systems, pHEN (Hoogenboom et al., 1991), pSEX (Dübel, 1993; single-chain antibody display only), and pCAN-
TAB (single-chain antibody display only, Pharmacia), utilize the entire g3p as a fusion partner. This approach necessitates additional steps in the selection process, first to shut down expression to allow superinfection and then to induce expression to allow for display.

Monovalency in g3p phagemid systems has important consequences in selection of the highest affinity antibody. The only study of the role of valency in the separation of clones of defined affinities has been reported with pComb3 (Barbas et al., 1991). In this study, phagemid vectors were constructed which where identical except for their antibiotic resistance. Enrichment could be monitored by simply plating on chloramphenicol and ampicillin plates. Enrichment of 253-fold with a single round of selection was observed in the separation of two human anti-tetanus toxoid Fabs with affinities that differed by two orders of magnitude. In an identical experiment, the multivalent g3p system, pComb8, yielded only 5-fold enrichment. Note that monovalency in the g3p system can only be ensured when Fab fragments are displayed. In contrast, single-chain antibodies, scFv, have been found in many cases to spontaneously dimerize (Holliger et al., 1993).

D. Cloning Strategies

Library construction begins with mRNA that is used as a template for cDNA synthesis. cDNA then becomes the template for the polymerase chain reaction. Two types of antibody fragments may be chosen for construction, Fab or scFv. Fab fragments, the natural antigen binding fragments of whole antibodies, retain the binding characteristics of the whole antibody with the exception of avidity. Single-chain antibodies reproduce the binding characteristics of the whole antibody to variable extents and may have greatly reduced binding affinity for antigen as compared to the whole antibody (Bird and Walker, 1991; Whitlow and Filipula, 1991). Detection and purification of the fragments should also be considered. Fab fragments may be detected with many commercially available reagents (anti-Fab, anti-κ etc.) with many reporter enzyme conjugates. scFv may only be detected by including a peptide tag at the tail of the protein and detection is limited by the anti-peptide antibody which must be used. Purification regimes are also more limited with scFv's. The properties of increased tissue penetration may, however, favor the use of scFv for particular applications. Thorough consideration of the end use of the antibody fragments should dictate the strategy to be taken.

The protocols for the construction of Fab or scFv libraries are quite distinct. Consider the construction of IgG1κ and IgG1λ Fab libraries (Fig 6). For IgG1 PCR, we commonly use eight 5'-VH and one 3'-IgG1 primers to amplify VH genes. For κ PCR, five 5'-Vk and one 3'-κ primer
are used. For λ, eight 5'-Vλ and one 3'-λ primer are used. Following PCR, all IgG1 products are pooled as well as all κ and λ products. The three pools are prepared for cloning by restriction digest. The prepared κ and λ products may be mixed and ligated with cut phagemid. Electroporation then allows for the efficient introduction of ligated products to form the light-chain library. From this library, phagemid is prepared for heavy-chain cloning and the combinatorial library is formed and ready for selection following helper phage rescue. A total of 21 PCR primers are required for completeness, a single PCR step is performed, and two ligation and transformation steps are performed. Libraries of about 10⁶ transformants can be economically prepared using electroporation in the transformation steps.

Now let's consider the construction of scFv libraries of IgG1κ and IgG1λ (e.g., Marks et al., 1991). A major difference in the construction of scFv libraries is that there is no antibody constant region in this construct. This fact requires the utilization of multiple J region primers while for Fab cloning a single primer would suffice. For scFv, V₇ construction requires six 5'-V₇ primers and four 3'-J₇ primers (J₇ 2 and 5 not reported). Vκ requires six 5'-Vκ and five 3'-Jκ primers. Vλ requires seven 5'-Vλ and three 3'-λ primers. PCR is performed with these sets of primers. Subsequently another round of PCR to create the linker regions is required with additional four Jλ, six Vκ, and seven Vκ-linker primers in 52 separate PCR reactions. Now in a third
round of PCR, VH is fused with Vk and VL and the linkers first without addition of primers and then in another step with the addition of primers. This fused product is then subjected to a fifth round of PCR with an additional new set of six VH, five Jκ, and three JA primers, which add restriction sites. The products are subjected to a restriction digest and ligated and cloned in one step. In five PCR steps with 62 different PCR primers and one ligation and transformation step, the library is constructed. In our estimation, the difficulty and cost of constructing representative scFv libraries can only be justified when scFv is required as the end product. Indeed, it may be more effective to first construct and select clones from a Fab library and convert the desired clones into scFv. This two-step strategy also circumvents the multivalency problem in the selection of clones of the highest affinity as the library is selected by way of Fab display.

An alternative approach to the construction of Fab libraries has been suggested (Hogrefe et al., 1993). This approach combines aspects of the original λ phage approach with phagemid Fab and scFv protocols. Light-chain amplification is performed as described in the Fab construction protocol with an additional PCR step to fuse the heavy-chain leader sequence to the light chain. Next, VH regions are amplified requiring the multitude of primers required in scFv construction. The two fragments are then cut with restriction enzymes, ligated, and subjected to another round of PCR after which the single light-chain leader VH fragment is again cut with restriction enzymes. This product is then ligated with an ImmunoZap 13 vector encoding a phagemid which is a close copy of pComb 3 containing a CH1 region. In vitro packaging then allows for the construction of a λ library. To obtain Fab phage display, helper phage is used in a mass excision step resulting in packaged phagemid. The rationale of this approach is to simplify library construction by taking advantage of the high efficiency of in vitro packaging of λ phage as an alternative to electroporation. The strategy assumes that PCR product is limiting in library construction although we would suggest that more product is easily produced with additional PCR reactions. Furthermore in vitro packaging, though efficient, is extremely expensive and limited to a small scale making the construction of libraries of 10^6 clones tedious, if not economically unfeasible. There is no report in the literature, to our knowledge, of the construction of λ libraries of this magnitude. The utilization of a VH cloning strategy further complicates library construction by necessitating more PCR primers and additional steps. An additional concern is the lytic nature of λ phage which frequently leads to serious problems of contamination in the laboratory.
Huse et al. (1992) suggest a modification of the original lambda phage approach for library screening. In this report M13 FF phage is utilized with a second copy of g8p to which a Fab is fused. The library is then screened utilizing essentially the same protocol described for λ phage. No selection was attempted and the g8p is utilized only to expose the Fab as it is secreted with the phage. A limitation of this approach is its requirement for screening.

E. Selection Strategies

Phage systems readily allow sorting of enormous libraries. This rapid sorting is a result of the linkage of recognition and replication. Only those phage that bear a functional peptide or protein expressed on their surface will be permitted to propagate through a procedure known as panning (Fig. 7). The display of the antibody on the surface of the phage allows for the selection of clones by panning against antigen in ELISA wells. This is analogous to an affinity chromatography step. Generally a single well of a 96-well ELISA plate is sufficient for selection making several micrograms of protein sufficient for multiple selection steps and subsequent binding assays to verify success. The efficiency of the selection process is due in part to oversampling of the library. This is a distinct advantage of phage since 10^12 phage in a volume of 50 μl may be applied to a single ELISA well. Thus for a library of a million clones, each type will be present on a million phage. Phage bind by way of the displayed antibody fragment. The approach is not limited by a requirement for purified antigen and is sensitive to rather rare components, for example, in viral lysates, allowing for the discovery in principle of biologically active antibodies to unknown antigens. Furthermore whole cells may be utilized for selection. Antigen immobilized on beads or whole cells may also be utilized. Chromatographic procedures may also be used for phage selection, however, high levels of background binding of phage make panning in microtiter plates the preferred mode of selection.

After vigorous washing, the bound phage is enriched for antigen-specific Fabs. Elution of the specifically bound phage is most generally achieved by acid elution. Alternatives to acid elution include base elution, antigen elution, direct infection by simple addition of E. coli, or reductive elution. This phage is then amplified and reselected by further rounds of panning. Each step selects for antigen-specific clones as well as for clones of the highest affinity, at least in the monovalent Fab display. In this way one can rapidly generate a panel of antigen-specific Fabs/scFvs.
lification of the original lambda phage. In this report M13 F1 phage is which a Fab is fused. The library of the same protocol described for d and the g3p is utilized only to h the phage. A limitation of this panning.

t of enormous libraries. This rapid ecognition and replication. Only side or protein expressed on their te through a procedure known as the antibody on the surface of the ones by panning against antigen an affinity chromatography step. El ELISA plate is sufficient for s of protein sufficient for multiple lling assays to verify success. Th s due in part to oversampling of age of phage since 10^{12} phage in a single ELISA well. Thus for a pe will be present on a million displayed antibody fragment. The rement for purified antigen and its, for example, in viral lysates, e of biologically active antibodies e whole cells may be utilized on beads or whole cells may procedures may also be used n levels of background binding er plates the preferred mode of d phage is enriched for antigen bly bound phage is most generally ves to acid elution include base tion by simple addition of E. coli, then amplified and reselected by selects for antigen-specific clones affinity, at least in the monovalent piddly generate a panel of antigen-

Fig. 7. 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 by, e.g., low pH or excess soluble antigen (C) and isolated (D). In practice after one round of panning, many irrelevant phage-Fabs are apparent 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 the cells, and then rescuing phage-Fabs using helper phage as in Fig. 4b. Finally the phage-Fabs from (D) are converted to the phagemid form, the DNA is prepared, and the gene for cpIII is excised. Religation then gives a reconstructed phagemid which can be used to transform bacterial cells for the production of soluble Fab fragment. After Burton, 1992.

F. Expression of Antibody Fragments

Selected clones must be characterized individually to assess their binding characteristics. The simplest route to soluble Fab is to rely on the proteases present in E. coli to cleave the g3p fusion protein to release Fab. Simple induction of expression overnight yields sufficient protein in the supernatant for specificity and competitive studies. For the production and purification of larger amounts of protein, the gene encoding the g3p may be removed in the pComb3 vector by restriction digest with NheI and SpeI followed by religation and transformation (Barbas et al., 1991). The removal and religation may be performed in a single step as religation destroys the restriction site. Purification
is performed most readily on a large number of clones by using an anti-Fab affinity column. As an alternative to removal of the g3p gene an amber mutation may be placed at the scFv or Fab/g3p junction (Clackson et al., 1991). Isolation of DNA and retransformation into a nonsuppressing strain of E. coli allow for the amber codon to be read as a stop and soluble protein to be produced. The amber codon strategy is a viable approach when cloning natural genes; however, when the libraries are constructed with random synthetic segments which encode 1 part in 32 as an amber codon this approach becomes less reliable (vida infra). As noted previously, the options for purification of scFv are more limited, although affinity chromatography is an option if large amounts of the anti-peptide monoclonal antibody are available. Fab are purified more economically with polyclonal reagents.

G. Whole Antibody Molecules

For most applications of antibodies in therapy, the whole antibody molecule is preferred. Expression of whole antibody molecules, at least in part because of the glycosylation requirement of the Fe part of the molecule, demands the use of eukaryotic cell lines. The favorites in this regard have been myeloma (Wright and Shin, 1991) or Chinese hamster ovary (CHO) cells (Bebbington, 1991) although baculovirus has also been used (Haseman and Capra, 1991). Modification of existing vectors enables the facile expression of whole antibodies utilizing Fab cassettes from the phage system. Using such an approach we have expressed a whole IgG1 molecule in CHO cells using the Fd and light chains derived from a phage clone (Bender et al., 1993). In principle any Fe can be linked to the Fd of the heavy chain from the phage system. This allows the experimenter to choose a suitable isotype for a given task. The reader is referred to Burton and Woof (1992) for a more complete discussion of human antibody effector function.

III. Antibodies to Non-Self-Antigens from Immune Donors

Humans typically have serum antibody titers to a range of non-self-molecules. Clinically the most significant are those to infectious agents, to vaccines, and to allergens. There is great interest in cloning antibodies to these antigens for prophylaxis and therapy and to monitor humoral responses.

In adopting a library approach, a number of choices need to be made including donor, tissue source, and antibody isotype. The choice of donor and isotype is dictated primarily by the serum titer against the antigen to be studied. A high serum titer is presumed to reflect rela-
number of clones by using an active to removal of the g3p gene for the scFv or Fab/g3p junction DNA and retransformation into a for the amber codon to be read for the amber codon strategy. However, when the n synthetic segments which ens approach becomes less reliable options for purification of scFv chromatography is an option if large monal antibody are available. Fabs polyclonal reagents.

Choice of tissue source is crucial in the library approach. Ideally one begins with the source richest in plasma cells secreting antibodies against the antigen of interest since this will contain the highest levels of specific mRNA. Richest can refer to numbers relative to irrelevant specificities and to diversity of clones secreting antibody to the given antigen. In practice the two probably go together. It appears from studies on tetanus toxoid boosting of humans that, immediately following secondary antigen contact, i.e., 3–10 days, the number of specific antibody secreting cells in the peripheral blood is high (Stevens et al., 1979; Thiele et al., 1981; Ershler et al., 1982; Volkman et al., 1982). Indeed human Fabs to tetanus toxoid have been derived from PBL libraries from boosted donors (Mullinax et al., 1990; Persson et al., 1991). However, the number of antibody secreting cells in the periphery declines very rapidly following the antigen boost to a low resting level (Stevens et al., 1979; Ershler et al., 1982). Consistent with this was our failure to detect anti-tetanus toxoid antibodies in a library prepared from an individual with a high serum titer to toxoid but no recent boost (Persson et al., 1991). Bone marrow has been shown in humans to be a major repository of antibody producing cells (Lum et al., 1990) and is the source we have used most frequently for libraries.

We have shown that antibodies to many different pathogens can be derived from bone marrow libraries constructed from a single donor (Williamson et al., 1993). Other tissue sources from which we have prepared libraries are spleen, lymph node, and tonsil. Antibodies to respiratory syncytial virus (RSV) have been selected from spleen and tonsil libraries (unpublished results).

A. Antibodies to Viruses

Specific human antibodies have been shown to prevent disease caused by a wide variety of viruses belonging to diverse RNA or DNA virus families that include the orthomyxoviruses, paramyxoviruses,
alphaviruses, flaviviruses, arenaviruses, lentiviruses, picornaviruses, hepatadnaviruses, and herpesviruses reviewed in Chanock et al., 1993). Examples from clinical medicine include the hepatitis A virus, measles virus, and poliovirus. Commercial human gamma globulin, which usually contains 16 to 18% IgG, of which only a very small proportion is specific for any given antigen, is highly effective in the prevention of hepatitis A disease and has been widely used for that purpose during the past 40 years (Krugman et al., 1960). A total of 5–10 ml of commercial human gamma globulin (hereafter referred to as IgG) provides effective protection for adults for about 6 months. Prior to licensing of the live measles virus vaccine, human IgG was successfully used for passive prophylaxis and was the mainstay for prevention of the disease (Janczewski, 1945). A double-blind prospective clinical trial performed in 1951–1952 demonstrated that human IgG was also effective in preventing paralytic disease caused by poliovirus (Hammon et al., 1953).

One of the more dramatic effects of viral antibodies was observed during a study (Beasley et al., 1983) in which human IgG was shown to be effective in preventing chronic hepatitis B virus (HBV) infection in high-risk infants. The IgG preparation, selected for having a high titer of antibodies to HBV, was administered to infants born to mothers chronically infected with HBV. In the absence of any treatment, most such infants (>90%) become chronic HBV carriers within the first few months of life. IgG given in three 0.5-ml doses at birth and 3 and 6 months was 71% effective in preventing the development of persistent HBV infection. A single dose given at birth was only 41% effective. In the group of successfully treated infants, 75% were in fact initially infected with virus but they resolved their infection and developed protective antibodies.

The prophylactic value of antibodies to cytomegalovirus (CMV) and varicella Zoster virus (VZV) is discussed below.

Conventional wisdom, while recognizing the prophylactic role of antibodies, tends to discount their importance in the resolution of established viral infections. Instead, clearance of virus is thought to be mediated primarily by CTLs. Several recent observations cast doubt on this generalization and suggest that in some situations antibody can independently bring about resolution of infection.

The most dramatic clinical therapeutic effect of viral antibodies has been observed in patients with Argentine hemorrhagic fever which is caused by Junin virus, an arenavirus (Enria et al., 1984). The disease has a high mortality but death can be prevented when a preparation of pooled human sera with a high titer of Junin virus neutralizing antibodies is administered within 8 days of the onset of symptoms.
Mucosal virus infections that are limited to the cells that line the lumen of the respiratory tract can also be cleared by specific antibodies that are delivered by parenteral inoculation or by direct instillation into the lungs. High therapeutic efficiency has been demonstrated for RSV neutralizing antibodies delivered to the lungs of cotton rats at the height of infection (Prince et al., 1987; see also our studies described below). In the case of influenza A virus, viral hemagglutinin-specific antibodies can clear the lungs of infected mice in the absence of other immune functions (Scherle et al., 1992). Complete clearance of virus from persistently infected SCID mice was achieved using physiologic amounts of hemagglutinin-specific antibodies inoculated parenterally.

Therapeutic efficacy of antibodies has been described for a number of neurotropic viruses. In SCID mice, persistent infection of neurons by Sindbis virus can be cleared rapidly by parenteral inoculation of Sindbis virus envelope glycoprotein-specific antibodies (Levine et al., 1991). Such neurons are not recognized by CD8+ CTLs because these cells are deficient in class I MHC molecules. Surprisingly this clearance appears to occur without causing obvious cell damage. The mechanism responsible for this dramatic effect is not understood. Other studies in mice indicate that antibody directed against the σ1 outer capsid protein of reovirus (the viral attachment protein) can interrupt the spread of established reovirus infection within the central nervous system (Virgin et al., 1988; Tyler et al., 1989). Monoclonal antibody against the reovirus σ1, σ3, or μ1 outer capsid protein can also inhibit the neural spread of virus from the brain to the eye. Therapeutic effects have also been described for antibodies to rabies and measles viruses in rodent systems. IgG has been used in the successful treatment of chronic enteroviral meningoencephalitis (Mease et al., 1981).

For some of the reasons outlined above and because current antiviral strategies have met with only limited success, we have put some effort into the generation of human antibodies to viruses as described below. Most of the published work to date has originated from our laboratories, although several groups are now working successfully in this area.

1. Antibodies to Human Immunodeficiency Virus Type 1 (HIV-1)

There are three principal reasons for producing human monoclonal antibodies from HIV-1-seropositive individuals. The first is to explore the humoral response to the virus and thereby increase understanding of the nature of infection and disease. The second is to aid in vaccine design. One of the goals of a vaccine is to elicit potent neutralizing antibodies and therefore study of such antibodies and the epitopes they recognize is of clear value. The third reason is to produce reagents
for passive immunization. The variability of the HIV-1 virus means that a cocktail of human monoclonal antibodies rather than a single antibody may be appropriate here. The ability of the library approach to provide large numbers of antibodies for evaluation may then be highly beneficial. Passive immunization is likely to find immediate application in the treatment of individuals accidentally infected with HIV-1, e.g., needle sticks and in prophylaxis of infected mothers to hinder transmission of virus to the fetus or newborn. Given the complexity of AIDS, the value of antibodies in therapy will remain uncertain until it is tried. However, it seems reasonable to suppose that reducing the viral load will always be a desirable goal and, even if antibodies alone do not arrest the disease, they may be valuable in conjunction with new generations of anti-retroviral drugs.

All of the antibody-mediated HIV-1 neutralizing activity in human sera has been associated with reactivity to the envelope glycoproteins gp120 and gp41 and in particular to the third hypervariable domain (V3 loop) and CD4 binding site of gp120 (for reviews see Nara et al., 1991; Moore and Nara, 1991; Laal and Zolla-Pazner, 1993; Moore and Ho, 1993; Neurath, 1993). The importance of antibodies to the V2 loop of gp120 in the neutralizing response has also been reported (McKeating et al., 1993; Moore et al., 1993). It is these epitopes which figure in the discussion below.

The first phage display library in these studies was prepared from a 31-year-old homosexual male who had been HIV positive for 6 years but had no symptoms of disease (Burton et al., 1991). Serological studies showed the presence of a high ELISA titer against the HIV-1 surface glycoprotein, gp120 (LAI strain). After securing informed consent, bone marrow cells were obtained by aspiration. Amplified antibody genes were then cloned into pComb3 to give a library of $10^7$ members.

The phage surface expression library was panned against recombinant gp120 (strain LAI) coated on ELISA wells. This choice of antigen was reasoned to encourage strain cross-reactivity because the library donor was of United States origin, where MN-like strains predominate, whereas the antigen was derived from the LAI strain which is very rare in the United States. Four rounds of panning produced an amplification in the number of eluted phage of a factor of about 100 compared to the first round of panning, indicating enrichment for specific antigen binding clones. Forty reconstructed clones secreting soluble Fab fragments were grown up and the supernates screened in an ELISA assay for reactivity with recombinant gp120. The supernates from 33 clones showed clear reactivity. The supernates did not react with BSA-coated
variability of the HIV-1 virus means that antibodies rather than a single virion is likely to find immediate victims. The ability of the library approach has for evaluation may then be judged by how effectively and broadly a library of 30-100 clones was found to be infected with prophylaxis of infected mothers to the extent of a fetus or newborn. Given the com-

odities in therapy will remain under-

seems reasonable to suppose that as be a desirable goal and, even if disease, they may be valuable in the treatment of anti-retroviral drugs.

IV-1 neutralizing activity in human sera to the envelope glycoproteins to the third hypervariable domain gp120 (for reviews see Narasimhan, 1993; Moore and Zolla-Pazner, 1993; and Zolla-Pazner, 1993). It is these epitopes which in these studies was prepared from 10 had been HIV positive for 6 years (Burton et al., 1991). Serologi-

zation of a high ELISA titer against the 20 (LA1 strain). After securing inc-

s were obtained by aspiration. Ampli-

oned into pComb3 to give a library

library was panned against recombi-

ELISA wells. This choice of antigen cross-reactivity because the library where MN-like strains predominate, I from the LA1 strain which is very

uts of panning produced an amplifi-

age of a factor of about 100 compared to the starting enrichment for specific antigen

ed clones secreting soluble Fab frag-

ternates screened in an ELISA assay of gp120. The supernates from 33 clones supernates did not react with BSA-coated

wells and anti-tetanus toxoid Fab supernates did not react with gp120-coated wells.

DNA from the 33 clones was used as templates for sequencing of thymidine nucleotides of the V H and V L regions to reveal that at least 10 clones had unique heavy chains and 20 clones unique light chains. A representative number of chains were then sequenced. Figures 15 and 16 indicate the diversity of the panel of antibodies cloned. The heavy chains fell into a number of groups with evidence of somatic mutation within a given group. The light chains showed even greater diversity. Chain promiscuity was observed in the sense that very similar or identical heavy chains were paired with different light chains. The sequences are discussed in detail below.

To estimate the affinities of the Fab fragments for gp120, inhibition ELISAs using soluble gp120 were performed (Fig. 8). The examination of 15 clones showed that most inhibition constants were less than $10^{-6} M$ implying monomer Fab-gp120 apparent binding constants of the order of or greater than $10^8 M^{-1}$. These values have now been confirmed by surface plasmon resonance using the Pharmacia BiaCore machine (Table I). Generally we find a good correlation between apparent binding constants deduced from inhibition ELISA data and binding constants determined by surface plasmon resonance.

The phage surface library was also panned against gp160 (LA1), gp120 (SF2), and a constrained peptide having the central part of the

![Graph](image_url)

**FIG. 8.** Relative affinities of Fab fragments for gp120 (LA1) as illustrated by inhibition ELISA. Fabs 12, 27, 6, 29, 3, and 3 are all prototype members of groups of CD4 binding site Fabs discussed in the text. Loop 2 is a Fab fragment selected from the same library as the other Fabs but which recognizes the V3 loop. In this case competition was carried out with gp120 from the SF2 strain.
TABLE I
Binding Constants for Recombinant Fabs to gp120 Determined by BiaCore

<table>
<thead>
<tr>
<th>Fab</th>
<th>$k_{on} (M^{-1} \text{sec}^{-1})$</th>
<th>$k_{off} (\text{sec}^{-1})$</th>
<th>$K_d (M^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>b3</td>
<td>$9.6 \times 10^3$</td>
<td>$1.8 \times 10^4$</td>
<td>$5.1 \times 10^7$</td>
</tr>
<tr>
<td>b6</td>
<td>$1.6 \times 10^4$</td>
<td>$1.6 \times 10^4$</td>
<td>$9.7 \times 10^7$</td>
</tr>
<tr>
<td>b12</td>
<td>$4.5 \times 10^4$</td>
<td>$4.3 \times 10^4$</td>
<td>$1.1 \times 10^8$</td>
</tr>
<tr>
<td>b13</td>
<td>$1.1 \times 10^4$</td>
<td>$1.4 \times 10^4$</td>
<td>$7.9 \times 10^7$</td>
</tr>
</tbody>
</table>

Note. Kinetic constants and calculated affinity constants for the binding of selected Fabs to LAI gp120 measured by BiaCore (from Roben et al., 1994).

MN V3 loop sequence. Fabs isolated by panning against gp160, and showing a strong ELISA signal with gp160, also cross-reacted strongly with gp120. The sequences of the Fabs obtained by panning against gp160 (LAI) or gp120 (SF2) were mostly closely related to those described above from panning against gp120 (LAI). Indeed regardless of the panning antigen (gp120 (LAI), gp120 (SF2), or gp160 (LAI)), the Fabs selected reacted with both gp120(LAI) and gp120 (SF2). Several Fabs were obtained by panning against the constrained peptide but only one also reacted with gp120. In fact this Fab reacted with gp120 (SF2) but only weakly with gp120 (LAI).

The ability of Fabs and soluble CD4 to compete for gp120 was investigated in competition ELISAs. All of the Fabs obtained by panning against gp120/160 were found to be competed by CD4 for binding to gp120. The Fab specific for the V3 loop was not completed. Therefore the predominant Fabs isolated from this donor by selection against gp120 (LAI) appear to be strain cross-reactive and CD4 inhibited. This is consistent with the observation that more than 50% of the reactivity of the donor serum with gp120 (LAI) is inhibitable by CD4.

Further, the Fabs appear to be directed to major epitopes on gp120, in that a cocktail of three of the Fabs was able to inhibit >50% of the serum reactivity with gp120 (LAI) of more than 90% of a selection of seropositive donors (R. Burioni, private communication).

A major question in relation to Fabs isolated from libraries is their functionality. To this end, a panel of 20 recombinant Fab fragments reacting with the surface glycoprotein gp120 of HIV-1 was examined
E 1
RECOMBINANT FABS TO D BY BIA CORE

<table>
<thead>
<tr>
<th>t</th>
<th>k_{eff} (sec^{-1})</th>
<th>K_{D} (M^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8 \times 10^{-4}</td>
<td>5.1 \times 10^{7}</td>
</tr>
<tr>
<td>1.6 \times 10^{-4}</td>
<td>9.7 \times 10^{7}</td>
<td></td>
</tr>
<tr>
<td>4.3 \times 10^{-4}</td>
<td>1.1 \times 10^{8}</td>
<td></td>
</tr>
<tr>
<td>1.4 \times 10^{-4}</td>
<td>7.9 \times 10^{7}</td>
<td></td>
</tr>
</tbody>
</table>

calculated affinity constants s to LAI gp120 measured by 64.

for their ability to neutralize MN and LAI strain of the virus (Barbas et al., 1992b). Neutralization was determined as the ability of Fab supernates to inhibit infection as measured in both p24 ELISA and syncytia assays. One group of closely sequence-related Fabs was found to neutralize virus in both assays (Table II, compare Figs. 15 and 16). The corresponding purified Fabs neutralized virus with a titer (50% neutralization) at approximately 1 μg/ml. The prototype of this group, designated Fab b12, has now been shown in a number of different laboratories to be, as the Fab fragment, a more potent neutralizer than most CD4 site whole antibodies.

The spectrum of neutralizing ability of a set of Fabs to the CD4 binding site of gp120 (Fig. 9) provides an opportunity to explore the

<p>| TABLE II |
| Neutralization of HIV-1 by Recombinant Fab Supernatants |</p>
<table>
<thead>
<tr>
<th>Clone</th>
<th>Fab concn. (μg/ml)</th>
<th>gp120 ELISA titer</th>
<th>p24 assay</th>
<th>Syncytia assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MN</td>
<td>IIIB</td>
</tr>
<tr>
<td>1</td>
<td>1.8</td>
<td>1.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
<td>1.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>1.32</td>
<td>—</td>
<td>—</td>
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<tr>
<td>4</td>
<td>25.0</td>
<td>1.16</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>2.4</td>
<td>1.128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
<td>1.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>9.6</td>
<td>1.256</td>
<td>20</td>
<td>20</td>
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<tr>
<td>8</td>
<td>14.0</td>
<td>1.256</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>11.0</td>
<td>1.128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>1.64</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>8.1</td>
<td>1.128</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>9.9</td>
<td>1.128</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>6.9</td>
<td>1.256</td>
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<td>—</td>
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<td>8.5</td>
<td>1.32</td>
<td>20</td>
<td>20</td>
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<tr>
<td>15</td>
<td>8.6</td>
<td>1.64</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>0.7</td>
<td>1.32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17</td>
<td>10.0</td>
<td>1.64</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>18</td>
<td>16.0</td>
<td>1.1024</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>19</td>
<td>9.3</td>
<td>1.128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>20</td>
<td>8.9</td>
<td>1.64</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: From Barbas et al., 1992b. In this experiment aliquots of the same supernate preparations were used in p24 and syncytia assays. Figures indicate neutralization titers. For the p24 assay the titer corresponds to the greatest dilution producing >90% reduction in absorbance in ELISA. For the syncytia assay, Fabs 4 and 12 produced >95% neutralization at a 1:4 dilution of supernate and 80 and 70% reduction at 1:128 dilution, respectively.—indicates no neutralization at 1:20 dilution in the p24 assay and 1:16 in the syncytial assay (with most clones showing no detectable neutralization at a 1:4 dilution). The plaque assay data were kindly provided by Michael Hendry.
Fig. 9. Neutralization of HIV-1 by a panel of recombinant Fabs using an envelope glycoprotein complementation assay (from Roben et al., 1994). The ability of recombinant Fabs to neutralize the HXBc2 molecular clone of the HTLV-III (LAI) isolate was assessed in an envelope complementation assay (Helseth et al., 1991). Briefly, COS-1 cells were cotransfected with a plasmid expressing envelope glycoproteins and a plasmid containing an eno-defective HIV-1 virus encoding the bacterial chloramphenicol acetyltransferase (CAT) gene. Equal fractions of the cell supernates containing recombinant virions were incubated at 37°C for 1 hr with varying concentrations of Fab prior to incubation with Jurkat cells. Three days postinfection, Jurkat cells were lysed and CAT activity measured.

mechanism(s) of virus neutralization. Clearly neutralization does not require either cross-linking of virion particles or cross-linking of gp120 molecules on the surface of the virion since the Fab is monovalent. Neutralization is not correlated either with Fab affinity for recombinant gp120 or with the ability of Fabs and soluble CD4 to compete for binding to recombinant gp120. However, there is evidence from radioimmunoprecipitation studies that the most potent Fab recognizes a native presentation of gp120 on the surface of infected cells more effectively than less potent Fabs (Roben et al., 1994). This finding may have important consequences for vaccine design.

Fab b12 has been linked to Fc to generate a whole IgG1 molecule using methods outlined above. IgG b12 is an outstanding neutralizer, clearly 2–3 orders more effective than other CD4 site antibodies sub-
mited to a recent NIH workshop (D'Souza et al., 1994). Furthermore the antibody has been shown to be capable of neutralizing a range of primary isolates of HIV-1 (D. D. Ho, unpublished observations; R. M. Hendry, unpublished observations).

Neutralizing antibodies to other epitopes on the HIV-1 envelope have been generated. These are generally broadly strain cross-reactive because of the design of the selection procedure. Antibodies to the tip of the V3 loop have been derived by panning libraries against a constrained peptide having a consensus sequence (GPGRAF) corresponding to this region. Neutralizing antibodies to an epitope dependent on the V2 loop of gp120 have been derived by a novel epitope masking procedure (Ditzel et al., manuscript submitted). Antibodies to the CD4bs predominate in sera from HIV-1-infected humans (Moore and Ho, 1993) and antibodies to the V2 loop are relatively rare (Moore et al., 1993). Therefore, panning was carried out after the CD4bs epitope(s) was masked with two CD4bs-directed Fabs. Three novel Fabs were derived, which did not compete with the CD4 bs antibodies and were sensitive to mutations in the V2 loop. This demonstrates the feasibility of selecting minor specificities from libraries by masking major specificities before panning. Neutralizing antibodies to gp41 have been derived by panning a library prepared from a gp160-immunized donor with a peptide corresponding to a known neutralizing epitope (M. A. A. Persson, personal communication).

2. Antibodies to Respiratory Syncytial Virus

RSV is the major pediatric viral respiratory tract pathogen outranking all others as a cause of pneumonia and bronchiolitis in infants under 1 year of age (reviewed in McIntosh and Chanock, 1990). It has been concluded that RSV infection in bone marrow transplant patients is a serious and life threatening infection with a high mortality rate once pneumonia develops (Harrington et al., 1992). Several lines of evidence indicate that antibodies mediate resistance to RSV infection and illness including clinical studies of pooled human IgG containing a high titer of RSV neutralizing antibodies which provided indications that these antibodies can exert a therapeutic effect in serious RSV infection in infants and young children (Hemming et al., 1987) and a prophylactic effect in high-risk infants and children (Groothuis et al., 1993). Given this evidence, there is considerable interest in having available human monoclonal neutralizing antibodies to RSV for prophylaxis in protecting infants at high risk of serious disease and for therapy in cases of serious RSV lower respiratory tract disease.

Since ELISA analysis of the serum of the donor used above also
indicated a high titer to RSV surface FC glycoprotein, it was decided to pan the same library against FG. Two classes of Fab fragments interacting with the F glycoprotein were identified (Barbas et al., 1992c). The predominant Fab neutralized the virus with a titer of approximately 1 μg/ml. The less abundant Fab (about 5% of the clones) neutralized both A2 and B strains of the virus highly effectively with a titer around 0.1 μg/ml. Furthermore, this Fab neutralized 19 field isolates of virus of various geographical and temporal origins with titers of approximately 0.1–1.0 μg/ml (Table III). Most recently, we have demonstrated a marked therapeutic efficacy for this Fab in vivo (Crowe et al., 1994). A single dose of 12.9 μg of Fab (RSV 19) administered to mice at the height of RSV infection is able to reduce the viral titer in the lungs of the mice by a factor of 5000 (Table IV). A nonneutralizing anti-F Fab (RSV 126) was ineffective. The therapeutic effect of RSV19 is not fully sustained and there is some rebound in pulmonary virus titer a day after Fab administration. However, the rebound could be prevented by two further doses of RSV19 (Fig. 10). The results are very promising for the development of an aerosol of specific recombinant Fabs for the treatment of RSV disease and possibly for virus-mediated respiratory disease generally.

<table>
<thead>
<tr>
<th>Antigenic subgroup</th>
<th>RSV Isolates Tested</th>
<th>Temporal distribution</th>
<th>Specific neutralizing activity of Fab RSV 19 (conc. of Fab(μg/ml) needed for 60% plaque reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>1959–1984</td>
<td>0.3, 0.3, 0.4, 0.7, 1.0, 1.1, 1.2, 1.2, 1.7, 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>1962–1990</td>
<td>&lt;0.2, 0.3, 0.4, 0.4, 0.4, 0.4, 0.4, 0.4, 0.5, 0.6, 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note. Neutralizing activity of RSV Fab 19 against diverse RSV isolates belonging to antigenic subgroup A or B. A purified preparation of Fab was used (from Barbas et al., 1992c).

<sup>a</sup> Washington/Bern/65, St. Louis/10865/84, Australia/AZ/81, St. Louis/863/84, Washington/343/67, Australia/A1/81, Washington/1657/80, St. Louis/10849/84, Washington/3199/86, Sweden/663/89, respectively.

<sup>b</sup> West Virginia (WV)/1/617/85, WV/17154/85, WV/4943/81, WV/20223/87, WV/401R/90, Washington/1857/62, WV/474r/90, WV/985R/90, WV/1293/79, respectively. (West Virginia strains kindly provided by Maurice A. Mufson, MD.)
'G' glycoprotein, it was decided that two classes of Fab fragments were identified (Barbas et al., 1994). The virus was identified with a titr of Fab (about 5% of the clones) and the virus highly effectively with Fab. This Fab neutralized 19 field trials and temporal origins with Fab (Table III). Most recently, we have shown that for this Fab in vivo 2.9 μg of Fab (RSV 19) administration is able to reduce the virus factor of 5000 (Table IV). A was ineffective. The therapeutic efficacy and there is some rebound in administration. However, the further doses of RSV19 (Fig. 10) development of an aerosol of RSV disease and poisons generally.

**TABLE IV**

<table>
<thead>
<tr>
<th>Fab</th>
<th>Dose (μg)</th>
<th>Virus titer (log_{10} pfu/g tissue) (six animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV19</td>
<td>13</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>RSV126</td>
<td>14</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>HIV D121</td>
<td>15</td>
<td>5.9 ± 0.05</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>8.1 ± 0.1</td>
</tr>
</tbody>
</table>

*Note: From Crowe et al. (1994).*

**FIG. 10.** Rebound of virus replication following administration of RSV Fab 19 on Day 4 postinfection is reduced by additional daily instillation of the Fab (from Crowe et al., 1994). BALB/c mice were infected intranasally with 10^6 pfu of wild-type RSV on day 0, then were treated intranasally with 50 μg RSV Fab 19 on either Day 4 alone (●), Days 4, 5, and 6 (○), or were not treated (●). The titers of virus in the lungs of RSV-infected mice treated on days 4, 5, and 6 with a control Fab (HIV-12) did not differ from that of untreated mice (data not shown). Four animals were sacrificed at each time indicated in the figure and lung tissue was obtained for determination of virus titer on the indicated day.
3. Antibodies to Hepatitis B Virus

Hepatitis B affects more than 200 million people worldwide (Hollinger, 1990). A dramatic example of the protective effect of antiviral antibodies is described above although any therapeutic effect in humans remains largely unexplored. In a library study (Zebedee et al., 1992), two individuals were vaccinated with recombinant hepatitis B surface antigen and PBLs extracted 7–14 days later (although 4–5 days is probably optimal). Libraries (IgG1κ and IgG1λ) were constructed on the surface of phage and panned against the hepatitis B surface antigen. Specific Fabs were identified and in this case the binding of phage expressing specific Fabs to virus antigen particles could be clearly visualized in the electron microscope (Fig. 3a). Sequencing of positive clones showed a limited diversity with a remarkable example of promiscuity in that a given heavy chain was shown to bind antigen with either a κ or λ light chain. Further, the light-chain partner appeared to affect specificity as measured by the ability of excess Fab to compete with mouse monoclonal antibodies for virus antigen.

4. Antibodies to Human Cytomegalovirus (HCMV)

CMV disease may affect virtually every organ system and is frequently the causative agent in retinitis and gastroenteritis in AIDS patients. Essentially all HIV-1-positive patients are at risk from both reactivation of endogenous infection as well as reinfection with a second strain. Similarly, immunosuppressed patients who have not been infected previously with CMV are at extremely high risk of developing severe life threatening disease when they receive an organ transplant from an infected donor. Studies by Snydman et al., (1987, 1990) show that administration of human IgG with high anti-CMV titer prevents approximately one-half of these serious CMV illnesses, but does not reduce the incidence of infection. As CMV is thought to be strongly cell associated during infection, control of infection is usually ascribed to class I MHC-restricted CD8+ CTLs. As a consequence, prevention of disease in the high-risk group by IgG is of some interest because it suggests that antibodies can also suppress infection by their action on CMV-infected cells.

Two major complement-independent neutralization antigenic targets have been identified on the viral envelope of CMV, i.e., glycoproteins gB and gH. gH, and more particularly gB in some individuals, has been determined to be the most immunodominant in studies with convalescent human sera (Marshall et al., 1992; Britt et al., 1990). These molecules have been implicated in viral attachment to host cells and in membrane fusion and penetration.
number of groups have described gB- and gH-specific mouse and human monoclonal antibodies capable of neutralizing virus (Utz et al., 1989; Urban et al., 1992; Meyer et al., 1990; Ohizumi et al., 1992; Aulitzky et al., 1991; Ohlin et al., 1993).

We have constructed two libraries from HIV-1-seropositive individuals with significant antibody titers against HCMV and panned them against lysates of HCMV-infected cells (Williamson et al., 1993). Of 25 clones examined from a single library, 6 different heavy- and light-chain sequence pairings were observed (Williamson et al., unpublished observations). These clones recognized infected cells and were variously studied in immunoprecipitation and Western blotting assays. One clone immunoprecipitated what we believe to be a novel banding pattern. The remaining clones were found to recognize either the 65-kDa lower matrix phosphoprotein or the 52-kDa nuclear DNA binding phosphoprotein. As both of these antigens have been shown to be strongly immunogenic in humans and are present in large quantities in viral lysates, it is not surprising that antibodies with these specificities should be recovered. Indeed these antibodies were able to strongly compete with donor serum for binding to viral lysate, confirming their high serum levels in this individual and the ability of library technology to reflect the donor's antibody response.

5. Antibodies to Varicella Zoster Virus

VZV is recognized as one of the most frequent viral opportunistic infections in HIV-infected individuals, classically presenting as reactivated multidermatomal zoster, with or without dissemination, although a recently recognized chronically progressive syndrome is increasingly being identified. Immune prophylaxis with large doses of human globulin is currently used in the clinic for the prevention of VZV infection in immunocompromised patients. Specifically, the administration of zoster immune globulin within 4 days after exposure to the virus will prevent or reduce the severity of subsequent disease (Zaia et al., 1983; Balfour, 1988). Moreover, Larkin et al. (1985) determined that disseminated herpes zoster is associated with significantly low levels of antibody to VZV glycoproteins, implicating antibodies recognizing particular viral determinants in the containment of virus postinfection.

The envelope glycoproteins I through IV have been shown to be powerful immunogens and have been shown to elicit antibodies capable of neutralizing virus in vitro (Forghani et al., 1984; Grose et al., 1983; Keller et al., 1984; Vafai et al., 1984).

Libraries from two HIV-1-positive individuals with significant anti-
body titers against VZV have been independently panned against VZV-infected cell lysate (Williamson et al., 1993). Amino acid sequences of the clones revealed that panning had isolated only four different sequences from the two libraries. None of these clones demonstrated any significant neutralization of virus in inhibition of plaque development assays.

6. Antibodies to Herpes Simplex Viruses Types 1 and 2

Herpes simplex virus (HSV) remains one of the most common viral maladies in man, achieving a worldwide distribution and causing a variety of infections (reviewed in Whitley, 1990). Two forms of the virus, HSV-1 and HSV-2, have been distinguished by clinical manifestations and biochemical and serological criteria. HSV-2 is more commonly implicated in genital infection, while HSV-1 is associated with oral and ocular disease. Both types of the virus may become latent following travel intraaxonally to sensory ganglia.

Primary and secondary herpes infections in those immunocompromised by underlying disease or immunosuppressive drugs are often more severe than in the healthy host. Such individuals, which include AIDS patients, those with hematological or lymphoreticular neoplasms, and organ and bone marrow transplant recipients, are also prone to increased frequency of secondary herpes episodes. In the transplant recipients, the severity of herpes infection correlates with the degree of immunosuppressive therapy employed.

Devastating illness may also result from HSV infection of the neonate. In the United States such infections are encountered in 1 in 2500 to 1 in 5000 deliveries per year, most being acquired following intrapartum contact with infected genital secretions. As compared to a recurrent episode, primary infection occurring late in pregnancy generally produces more frequent and more severe disease in the newborn. This correlates with a greater maternal viral load at delivery (Corey et al., 1983), probably arising because the immune response to the virus is only in its early stages.

Current therapy of herpes infections is limited, although acyclovir, vidarabine, and related drugs have proven useful for the management of specific infections such as mucocutaneous herpes infections in the immunocompromised host, herpes simplex encephalitis, and neonatal herpes. Recurrent episodes, however, are less responsive. Moreover, viral strains resistant to these drugs have been isolated from immunocompromised patients.

There is evidence of a significant protective role for antibody in human infection in vivo. The presence of neutralizing antibody in
Acute phase serum during primary infections has been associated with reduced severity and duration of the primary genital herpes episode (Corey, 1982). Further, it has been shown that the development of recurrent genital herpes in an individual following a primary infection with homologous virus is inversely correlated with the titer of HSV-2 neutralizing antibody in the convalescent serum (Reeves et al., 1981). Moreover, the titer of anti-HSV antibodies in bone marrow transplant recipients is predictive of the risk of infection (Pass et al., 1979). In vitro, human serum antibody has additionally been shown to neutralize extracellular virus and lyse certain HSV-infected cells.

HSV is a complex virus: over 50 virus-encoded polypeptides including both structural (envelope and core) and regulatory proteins have been identified in infected cells. Analysis of the humoral response against HSV and the identification and characterization of potentially protective antigens have been undertaken largely using human sera and mouse monoclonal antibodies. The main targets of the humoral and cellular responses appear to be the seven well-characterized HSV envelope glycoproteins, gB, gC, gD, gE, gG, gH, and gI, which are found both on the virion and on the infected cell surface where they are thought to act to promote viral attachment and penetration through multiple interactions between themselves and the cell membrane. Only gB, gD, and gH have been found to be indispensable for viral growth in cell culture. Virus mutants defective in these molecules will bind to the host cell surface but are unable to penetrate into the cytoplasm. Glycoproteins mandatory for virus attachment have not yet been identified.

Each envelope glycoprotein is capable of eliciting mouse monoclonal antibodies able to neutralize virus in vitro. Passive immunization with monoclonal antibodies specific for gB, gC, gD, gE, and gH has also been shown to protect animals from infection (Kumel et al., 1985; Simmons and Nash, 1985; Balachandran et al., 1982; Dix et al., 1981). In addition, polyclonal immune sera and gD-specific monoclonal antibodies have been shown to protect mice from recurrent disease (Simmons and Nash, 1985). It has also been demonstrated that monoclonal antibodies against gB and gE suppress the replication of HSV-1 in trigeminal ganglia (Oakes and Lausch, 1984). Glycoproteins B and D appear to elicit a major part of the antibody response to virus in humans and also appear to be the major targets of neutralizing antibodies (Kuhn et al., 1987).

A combinatorial library constructed from an HIV-1-seropositive individual with serum antibody titers against HSV-1 and -2 was independently probed using lysates of cells infected with each of these viruses.
(Burioni et al., 1994). The antibodies were sequenced and characterized by their ability to react in ELISA with the antigen against which they were panned and in immunofluorescence studies with infected cells. A diverse panel of Fab clones was derived from the two pannings, with 9 of 18 heavy-chain sequences obtained from the HSV-2 panning being largely unrelated. Five of eight heavy chains taken from the HSV-1 panning were different from each other. However, although each lysate selected two diverse and largely distinct (only one heavy-chain sequence was common to both pannings) sets of clones, all of the antibodies examined were found to bind to both HSV-1 and -2. The generation of these different yet cross-reactive sets of clones using two similar virions probably arises from differences in affinity for the two virions, reflecting the sensitivity of the panning procedure to antibody affinity.

Of 17 different clones tested for their ability to neutralize HSV-1 and -2 in plaque reduction and inhibition of plaque development assays only one was able to significantly interfere with virus infectivity when tested as a crude bacterial supernatant. Accordingly this antibody, HSV Fab8, has been thoroughly characterized. We have determined the antibody binds specifically to the envelope glycoprotein D and is able to neutralize HSV-2 (50% plaque reduction at 0.05 μg/ml and 80% inhibition at 0.1 μg/ml) somewhat more efficiently than HSV-1 (50% inhibition at 0.25 μg/ml and 80% inhibition at 0.6 μg/ml) (Fig. 11). Figures comparable to these have been obtained in the neutralization of several clinical isolates of both HSV-1 and -2.

HSV Fab8 has further been shown to completely abolish HSV-2 plaque development in virus-infected monolayers at a concentration of 25 μg/ml, illustrating that Fab efficiently prevents cell-to-cell transmission of the virus (Fig. 12). In addition this antibody strongly reduced infectivity after attachment of the virus suggesting its inhibitory action takes place either at the level of membrane fusion or during virus penetration or uncoating and not by blocking attachment of virus to the target cell. Consistent with this supposition, we have demonstrated that neutralization obeys single-hit kinetics.

The data indicate that Fab8 is approximately 1 order of magnitude more potent than most murine whole antibodies described so far, and further underlines the potential of Fab in the containment of virus infectivity.

It is, however, significant that all but two of the antibodies isolated above by probing of libraries using HSV-1 and -2, HCMV and VZV viral lysates were found to be nonneutralizing. In using whole viral lysate as a selecting agent it may be expected that the breadth of the humoral response to the pathogen will be accessed, of which high-
were sequenced and characterized with the antigen against which resonance studies with infected derived from the two pannings, aimed from the HSV-2 panning for heavy chains taken from the each other. However, although generally distinct (only one heavy chain of clones, all of to bind to both HSV-1 and -2, non-reactive sets of clones using in differences in affinity for the the panning procedure to anti-
eir ability to neutralize HSV-1 bition of plaque development y interfere with virus infectivity property. Accordingly this anti-
characterized. We have deter-
the envelope glycoprotein D plaque reduction at 0.05 μg/ml what more efficiently than HSV-
1 80% inhibition at 0.6 μg/ml) have been obtained in the tests of both HSV-1 and -2.

To completely abolish HSV-2 D monolayers at a concentration ently prevents cell-to-cell transmission this antibody strongly reduces virus suggesting its inhibitory of membrane fusion or during by blocking attachment of virus is supposition, we have demon-
le-hit kinetics.

Approximately 1 order of magnitude antibodies described so far, and Fab in the containment of virus put two of the antibodies isolated HSV-1 and -2, HCMV and VZV neutralizing. In using whole viral expected that the breadth of the will be assessed, of which high-

affinity neutralizing antibodies may typically be only a small part. In response to our experiences with these and other herpesviruses we have successfully developed a simple “antigen-capture” technique to overcome this problem by more accurately selecting Fab specifically binding to antigens known to be important targets for neutralizing antibodies, even when a recombinant form of the molecule is unavailable (Fig. 13). Here, antibody specific for nonneutralizing epitopes of a chosen antigen is attached to a solid surface and used to “capture” (typically from a viral lysate) that antigen. Unbound material is then washed away and the panning procedure is performed over the captured antigen. This method has enabled us to more accurately direct the panning procedure toward isolation of antibodies against a number of virus-neutralizing epitopes, including glycoproteins D and B of HSV (Sanna et al., unpublished data). In the case of gD, ELISA wells
Figure 1 illustrates the inhibition of HSV plaque development by HSV E4d (from the E4d+ or E4d- strains) at different concentrations. The graphs show the number of plaques and plaque size over time with and without the presence of the HSV E4d protein. The data indicates a dose-dependent inhibition of plaque formation. The figure includes images of plaque development at different concentrations and a graph with a dose-response curve.
Fig. 13. Directed antigen capture for library panning. In order to focus selection to a neutralizing epitope on a given protein, the protein is captured by a mouse antibody or recombinant Fab to a non-neutralizing epitope prior to panning. The method has the advantage that it does not require recombinant or purified antigen.

were independently coated with recombinant Fab8 and a non-neutralizing gD-specific mouse monoclonal antibody. HSV-2-infected cell lysate was then overlayed into the wells and the library panned. When gD was captured from infected cell lysate using the non-neutralizing murine antibody, all 10 clones examined were of the same sequence as Fab8. However, when antigen was captured by Fab8, the antibodies derived possessed a different sequence and did not neutralize the virus. This example clearly shows the ability to fine tune antibody selection against chosen regions of a given antigen by capturing and masking epitopes using existing antibodies.

7. Antibodies to Measles Virus

Currently there is a renewed interest in the characterization of measles virus immunobiology. There are two principal reasons for this.
The first is the incomplete success of some vaccination programs in industrialized countries. To a major extent, the problems in this situation are of a logistic nature. However, there is a need to understand vaccine failures and to interpret the effect of revaccinations (a two-dose scheme of immunization is used in some countries). The second reason is the problem of using live vaccines for vaccination at the age of 3–6 months when infants can be at risk of infection. There is a need to define the possibility of generating an attenuated variant of the virus that displays an improved capacity to replicate in the presence of a maternal immune response. These problems would clearly benefit from enhanced understanding of human antibody responses to virus and vaccine.

We have carried out a single study on a 22-year-old donor who was vaccinated against measles in his childhood (Bender et al., 1994). An IgG1κ bone marrow library was generated and panned against a measles antigen preparation. A single high-affinity Fab was recovered which was subsequently shown to be specific for measles virus nucleoprotein. Interestingly, two other Fabs were recovered which showed lower affinity for the measles antigen preparation and polyreactivity. The heavy chains of both of these Fabs belonged to the Vκ6 family which has been associated with polyreactivity.

B. Antibodies to Bacteria

The only reports of which we are aware describe the generation of human Fabs to tetanus toxoid (TT) from libraries using peripheral blood lymphocytes of recently boosted donors. In one study (Mullinax et al., 1990), the frequency of TT binding clones in a λ phage library was 1:500 and the apparent antigen binding affinities were in the range 10⁷–10⁹ M⁻¹. In our study (Persson et al., 1991), a λ phage library prepared from a donor with a serum titer of 1:14,000 seven days after a boost showed antigen-specific clones at a frequency of 1:5000. Apparent affinities for antigen were in the range of 10⁷–10⁹ M⁻¹ and sequence diversity was observed in that six of eight heavy chains from binders were clearly clonally unrelated.

C. Antibodies to Allergens

One report has described the PCR amplification of human IgE heavy chains (Walker et al., 1992). It was not found possible to obtain amplification from PBL mRNA directly. Rather it was found necessary to first enrich for IgE-positive B cells by a sorting procedure using an anti-IgE reagent. We are not aware of any specific IgE antibodies prepared by the library approach at this time.
D. Antibodies to Unconventional Antigens

The binding sites on antibodies for certain antigens occur with a single type of \( V_h \) gene segment with little conservation of other elements in the V regions. For one of these, staphylococcal protein A (SpA), binding clearly occurs at a site distinct from the conventional antigen binding pocket since SpA binding is not inhibited by binding of the appropriate conventional antigen. Molecules of this type have been dubbed unconventional antigens by Silverman (1992, 1994). HIV-1 gp120 is another molecule which may fall into this category (Berberian et al., 1993).

The interaction of SpA and Fab has been explored using the library approach (Sasano et al., 1993). An IgGk library was constructed from the peripheral blood lymphocytes of a healthy donor with no history of staphylococcal infection. About 17% of antibody producing clones in the unselected library were found to bind SpA. SpA binding was completely restricted to Fab with \( V_{H3} \) heavy chains and about 60% of the \( V_{H3} \) Fab in the unselected library had SpA binding capacity. Analysis of 21 \( V_h \) and 6 \( V_l \) sequences showed that the SpA binders used diverse \( V_{H3} \) genes and L chains deriving from a variety of \( \kappa \) and \( \lambda \) gene families. Chain shuffling experiments showed that capacity to bind SpA was dictated by the heavy chain but different light chain usage could result in up to a fourfold change in affinity. The apparent affinities of the different Fabs for SpA varied from \( 4 \times 10^5 M^{-1} \) to \(<10^5 M^{-1} \). Repeated rounds of panning selected for antibodies of higher affinity as for conventional antigens.

The authors speculated that SpA could function as a B-cell superantigen. Since this and a flow cytometric study (Silverman et al., 1993) indicate that SpA can interact with most \( V_{H3} \) Fabs, in vivo exposure may result in a broad-based expansion of \( V_{H3} \) expressing B cells. However, because there is a hierarchy within the SpA binding affinities of the \( V_{H3} \) Fabs, in certain settings in vivo exposure might lend a clonal advantage to B cells that express antibodies with higher affinity for SpA. The authors suggested that the antibody repertoires expressed in chronic infections and lymphoproliferative and autoimmune diseases should be evaluated for the footprints of superantigen-directed B-cell clonal selection.

E. Rescue of Human Antibodies from Hybridomas and EBV-Transformed Cell Lines

EBV transformation and fusion have been successfully used to generate human cell lines secreting monoclonal antibodies against a number
of antigens (James and Bell, 1987). Such lines can be unstable or low secretors of antibody. Alternatively, the antibody may not be of the isotype desired. In any of these cases, it may be appropriate to rescue heavy- and light-chain genes from mRNA from the cell lines and express the Fab in bacteria. We have described this process for the cloning and expression of a human anti-rhesus D antibody from an EBV-transformed cell line using a λ phage system (Williamson et al., 1991). In this case five of five clones examined had the correct heavy and light chains. This may not be the case for all hybridomas which can contain mRNA from other chains which are PCR amplified and cloned. For instance, for one mouse hybridoma only 1 in 1000 recombinants had antigen binding activity (L. Sastry, private communication). Therefore, the most prudent general strategy may be to clone from the cell line into a phage display vector such as pComb3 and then pan against antigen to select positive clones.

More recently, plasmids derived from the λ phage vectors were used to rescue human Fabs to rabies virus (Cheung et al., 1992) and to HIV-1 gp120 and gp41 (Takeda et al., 1994) from hybridoma lines. The recombinant Fab to rabies was able to neutralize the virus about as effectively as the whole hybridoma-derived antibody. The anti-D cell line described above has also been rescued using the pComb3 phage display system (Siegel and Silberstein, 1994). In this case, it was shown that the anti-D specificity could be retrieved from a library comprising anti-D Fab expressing phage and anti-tetanus toxoid expressing phage at a ratio of 1:10⁴ by affinity selection using intact Rh(D)-positive red blood cells.

F. Rescue of Human Antibodies from HuSCID Mice

SCID mice populated with human cells offer the possibility of antigen boosting of human responses outside the human body. We have shown that SCID mice can be used in conjunction with the combinatorial library approach to give human monoclonal Fabs (Duchosal et al., 1992). PBLs taken from donors who had not been boosted with tetanus toxoid for very long periods (15–20 years) could be boosted in vitro, introduced into SCID mice, and reboosted, and strong secondary responses could be observed in the mice (Fig. 14). The spleen from such a mouse was used as a source of RNA from which libraries were constructed in λ phage and on M13 phages. Antigen-specific high-affinity human Fab fragments were readily derived from these libraries. For seropositive donors this sort of approach might be useful in stimulating antibodies essentially relegated to the memory compartment. One also has the advantage of being able to boost with smaller
rich lines can be unstable or low the antibody may not be of the it may be appropriate to rescue RNA from the cell lines and ex-described this process for the anti-rhesus D antibody from an phage system (Williamson et al., examined had the correct heavy case for all hybridomas which s which are PCR amplified and hybridoma only 1 in 1000 recombi-Sastry, private communication). l strategy may be to clone from vector such as pComb3 and then clones.

on the λ phage vectors were used (Cheung et al., 1992) and to HIV-94) from hybridoma lines. The to neutralize the virus about as derived antibody. The anti-D cell rescued using the pComb3 phage 1, 1994). In this case, it was shown trievied from a library comprising -tetanus toxoid expressing phage using intact Rh(D)-positive red

FROM HU-SCID MICE
cells offer the possibility of antiside the human body. We have in conjunction with the combinatorial monoclonal Fabs (Duchosal et al., ad not been boosted with tetanus years) could be boosted in vitro, oozed, and strong secondary re-noise (Fig. 14). The spleen from RNA from which libraries were 3 phages. Antigen-specific high-ready derived from these librar-t of approach might be useful in digated to the memory compart-being able to boost with smaller

Fig. 14. Generation of secondary antibody responses in hu-PBL-SCID mice. ELISA comparison of tetanus toxoid (TT) serum titer for a donor not boosted for 20 years (△) with that (+SEM) obtained by boosting the donor's PBLs in six Hu-PBL-SCID mice (○). The mean values for three similarly populated, but nonboosted mice are shown (◊).

units than the original immunogen so for instance a response to a particular peptide could be stimulated and human monoclonal Fab fragments rescued. In the future it may also become possible to create secondary responses from seronegative donors.

G. ANTIBODIES FROM PRIMATES

The constraints applied to immunization of primates are less than those applied to humans. However, antibody sequences are very similar suggesting that immunized primates may be an attractive source for the library approach. Chimpanzees are especially close to humans; differences between the two species in the constant domains are of the order of the differences found between human allotypes (Ehrlich et al., 1990, 1992). We have indeed used PCR primers designed for amplification of human sequences to prepare IgG1κ display libraries from HIV-1-infected chimpanzees (M. Zeidel et al., unpublished). The libraries have been panned against gp120 to yield antigen-specific high-affinity chimp Fabs. There are greater sequence differences between macaques and humans necessitating some modification to the
human PCR primers (M. A. A. Persson et al., private communication). Libraries have been constructed from simian immunodeficiency virus (SIV)-infected macaques and high-affinity Fabs specific for envelope prepared (Samuelsson et al., 1994).

H. THE USE OF THE LIBRARY APPROACH TO STUDY ANTIBODY RESPONSES

A major issue in library-derived antibodies from immune donors is their relevance to the in vivo response. Two studies have attempted to directly evaluate the relation of cloned to natural response, both in the mouse. The results led the authors to different conclusions. In the first study (Caton and Koprowski, 1990), Fab fragments-specific for influenza virus hemagglutinin were isolated from a λ phage library prepared from an immunized donor mouse. Sequence analysis indicated that the V\textsubscript{H} regions were derived from members of an expanded hemagglutinin-specific B-cell clone, in conjunction with one of two V\textsubscript{L} regions. The most frequently identified V\textsubscript{H}/V\textsubscript{L} combination was very similar to a V\textsubscript{H}/V\textsubscript{L} combination that had been identified previously among hemagglutinin-specific hybridoma antibodies. The authors concluded that the antibodies isolated bore a close relationship to the immune status of the donor mouse. In the second study, Gherardi and Milstein (1992) compared antibodies to 2-phenyloxazolone (PhOx) obtained by hybridomas with those obtained from a single-chain Fv phage display library using the same pool of spleen cells. The library approach yielded binders arising from eight individual V\textsubscript{H} genes and seven V\textsubscript{L} genes. The V\textsubscript{H} and V\textsubscript{L} genes which dominate the response as assessed by the hybridoma approach were found in only two cases in the library approach and then not in combination. The authors concluded that the antibodies isolated from the library were a poor guide to the natural response.

One consideration here is that different approaches are sensitive to different parameters and so will report on a different facet of an antibody response. The library approach begins with mRNA and is therefore probably reflecting activated and differentiated B cells, i.e., plasma cell populations. In contrast, hybridomas are thought to reflect activated but not terminally differentiated B-cell populations and EBV transformation to reflect resting B-cell populations. Therefore even experiments carried out on the same human tissue using the library approach and, for example, EBV transformation will not reveal how faithfully the former is reporting on the natural response. A possible alternative to at least maintain H–L pairings is to carry out the PCR of the library approach in situ, "in cell-PCR," as described for a mixture
of two hybridomas (Embleton et al., 1992). In this study, the authors fixed and permeabilized cells from the two hybridomas, reverse transcribed mRNA to cDNA, and amplified by PCR. After cloning, it could be shown that the original hybridoma $V_H-V_L$ combinations had been maintained. At this time, we are not aware of an extension of this approach to a diverse mixture of B cells.

It is instructive in relation to the authenticity of cloned antibodies from libraries to consider some of the properties of these antibodies. In principle, heavy–light chain combinations responsible for binding an antigen could arise fortuitously during library construction, i.e., neither chain is involved in binding antigen in vivo but the (random) combinations bind antigen in vitro. The difficulties of obtaining high-affinity antibodies from nonimmune compared to immune libraries of comparable size indicate this is unlikely to be a common occurrence. Furthermore, where it has been examined (Barbas et al., 1993b; Williamson et al., 1993; Portolano et al., 1992; Hexham et al., 1994), a single or a small number of cloned antibodies have been able to inhibit antigen binding of a large proportion of serum antibodies from the donor. This implies that broad features of the in vivo response are reproduced in the cloned repertoires in these instances.

From detailed analyses of cloned Fabs from a single library, we concluded that the cloned heavy chains probably corresponded to those used in vivo, whereas the status of the light chain was unclear (Barbas et al., 1993b). Figures 15 and 16 show the $V_H$ and $V_L$ sequences of 33 Fabs derived from panning the library against recombinant gp120 from LAI or SF2 strains of HIV-1 or gp160 from LAI HIV-1. All of the Fabs were shown to be directed to the CD4 binding site of gp120 and most had apparent affinities of the order of $10^8$ M$^{-1}$. The analysis immediately established that similar or identical antibodies were derived from panning against the different presentations of gp120. The heavy chains could be organized into seven groups based on sequence similarities. For some of these groups, e.g., the b8 group, differences between members of the group were so small as to be attributable to PCR or reverse transcription artifacts, i.e., the clones could have arisen from a single B cell in vivo. For others, e.g., the b3 group, the differences were more compatible with the cloning of somatic variants arising in vivo. For one group, the b1 group, the CDR3 region was conserved against a background of considerable variation in the $V_H$ gene segment. It is unclear whether the pattern in this group has arisen from somatic mutation, convergent evolution, or multiple cross-overs (in vivo or in vitro during PCR). The light chains in the antigen-selected Fabs also contained some groups of related sequences. In
### Table 15

Amino acid sequences of variable heavy (VH) domains of Fe8s binding to gp120 from Barbas et al. (1993a, b) and LESC.

**Note:** The N-terminal sequence LESC arises from the VH domain and could be argued to be intradiscal variants within the group. They are placed in their own group because of differences at the V-D-J interface.
Identity with the first sequence in a group is indicated by dots. The N-terminal sequence LEOSG arises from the V1a and LEESG from the V2a, primers. The b1 and b29 sequences are very similar to the b3 group and could be derived from intradiscal variants within that group or may be responsible for differences at the V3-D1 interface.
many cases, the light chains in a group were each paired with heavy chains from the same group and defined predominant H–L combinations, e.g., the b4 group and most of the b8 group (Fig. 16). However, many of the light chains were less easily grouped together on the basis of sequence. If constrained into the groupings defined by the heavy-chain sequence similarities, evidence of heavy-chain promiscuity was clearly revealed. For instance the b3 group shows seven closely related heavy chains (Fig. 15) paired with seven very different light chains (Fig. 16). Such extensive promiscuity is suggestive of some preeminence of the heavy chain in determining antigen binding in these cases.

To further explore chain pairing, the heavy chain from a single clone (b12, a potent neutralizing Fab) was recombined with the entire original light-chain library and the resulting library (H12-Ln) panned against gp120 LA1. Similarly the L12-Hn library was constructed and panned. Figures 17 and 18 show sequences of resulting binders. The apparent affinities of these binders were roughly similar to those for the original Fab (b12 = H12 L12). Among the light chains selected using H12, three are very similar to the original light chain whereas the rest arise from several different V-J rearrangements. In contrast, the shuffled heavy chains probably all arise from the same V-D-J rearrangement although there are several differences in the V gene segments. Therefore the shuffling data suggest some preeminence of the heavy chain in antigen binding for b12 Fab.

Chain shuffling enables pairings to be evaluated in a competitive situation. Pairing has also been examined in a forced situation using a binary plasmid system (Collet et al., 1992). All 441 H–L combinations arising from 21 anti-gp120 Fabs (from Figs. 15 and 16) were examined for reactivity in ELISA with gp120. The results are shown in Table V and indicate extensive chain promiscuity. The degree to which a given heavy chain paired productively with any light chain to bind antigen varied from 43 to 100% for antibodies selected on gp120 LA1. It should be borne in mind that all of these antibodies bind to the CD4 binding site of gp120. For two clones selected on other antigens, gp120 SF2 (s8) and a V3 loop peptide (p35), heavy-chain promiscuity is notably absent. This topic was further probed by directed crosses of heavy and light chains originating from several of the anti-gp120 Fabs with an anti-TT Fab. The heavy chains from several of the anti-gp120 Fabs in combination with the light chain from the anti-TT Fab retained binding to gp120. The heavy chain from the anti-TT Fab retained toxoid binding in combination with several of the light chains from anti-gp120 Fabs. None of the light chains from the gp120 binders
p were each paired with heavy predominant H-L combinations b8 group (Fig. 16). However, groupings defined by the heavy-chain promiscuity was supportive shows seven closely related ven very different light chains is indicative of some preeminent antigen binding in these
the heavy chain from a single was recombined with the entire ulting library (H12-Ln) panned Hn library was constructed and ences of resulting binders. The ere roughly similar to those for mong the light chains selected be original light chain whereas 𝜂-Δ rearrangements. In contrast, l arise from the same V-D-J rel differences in the V gene seg suggest some preeminence of the .2 Fab.

be evaluated in a competitive ined in a forced situation using 992). All 441 H-L combinations Figs. 15 and 16) were examined. The results are shown in Table iscuit. The degree to which a ly with any light chain to bind antibodies selected on gp120 LAI. of these antibodies bind to the ones selected on other antigens, (p35), heavy-chain promiscuity her probed by directed crosses from several of the anti-gp120 chains from several of the anti-light chain from the anti-TT Fab avy chain from the anti-TT Fab n with several of the light chains at chains from the gp120 binders

Fig. 17. Amino acid sequences of Vκ domains from Fabbs binding to gp120 and generated by shuffling the heavy Fab from clone
BLT Mixture a library of heavy chains (L/Lg+HC, Pab).

**Fig. 1B.** Amino acid sequences of V H domains from Pabs binding to gp120 and generated by shifting the light chain from clones

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>gp120 Binding</th>
<th>Light Chain Shifting</th>
</tr>
</thead>
<tbody>
<tr>
<td>V H1</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H2</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H3</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H4</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H5</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H6</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H7</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>V H8</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Note: gp120 binding and light chain shifting are indicated by 'Y'.
|     | b11 | b6  | b4  | b12 | b7  | b21 | b3  | b13 | b22 | b26 | b8  | b18 | b27 | b35 | s4  | b1  | b14 | b24 | s8  | p35 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| b11 | +   | +   | w   | +   | +   | -   | +   | -   | +   | -   | -   | -   | -   | +   | -   | -   | -   | -   | -   | -   |
| b6  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | +   | -   | +   | -   | +   | -   | -   | -   | -   | -   |
| b4  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | +   | +   | -   | -   | -   | -   | -   | -   |
| b12 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b7  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| b21 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b3  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b13 | w   | w   | w   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| b22 | w   | w   | w   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| b26 | w   | w   | w   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| b8  | +   | +   | +   | +   | -   | w   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b18 | +   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b27 | +   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b8  | +   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| b35 | +   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| s4  | +   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| h1  | +   | +   | +   | +   | -   | +   | w   | -   | w   | -   | -   | -   | +   | +   | -   | +   | -   | +   | -   | +   |
| b14 | w   | +   | +   | w   | -   | -   | +   | +   | +   | -   | -   | -   | +   | +   | -   | +   | -   | +   | -   | +   |
| b24 | +   | +   | +   | +   | -   | -   | -   | -   | -   | -   | -   | -   | +   | +   | -   | +   | -   | +   | -   | +   |
| s8  | w   | +   | +   | +   | -   | w   | +   | w   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| p35 | +   | +   | +   | +   | -   | +   | w   | w   | w   | w   | w   | w   | w   | +   | w   | -   | -   | -   | -   | -   |

From Collet et al., 1992.

* Directed crosses between heavy and light chains of Fab fragments isolated from a single library by panning with glycoprotein gp120(b1-b27) or gp160(B8-B35) of HIV-1 strain IIIb, gp120 (s4-s8) of HIV-1 strain SF2, and the loop peptide (p35), assayed by ELISA against IIIb gp120. Heavy chains are listed horizontally and light chains are listed vertically. Clones are sorted according to the grouping established in Barbash et al. (1993b). Different groups are separated by horizontal and vertical lines. ELISA results: −, negative (a signal of three times background or less); +, positive (comparable to the original heavy- and light-chain combination); w, intermediate value; *, the HCP35/LCP35 combination was negative with IIIb gp120 but positive with SF2 gp120. Identical chains carry the same identifier (either *, |, $, or 2).
were able to confer gp120 specificity in combination with the anti-TT heavy chain. Similarly, the light chain from the anti-TT Fab was unable to generate TT specificity in combination with any of the heavy chains originating from the gp120 binders. These results again support heavy-chain dominance in antigen binding.

Heavy-chain promiscuity has now been described in several reports using combinatorial libraries (e.g., Caton and Koprowski, 1990; Persson et al., 1991; Clackson et al., 1991; Kang et al., 1991c). Remarkable examples of chain promiscuity include those where κ and λ light chains combine with the same heavy chain with maintenance of antigen binding (e.g., Zebedee et al., 1992; unpublished observations). The negative side of this promiscuity with respect to the ability of libraries to report on antibody responses is that the in vivo light chain can probably not generally be identified. The positive side is that this assumes rather less importance if the heavy chain is dominating antigen binding.

Light-chain promiscuity, i.e., the ability of the same light chain to pair with different heavy chains with retention of antigen binding, has also been described. We argue that, in most cases, this behavior is most appropriately viewed as light-chain plasticity, rather than light-chain dominance. The reason is that the evidence indicates that the heavy chains in these cases are dominating the binding specificity with the light chain acting as a pluripotent or plastic partner. For instance, a comparison of sequences from B-cell lines (Kabat and Wu, 1991) shows identical light chains pairing with different heavy chains in antibodies of widely varying specificities. This phenomenon is a relatively common occurrence in library studies. κ light chains deriving from two germline genes, kv325 and vk02/012, are particularly prominent. The kv325 light chain in a near germline configuration has been described in library-derived antibodies to thyroglobulin (Hexham et al., 1991, 1992), hepatitis B surface antigen (Zebedee et al., 1992), HIV-1 gp120 (Barbas et al., 1993b), and thyroid peroxidase (Hexham et al., 1994). A dramatic example of kv325 light-chain plasticity is provided by changing the specificity of an antibody from tetanus toxoid to fluorescein solely through changes in the heavy-chain CDR3 region (Barbas et al., 1992a).

We would similarly tend to invoke plasticity as the most likely cause of the involvement of a similar light chain with different heavy chains in binding the same antigen. For instance the light chains B20 and s6 in Fig. 16 are virtually identical. One interpretation would be that this light chain is dominating binding to gp120 and the two heavy chains are of lesser importance. However, when the B20 heavy chain is paired with six other distinct light chains, the retention of gp120 binding
combination with the anti-7T1 on the anti-7T1 Fab was unable to activate any of the heavy chains on the Fab results again support heavy chain observations in several reports by Koppowski, 1990; Pershan et al., 1991c). Remarkable was the ability of libraries to in vitro light chain can probably be derived from a number of studies on antibodies to thyroid peroxidase. This set of studies is taken up in more detail below. In one chain shuffling investigations (Portolano et al., 1993a), the authors found the same light-chain (vk02/12 derived) pairing to two different heavy chains with maintenance of antigen binding and suggested that the light chain may be defining epitope specificity. Our feeling is that a "plastic" light chain is a good tool to go fishing for heavy chains in the chain shuffling experiment.

It is worth emphasizing strongly that we do not suggest that heavy-chain dominance, heavy-chain promiscuity, and light-chain plasticity are attributes which will apply to every heavy–light chain combination. For instance, there will undoubtedly be cases where light chains dominate antigen binding. Further, plastic behavior strictly requires an identical light chain in different antibody specificities although "very similar" (typically 95–99% identity) light chains may merit this description with the caveat that single amino acid substitutions can have profound effects on binding affinity and specificity. We also emphasize that heavy-chain dominance does not imply that heavy chains alone could function as isolated binding units or that light chains in such cases make no contribution to binding.

Since libraries are random combinations of heavy and light chains, it can be argued that combinations using promiscuous heavy chains and/or plastic light chains will be recovered from libraries with greater frequency than their occurrence in natural responses. Similarly, antigen-binding sites dependent on very restricted heavy–light chain pairings may be underrepresented in antibodies obtained from the library approach. Shuffling experiments using heavy chains isolated by conventional means would be of interest here. For κ light chains, there is a growing body of evidence to indicate that certain light chains are overrepresented in the expressed repertoire. Using our PCR primers, we find a strong overrepresentation of κ chains with kv325 or vk02/12 as the closest germline gene in unselected libraries from peripheral blood lymphocytes of a healthy donor (G. R. Pilkington, unpublished observations) or from thyroid lymphocytes from a donor with autoimmune disease (Heath et al., 1994). This overrepresentation is reflected in the light chains from Fabs binding gp120 in Fig. 16.

A number of factors, apart from considerations of chain pairing, might also skew the cloned response relative to the natural response. These
include unequal PCR priming, the presence of restriction sites in certain antibody genes, or the preferential expression of certain antibodies on the phage surface or in E. coli. The more PCR primers are used the more complete the captured repertoire is likely to be. However, using a relatively small and manageable set of primers (Persson et al., 1991; Kang et al., 1991b; Williamson et al., 1991), we have obtained members of most of the heavy- and light-chain families and a wide diversity of genes. As regards restriction sites, one systematic loss is currently incurred in our system, for example: a family of \( \lambda \) light chains possesses a restriction site used in the cloning process in the constant domain exon. From known sequences we estimate we presently lose about 5% of the total repertoire because of internal cloning sites. The effects of antibody sequence on bacterial expression levels are great ranging over at least 2 orders of magnitude and so this is clearly a factor which could skew cloned repertoires.

IV. Antibodies to Self-Antigens from Human Donors

Combinatorial libraries have been applied to human thyroid disease, primary biliary cirrhosis, and Graves’ ophthalmopathy. They have also been applied to the autoimmune repertoire in HIV-1 infection. These cases are now considered in turn.

A. Thyroid Disease

Autoantibodies to thyroid peroxidase (TPO), to thyroglobulin (Tg), and to the thyroid stimulating hormone (TSH) receptor are important features of thyroid autoimmunity (for reviews see Furmaniak and Rees-Smith, 1990; McLachlan and Rapoport, 1992; Bigazzi, 1993; Kendler and Davies, 1993). Autoantibodies to TPO and Tg are associated with hypothyroidism while anti-TSH receptor antibodies generally cause hyperthyroidism.

A set of pioneering studies have used the library approach to probe the autoimmune repertoire to TPO in Graves’ disease. Prior to these studies, despite numerous attempts using EBV transformation and/or cell fusion, only one cell line secreting an IgG anti-TPO antibody had been produced and this human–mouse hybridoma was unstable (Portolano et al., 1991; Fukuma et al., 1990). The studies have used thyroid tissue from Graves’ patients as the source of RNA. Initially an IgG1\( \times \) Fab library was constructed in \( \lambda \) phage and this was screened to identify one (Portolano et al., 1991) and then three (Portolano et al., 1992) Fabs with high apparent affinities (around \( 10^{10} M^{-1} \)) for TPO. The heavy-chain sequences of the three Fabs were identical. Two of
The essence of restriction sites in initial expression of certain anti-
cols. The more PCR primers
ed repertoire is likely to be.
manageable set of primers (Per-
lliamson et al., 1991), we have
- and light-chain families and
striction sites, one systematic
m, for example: a family of λ
used in the cloning process in
 sequences we estimate we
 repertoire because of internal
uence on bacterial expression
 orders of magnitude and so this
ened repertoires.

from Human Donors
plied to human thyroid disease,
thallomopathy. They have also
oire in HIV-1 infection. These

(TPO), to thyroglobulin (Tg),
(TSH) receptor are important
views see Furmaniak and Bees-
t, 1992, Bigazzi, 1993; Kendler
PO and Tg are associated with
or antibodies generally cause

d the library approach to probe
Graves’ disease. Prior to these
ing EBV transformation and/or
ng an IgG anti-TPO antibody
ouse hybridoma was unstable
90). The studies have used
 source of RNA. Initially an
 phage and this was screened
) and then three (Portolano et
ities (around \(10^{10} M^{-1}\)) for TPO.
ee Fabs were identical. Two of
the light chains were very similar and a third was more distant. The
closest germline gene to all three light chains was vκ02/012, although
for the more distant clone homology was only 92%. These results
therefore again indicate heavy-chain promiscuity. Importantly, the
Fabs were shown capable of inhibiting a high proportion (36–72%) of
 serum TPO autoantibodies from 11 patients with autoimmune thyroid
disease, implying the epitope recognized is of major significance. This
finding also applied to patients whose response was predominantly
IgG4 as well as those for which IgG1 antibodies were prevalent. The
epitope recognized was shown to be conformational.

The heavy and light chains from one of the above Fabs were then
used in chain shuffling experiments (described as “roulette” by
the authors) (Portolano et al., 1993a). The fixed heavy chain found a set
of light chains with which antigen binding could be generated, all
of which were most closely (92–95%) related to the vκ02/012 gene.
However, there were many differences between some of the chains
in the Vκ gene and at the Vκ-J joining region and at least two Jκ regions
were used. The fixed light chain found five heavy chains closely related
to the original and one new heavy chain which clearly arose from a
different V-D-J rearrangement. The Fab with the new heavy chain
competed with the parent Fab (which has an identical light chain).
The authors therefore raised the possibility that the light chain was
dominating binding and defining epitope specificity. We prefer an
alternative explanation. The original heavy chain did pair with several
quite different light chains, albeit that these were most readily related
to the same germline gene, suggestive of heavy-chain dominance. The
competition of two Fabs with different but dominant heavy chains is
common (compare Fig. 15 where all of the Fabs except one are cross-
competitive). As above, we suggest light-chain plasticity or pluripo-
tency is likely to be a better explanation of the finding of the same
light chain in two antibodies than light-chain dominance.

In their next study, the investigators identified TPO binders in phage
λ libraries prepared from three further Graves' disease donors to yield
a panel of 30 cloned high-affinity IgG Fabs (Chazenbalk et al., 1993).
The heavy chains could be organized into four groups with many
closely sequence-related chains within a group as described above for
the cloned response to gp120. Here, however, closely related heavy
chains were derived from libraries from different donors. This is a
potentially very important finding. However, the possibility of cross-
library phage contamination should be borne in mind (we have found
this to be a considerable problem) although considerable precautions
were taken in library construction (S. M. McLachlan, private communi-
cation). There was a very strong bias among the binders toward light chains having vk02/012 as the closest germline gene. Some responsibility for this bias may lie in the fact that unselected libraries have a very high (85%) representation of this light chain (Jaume et al., 1994). It is unclear at this time whether this is due to a real skewing of the repertoire in the tissue source or to the PCR.

Next the investigators subcloned heavy and light chains, maintaining pairing, from one of the λ phage libraries to the phage display vector pComb3 (Portolano et al., 1993b). This library was panned against viable, stably transfected CHO cells expressing human TPO on their surface. Several high-affinity Fabs were derived. The heavy chains fell into four groups, two corresponding to rearrangements described previously using phage λ and two to apparently different rearrangements. Two rearrangements found in the λ phage system were not found using the phage display system. The light chains again showed a bias toward vk02/12 and a tendency to be closer to the germline than in Fabs derived from the λ phage library. Therefore the phage display system recapitulated some of the features of the λ phage system but there were important differences.

Very recently, directed crosses have been used to explore the promiscuity of heavy and light chains in binding TPO (Concante et al., 1994). Three heavy chains clearly originating from different germline genes were combined with one or two light chains clearly originating from germline genes different from that of the original partner. Five new combinations were produced. None of these bound TPO providing no evidence of promiscuity among the chains examined.

A second group of investigators have carried out a more limited set of studies using libraries on anti-Tg and anti-TPO antibodies isolated from donors with Hashimoto’s disease. Initially a high-affinity (apparent K_0 = 5 \times 10^9 \text{ M}^{-1}) antibody to Tg was isolated from an IgGκ λ phage library (Hexham et al., 1991, 1992). The light-chain V gene from this Fab was identical to the kv325 germline. Using the pComb3 phage display system, the investigators have reported three Fabs with high affinity (apparent K_0 = 10^9 \text{ M}^{-1}) for TPO (Hexham et al., 1994). The heavy chains were unique while two of the light chains appeared derived from the vk02/012 germline. In this case the unselected library had 18% vk02/012 and 36% kv325.

B. PRIMARY BILIARY CIRRHOSIS (PBC)

PBC is a chronic autoimmune disease of the liver of unknown etiology and is characterized by the presence of high-titer antimitochondrial antibodies, inflammation of the septal and interlobular bile ducts,
among the binders toward light chain gene. Some responibilities selected libraries have a very chain (Jaume et al., 1994). It due to a real skewing of the PCR.

and light chains, maintaining the phage display vector is library was panned against expressing human TPO on their re derived. The heavy chains to rearrangements described to apparently different rend in the λ phage system were system. The light chains against a tendency to be closer to the be λ phage library. Therefore exposed some of the features of the λ st differences.

been used to explore the promising TPO (Constance et al., 1994). from different germline genes chains clearly originating from the original partner. Five new if these bound TPO providing chains examined.

carried out a more limited set of anti-TPO antibodies isolated. Initially a high-affinity (apparar g was isolated from an IgGλ λ 2). The light-chain λ gene from phine. Using the pComb3 phage reported three Fabs with high PO (Hexham et al., 1994). The 3 of the light chains appeared in this case the unselected library

case of the liver of unknown etiology of high-titer antimitochond ral and interlobular bile ducts, followed by necrosis, and ultimately cirrhosis (reviewed in Rowley et al., 1993). In recent years, the mitochondrial autoantigens have been identified as components of the 2-oxo acid dehydrogenase enzymes including the E2 subunits of the pyruvate dehydrogenase complex (PDC), the branched-chain oxo acid dehydrogenase complex (BCKD), the 2-oxo acid glutarate dehydrogenase complex, and other protein components. Murine monoclonal antibodies to the mitochondrial autoantigen PDC-E2 have been isolated. Although these monoclonal antibodies were able to inhibit PDC-E2 enzymatic activity, they recognized a different region of the target autoantigen than did patient sera (Surh et al., 1990), suggesting that monoclonal antibodies of rodent origin differ in fine specificity from human autoantibodies.

mRNA from a regional lymph node of a patient with PBC has been used to construct a combinatorial immunoglobulin library in the λ phage vector system (Cha et al., 1993). Six human monoclonal IgG Fabs specific for the major autoantigen of PBC, PDC-E2, were isolated, appearing at a frequency of 0.01% in the combinatorial library. These Fabs recognize human PDC-E2 with high affinity ($K_d = 10^7$–$10^6$ M$^{-1}$). Using both immunoblotting and ELISA, the Fabs showed little cross-reactivity to any of the other autoantigens commonly recognized by PBC sera or to other antigens commonly recognized by PBC sera or to other antigens. The Fabs showed a typical antimitochondrial staining pattern in HEP-2 cells but reacted strongly with the luminal aspect of biliary epithelial cells of patients with PBC. It was argued that the Fabs have similar specificities to those found in human PBC sera and in human monoclonal antibodies prepared by conventional means.

C. Graves' Ophthalmopathy

Graves' ophthalmopathy is a common disfiguring autoimmune disorder of unknown etiology. Despite extensive investigations over many years, there is still no consensus on the target cell(s) or autoantigen(s) involved (Weetman, 1992; Wall et al., 1993). The inflammatory cells infiltrating Graves' orbital muscle and fat/connective tissue are predominantly T cells, but plasma cells are also present. Hypothesizing that antibodies generated within Graves' orbital tissue may include autoantibodies against orbital antigen(s), Jaume et al., 1994 constructed an IgGLκ library in λ phage from the orbital tissue of a patient with active Graves' ophthalmopathy. Sequencing of 15 unselected heavy and light chains revealed an extreme bias (14/15) toward the Vk02/012 germline among the light chains and some restriction in heavy chain gene usage. The authors raised the possibility that particular germline genes may be associated with autoimmunity in humans.
D. Autoantibodies in HIV-1 Infection

Although most interest concerning the pathogenesis of HIV-1 infection has been focused on T cells, abnormal B-cell function is also a feature of the infection. Individuals infected with HIV-1 have serum antibody titers to a wide range of self and exogenous antigens. The origin of the autoantibodies and their effects are unknown, although speculations as to a pathogenic role have been made. The autoantibodies in HIV-1 infection appear early postinfection and persist throughout the disease course. In general, HIV-positive patients and in particular patients with AIDS have elevated numbers of spontaneous immunoglobulin secreting B cells in peripheral blood giving rise to hypergammaglobulinemia.

Several hypotheses for the retroviral induction of autoantibodies have been suggested (reviewed in Amadori, 1993). One hypothesis is that the virus or viral proteins themselves, directly or indirectly, induce generalized polyclonal B-cell activation that leads to elevated levels of all antibodies, including those to self-antigens. Another hypothesis invokes molecular mimickry whereby a viral epitope elicits antibodies that cross-react with a self-antigen. Sequence similarities have been reported for MHC class II, particular HLA-DR and HLA-DQ, and gp120, HIV-1 Nef, and gp41, and between the major retroviral capsid protein (CA) and regions in the SM-BB' autoantigen, the SSB/La antigen, the 70-kDa ribonucleoprotein, topoisomerase I, and the acetylcholine receptor. Finally, an autoimmune model of AIDS pathogenesis that involves both an immune response to HIV and to allogeneic stimuli has been proposed. A synergistic effect of the two responses is postulated to cause collapse of the immune system.

To characterize the autoantibodies seen in association with HIV-1 infection, we decided to use several libraries to select a series of antibodies against a range of autoantigens (Ditzen et al., 1994).

The sera from eight HIV-1-seropositive male donors were first tested in ELISA for binding to the purified autoantigens: MHC class II, double-stranded DNA, CD14, EGF receptor, and the ganglioside GD2. As shown in Fig. 19, higher average serum IgG titers against all five antigens were found in the eight HIV-1-positive donors compared to eight healthy seronegative control male donors. Serum IgG responses to the autoantigens varied significantly between HIV-1 donors, and some had serum levels comparable to those of healthy controls. Bone marrow IgG combinatorial libraries were constructed from the eight HIV-seropositive donors and selected libraries panned against antigens from the set above in order to retrieve a panel of 38 monoclonal autoantibodies.
A pathogenesis of HIV-1 infection is also affected with HIV-1 have serum and exogenous antigens. The effects are unknown, although some have been made. The autoantibody infection and persist throughout positive patients and in particular numbers of spontaneous peripheral blood giving rise to the induction of autoantibodies (dori, 1993). One hypothesis is that directly or indirectly, induces that leads to elevated levels of antigens. Another hypothesis is that viral epitope elicits antibodies that have similarities been HLA-DR and HLA-DQ, and the major retroviral capsid autoantigen, the SSB/La antigen, and the acetylcholinesterase model of AIDS pathogenesis to HIV and to allogeneic stimulatory effect of the two responses is due to system.

In association with HIV-1 libraries to select a series of donors (Ditzel et al., 1994). Six male donors were first tested autoantibodies: MHC class II, receptor, and the ganglioside IgG titers against HLA-DR, HIV-1-negative donors control male donors. Serum IgG significantly between HIV-1 donors and those of healthy control libraries were constructed from and selected libraries panned order to retrieve a panel of 38

**Fig. 19.** Reactivity of sera (diluted 1:100) from eight asymptomatic HIV-1-seropositive male donors and eight healthy males against five selected autoantigens individually assessed by ELISA and then averaged as shown (from Ditzel et al., 1994). Several HIV-1-positive donors (e.g., donor L) had strong serum responses to all of the autoantigens tested compared to those of the healthy donors. Serum samples were taken concomitantly with bone marrow aspiration for library construction.

The specificities and affinities of the Fabs for a panel of autoantigens were determined using ELISA. As exemplified in Fig. 20, the selected Fabs showed moderate affinity for antigen and cross-reactivity with several other autoantigens as well as exogenous antigens. The pattern of cross-reactivity was individual to a given Fab. These findings contrasted with antibodies selected to infectious agents from the same libraries. In a number of cases, the serum titers to the exogenous antigens were very similar to those to self-antigens. For example, the serum from one donor had titers against double-stranded DNA and gp120 of 1:500 and 1:400, respectively. However, as depicted in Fig. 20B, two human anti-gp120 Fabs retrieved from this library were of high affinity and monospecific, in contrast to the autoantibodies selected by panning against DNA, which were polyspecific and of lower affinity. Similarly, there was a marked contrast between the antibodies derived from HIV-1-seropositive donors with those derived from donors with established autoimmune disease. From a library constructed from a systemic lupus erythematosus (SLE) patient (S. M. Barbas, E. M. Salonen, H. J. Ditzel, G. J. Silverman, and D. R. B., unpublished data, Fig. 20A), we have isolated high-affinity
Fig. 20. The polyspecific nature of autoantibodies isolated from an HIV-1-seropositive donor by the library approach (from Ditzen et al., 1994). (A) Comparison of monospecific anti-DNA antibodies from an SLE donor library (S1) and polyspecific anti-DNA antibodies from an HIV-1-seropositive donor library (donor L). The solid lines indicate the binding properties of a Fab [LNA3; donor L, antigen DNA (NA), clone 3] selected by panning the L library against human placental double-stranded DNA (dsDNA). LNA3 was assayed for binding to solid phase dsDNA (○), ovalbumin (●), human transferrin (●), BSA (●), human IgG Fc (△), and ganglioside GT1 (●). The dashed lines indicate the binding properties of two Fabs (ds3-40 and ds3-32) selected by...
anti-DNA antibodies using the library approach. These Fabs showed no cross-reactivity with the same panel of autoantigens.

The polyreactivity of the Fabs from the HIV-positive donors was confirmed in ELISA competitive inhibition assays in which the binding of a given Fab to a solid phase antigen was tested in the presence of both homologous and heterologous soluble antigens. The apparent affinities of these antibodies were of the order of $10^6-10^7 \text{ M}^{-1}$.

Clearly it is not possible to examine all autoantigens and therefore the study described could not unequivocally eliminate the presence of specific autoantibodies in HIV-1 infection. However no support for this hypothesis was found, particularly in cases where sequence similarities between HIV-1 proteins and self-antigens have been reported. Instead, the results suggest that an increased level of polyreactive antibodies is probably responsible for the elevated serum titers to autoantigens in the serum of HIV-1-seropositive donors.

A role for polyreactive antibodies in the pathogenesis of HIV-1 infection is unclear. However, the affinities of the monovalent Fab fragments described were of the order of $10^6-10^7 \text{ M}^{-1}$ for a range of antigens. As bivalent IgG molecules, the affinity for antigen (avidity) may well be an order of magnitude higher, which would be expected to permit in vivo antibody–antigen interactions. The polyreactivity of the combining site may introduce novel features to the modus operandi of the antibodies isolated from an HIV-1-infected donor library (SL) and polyclonal donor library (L). The solid line (LNA3; donor L, antigen DNA (NA), anti-28). The two Fabs (ds3-40 and ds3-32) selected by panning the SL library against dsDNA. The steep dashed curves show the concentration dependence of ds3-40 (△) and ds3-32 (○) binding to dsDNA. Also shown is the binding of ds3-32 to human IgG Fc (×), ovalbumin (+), and human transferrin (□). The binding of Fab ds3-40 to these latter antigens is essentially identical to that of Fab ds3-32. The figure illustrates the much higher affinity (half-maximal binding at $10^5 \text{ M}$ compared to $10^6 \text{ M}$) and lower cross-reactivity of the anti-DNA Fabs from the SL library compared to the Fab from the HIV-1-seropositive donor. (B) Comparison of mono- and polyclonal antibodies isolated from the same HIV-1 library (L). The solid lines indicate the binding properties of a Fab (LNA12) selected by panning the library against dsDNA. LNA12 was assayed for binding to the same panel of antigens as in A. The dashed lines indicate the binding properties of two Fabs (L21 and L41) selected by panning the library against gp120. The steep dashed curves show the concentration dependence of Fab L21 (△) and Fab L41 (○) binding to gp120. Also shown is the binding of Fab L21 to human dsDNA (×), ovalbumin (+), and human transferrin (□). The binding of Fab L41 to these latter antigens is essentially identical to that of Fab L21. The figure illustrates the much higher affinity (approximately 100-fold, half-maximal binding at $10^5 \text{ M}$ compared to $10^6 \text{ M}$) and lower cross-reactivity of the Fabs selected by panning against gp120 compared to the Fabs selected by panning against dsDNA. The IgG titers in the serum of the donor to gp120 and dsDNA were essentially equivalent (1:500) at the time of bone marrow aspiration for library construction. Comparison of Figs. 2A and 2B also shows that polyreactivity is more pronounced for Fab LNA3 and LNA12.
antibody molecule, e.g., self-association, cross-linking of unrelated antigen molecules, and focusing of diverse species to the surface of Fc receptor bearing cells. Given considerations such as these, the study concluded that the effects of high serum levels of antibodies capable of interacting with diverse molecular species were difficult to predict and warranted further investigation.

V. Antibodies without Immunization

A direct consequence of the ability to sort through vast libraries of antibodies is the isolation of antibodies without immunization (Lerner et al., 1992; Marks et al., 1992). Phage display allows for the construction and sorting of libraries whose size matches or exceeds those displayed by an animal at a given moment. Two such approaches have been described. The first takes advantage of the combinatorial strategy and the observation made with hybridomas that naive IgM antibodies can recognize a variety of antigens. Prior to the development of hybridoma technology, myelomas had been screened for their reactivity with small molecules. An extension of this approach is to search through natural libraries and to use phage display to select antibodies against a defined antigen. This strategy, termed the naive repertoire approach, has been demonstrated for both human (Marks et al., 1991) and murine (Gram et al., 1992) antibodies. The second strategy involves introducing synthetic diversity into antibody CDR regions (Barbas et al., 1992a; Hoogenboom and Winter, 1992) and takes advantage of our more sophisticated understanding of antigen–antibody interactions.

A. Naive Repertoire Approach

The term "naive" in this sense refers to a library of antibodies which has not been educated or biased by the immune system toward the recognition of any particular antigen or antigens. Preparation of naive libraries involves the use of RNA from nonimmune sources and amplification of μ or δ heavy chains which are the starting point in the natural response. Naive libraries have been constructed from both humans (Marks et al., 1991) and mice (Gram et al., 1992) and selection attempts have had some success for the production of low or medium affinity antibodies.

Marks et al. (1991) have reported the construction of naive scFv libraries. In this report FBLs from two donors served as the source of mRNA which was used to prepare cDNA using IgM, IgG1, κ, and λ oligonucleotides for first strand synthesis. This cDNA was then used
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**Immunization**

to sort through vast libraries of ; without immunization (Lerner display allows for the construct matches or exceeds those dist- ant. Two such approaches have age of the combinatorial strategy omas that naive IgM antibodies or to the development of hybrid screened for their reactivity of this approach is to search stage display to select antibodies g, termed the naive repertoire both human (Marks et al., 1991) bodies. The second strategy into antibody CDR regions (Bar- inter, 1992) and takes advantage ng of antigen–antibody interac-

s to a library of antibodies which the immune system toward the r antigens. Preparation of naive nonimmune sources and amplifi- ams the starting point in the ve been constructed from both (Gram et al., 1992) and selection the production of low or medium the construction of naive scFv o donors served as the source of DNA using IgM, IgGl, κ, and λ esis. This cDNA was then used as the template for scFv construction. The construction of this library has already been considered (vida supra). From libraries of 2.9 × 10⁷ VHM-VL and 1.6 × 10⁸ VHG-VL scFv clones, several scFv’s were selected. Clones which had affinities amenable to ELISA studies bound turkey egg white lysozyme, TEL, (3 clones), bovine serum albumin, BSA, (1 clone), and the hapten 2-phenyloxazol-5-one, phOX, (1 clone). The BSA binding clone was an artifact from the selection of phOX clones as BSA had been used as a carrier for this hapten. All clones were tested for cross-reactivity with a panel of other antigens and demonstrated good selectivity albeit good to poor signal on ELISA. The affinities of the best TEL clone and the phOX clone were 1.2 × 10⁷ M⁻¹ and 1.8 × 10⁹ M⁻¹, respectively.

Two subsequent papers have appeared since this initial report and describe additional antibodies isolated from these libraries. Griffiths et al. (1993) describe the isolation of antibodies reactive with self-antigens. The antigens chosen for investigation were thyroglobulin, a human antibody, TNF-α, CEA, MUC1-peptide, and rsCD4. The scFv’s selected were of such low affinity only those which dimerized on the phage and subsequently in solution were selected. Affinities of multimeric forms were determined and in one case monomer could be isolated by gel filtration and independently examined. Avidity constants for one anti-TNF-α, three anti-human antibodies, and one anti-thyroglobulin were reported and range from 0.6 to 1.4 × 10⁷ M⁻¹. A fivefold effect was reported to be due to avidity yielding a corrected monovalent affinity of 1.3–2.9 × 10⁶ M⁻¹. ELISA signals for clones selected against other antigens were extremely weak and in one case did not exceed an OD405 of 0.12. To select these clones a helper phage which was deleted in gIII was utilized in the phagemid system. This resulted in a multivalent system to aid the capture of these very low-affinity clones. Additional valency is added by dimerization g3p-fused scFv and soluble scFv released by proteolysis. This suggests that Fab fragments which are clearly monomeric in nature (according to all published reports) may be superior to scFv in phage display for the isolation of high-affinity clones. Alternatively, this may have resulted from selecting from a library which contained only very low-affinity clones.

Marks et al. (1993) later reported the isolation of six scFv’s selected to bind blood group antigens by way of selecting on fixed and sus- pended cells. This report demonstrates the sensitivity of the phage display approach to select clones from mixed antigen sources as had been done with viral lysates and in particular extends the approach to the cell surface. The isolated scFv’s were functional in immunoefluo-
rescence, however, no affinity measurements were reported. Characterization of conditions for the selection of cell-surface antigens has also been performed by Portolano et al. (1993b) and by Siegel and Silberstein (1994).

Thus several reports have demonstrated that scFv of modest to low affinity can be isolated from naive libraries. The main problem with the naive approach is the difficulty in ensuring the library is diverse and unbiased. This is due to the fact that the libraries will be influenced or edited in the human and that library construction is extremely susceptible to contamination from mRNA derived from activated B cells or plasma cells.

B. Synthetic Repertoire Approach

This approach allows for the construction of more diverse and defined libraries. The ability to design and construct synthetic antibody repertoires reflects our increased understanding of antibody/antigen interactions and in particular the contributions to binding of the complementarity determining regions (CDRs). In 1970, the CDRs were predicted by Wu and Kabat (1970) to be the regions responsible for antigen recognition. Numerous crystallographic and protein engineering studies have since supported this hypothesis.

In the first synthetic antibody repertoire experiment (Barbas et al., 1992a) a single human anti-tetanus toxoid binding antibody was utilized as the backbone of an antibody library which was varied in sequence only over the heavy-chain CDR3 region. The variants contained an HCDR3 of 16 amino acids which was the length in the parent antibody. This region was completely randomized using a synthetic oligonucleotide with an NNS doping strategy, where N is any of the four nucleotides and S is G or C. NNS encodes all 20 amino acids and a single amber stop codon in a total of 32 codons. The rationale for limiting diversity to this region was based on the observation that this loop contributes most in terms of molecular diversity in the antibody combining site. Genetic considerations in the human system have led to estimates of 10^{14} naturally occurring sequences in this region (Sanz, 1991). Diversity in this region is not limited to sequence but includes length which may vary from 2 to more than 26 residues (Wu et al., 1993). In this case the extended length of 16 residues was utilized to ensure structural diversity even though such a library would be incomplete as more than 10^{20} clones would be required for each possible sequence to be represented. The synthetic genes were constructed by PCR and cloned into pComb3 to yield a library of 5 x 10^{7} clones, a size on the order of the number of B cells in a mouse. The library
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of B cells in a mouse. The library

of Fab fragments was selected to bind either fluorescein-BSA conjugate or free fluorescein. Selective pressure for binding free fluorescein was provided by eluting bound phage with fluorescein. Seven unique clones from the conjugate selection were obtained. The affinities of these clones were in the \(10^6 M^{-1}\) range for free fluorescein and as high as \(2 \times 10^7 M^{-1}\) for the conjugate on which they were selected. Three unique clones were obtained in the selection for binding free fluorescein and the affinities of these were approximately \(10^7 M^{-1}\) for free fluorescein and approximately \(5 \times 10^7 M^{-1}\) for the conjugate (Fig. 21). The most striking observation was the appearance of a Ser-Arg-Pro sequence near the central position of the HCDR3 of these three clones. Codon usage suggested that this consensus was the result of selection at the level of the protein and not due to bias in the initial oligonucleotide sequence. Another structurally significant observation was the selection of Asp-101 in the HCDR3. In naturally occurring antibodies Asp-101 has been shown to participate in a structurally important salt bridge with Arg-94, the last residue in FR3. Thus, these synthetic antibodies have recapitulated a structurally conserved feature of natu

<table>
<thead>
<tr>
<th>Clone</th>
<th>HCDR2 Sequence</th>
<th>(K_d [\text{FL}] \text{ M}^{-1})</th>
<th>(K_d [\text{FL-BSA}] \text{ M}^{-1})</th>
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<tbody>
<tr>
<td>7E</td>
<td>GDFWTGYSDKYA M</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>F32</td>
<td>GNRMGRLSRPVM DL</td>
<td>6.0 (\times 10^{-7})</td>
<td>1 (\times 10^{-7})</td>
</tr>
<tr>
<td>F34</td>
<td>GNRMGRLSRPVM DL</td>
<td>1.4 (\times 10^{-7})</td>
<td>2 (\times 10^{-8})</td>
</tr>
<tr>
<td>F1</td>
<td>GNVLFVRNRSRPLDM</td>
<td>1.3 (\times 10^{-4})</td>
<td>3 (\times 10^{-4})</td>
</tr>
<tr>
<td>F9</td>
<td>GNVLFVRNRSRPLDM</td>
<td>1.5 (\times 10^{-4})</td>
<td>5 (\times 10^{-4})</td>
</tr>
<tr>
<td>F22</td>
<td>GNVLFVRNRSRPLDM</td>
<td>1 (\times 10^{-7})</td>
<td>8 (\times 10^{-6})</td>
</tr>
<tr>
<td>F31</td>
<td>GNVLFVRNRSRPLDM</td>
<td>1.6 (\times 10^{-7})</td>
<td>1 (\times 10^{-7})</td>
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Fig. 21. Amino acid sequences of the heavy-chain CDR3 of a tetanus toxoid binding Fab (starting clone, 7E) and fluorescein binding Fabs derived mutagenesis of H-CDR3 and selection on BSA fluorescein. \(K_d's\) for binding to fluorescein (FL) were determined by fluorescence quenching and apparent affinities for binding to FL BSA by inhibition ELISA. From Barbas et al., 1992a.
ral antibodies supporting the hypothesis for the role of this conserved sequence. This leads to the suggestion that synthetic antibodies will not be artificial in their character and will be constrained in some extent to sequences found in natural antibodies.

Subsequent reports have investigated the extent to which the introduction of structural diversity into the antibody combining site is more important than simple sequence diversity as well as the role of Asp-101 in the HCDR3. For practical reasons it is very difficult to prepare libraries containing more than $10^9$ heavy or light chains. This imposes a practical limit to the sequence diversity which may be examined. For example, to survey a library with 99% confidence, a library with six amino acids randomized requires $>10^9$ clones. Randomization of six residues results in a library diverse in sequences but constrained in structure. The remodeling of a single combining site to bind a variety of antigens might best be achieved by focusing on structural diversity, i.e., randomizing stretches which are longer than can be completely surveyed and display a greater degree of structural freedom. This hypothesis is testable using a competitive selection scheme. Barbas et al. (1993d) utilized three HCDR3 libraries of length 5, 10, and 16. This report focused on developing a strategy for the iterative selection of catalytic antibodies which utilized metal cofactors. Fabs were selected to bind a variety of metal ions by immobilizing the metal with an iminodiacetic acid support. Success of the selection strategy could be verified with some of the metals, such as Cu$^{2+}$ and Zn$^{2+}$, by knowledge of side chains which have been shown to be important in chelating these metals. The Cu$^{2+}$ and Zn$^{2+}$ selected clones were abundant in His, Cys, Met, and Asp as would be predicted. Binding to free Cu$^{2+}$ was verified by fluorescence quench experiments. The selective panning was also applied to the selection of Fab which could bind the surface of the metal oxide magnetite. This experiment produced four clones which were similar to other loop sequences which had previously been reported to bind magnetite. No clones from the five-residue-length HCDR3 library were observed following analysis of 49 selected clones.

In another report (Barbas et al., 1993a), libraries of HCDR3, HCDR3 and LCDR3, and LCDR3 alone were examined using a competitive selection scheme for the selection of anti-hapten Fab. In this report, selection for binding three different haptens resulted in 18 anti-hapten antibodies; additional clones were available but were not sequenced. The specificities of a number of these were examined and are shown in Fig. 22. Various degrees of specificity were observed with binding constants in the range $1-3 \times 10^7 M^{-1}$ as determined by surface plasmon resonance. Again no clones from the five-residue HCDR3 were se-
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site in that synthetic antibodies will sub-
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ed the extent to which the intro-
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3a), libraries of HCDR3, HCDR3
examined using a competitive
anti-hapten Fab. In this report,
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available but were not sequenced.
ere examined and are shown
ity were observed with binding
determined by surface plasmon
five-residue HCDR3 were se-
lected with the competitive selection scheme. Additionally none were
selected from the LCDBD only randomized libraries where a maximum
of six residues were randomized. The selected clones were derived
mostly from HCDR3/LCDR3 libraries. Furthermore, all clones
selected were derived from HCDR3 libraries wherein Asp-101 was fixed.
These results support the notion that structurally diverse libraries are
a requirement for remodeling a given framework. Extrapolation of
these results may be used to rationalize the utilization of shorter
HCDR3 lengths in mice as compared with humans. Structural diversity
lacking in HCDR3 of the mouse may be compensated by the use of
a far greater number of V segments.

Synthetic libraries can be utilized as probes for molecular recog-
nition of antigens from which it is difficult to prepare antibodies for
reasons of tolerance, toxicity, or reactivity. To this end, S. M. Barbas
et al. (1994) have utilized the aforementioned libraries to study the
recognition of double-stranded DNA. Two Fabs were isolated which
bound double- and single-stranded DNA with moderate to high affin-
ity. The Fabs did not bind the negatively charged polyelectrolyte dextran sulfate or lipid A. The ability of one of the Fabs to perform as a naturally occurring DNA binding protein was investigated in electrophoretic mobility shift assays. The ability to form nucleoprotein complexes was clearly demonstrated (Fig. 23). The two Fabs isolated contained HCDRs of 10 and 16 residues in length. Interestingly, although the CDRs differed in length, the amino- and carboxy-ends of the HCDR3 were virtually identical (Fig. 24). The clone with the 16-residue HCDR3 demonstrated a clear preference for poly(dG-dC).poly(dG-dC), whereas the other clone bound different oligonucleotides with similar affinity (Fig. 25). This observation and the fact that the two clones differ only in the central portion of HCDR3 suggest this region is crucial for the sequence preference displayed by one of the clones and may provide the basis for the design and selection of antibodies capable of sequence-specific recognition.

In unpublished studies, these synthetic libraries have further been utilized in the selection of Fabs which bind a number of protein antigens and in one case neutralize HIV.

Fabs with catalytic activity may also be selected from the aforementioned synthetic antibody libraries. The direct covalent selection of Fabs from phage display libraries was proposed in the original Fab display report of Barbas et al. (1991). The strategy proposed was to use mechanism-based inhibitors or affinity labels to select for appropriately positioned functionalities within the combining site of an antibody. These functionalities, amino acid side chains with the appropriate chemical characteristics and geometries, would then catalyze a chemical reaction with the appropriate substrate. To this end, Janda et al. (1994) have utilized an pyridyl disulfide affinity label to trap an appropriately positioned thiol in the active site of an synthetic antibody. Phage which covalently bind to the support via a disulfide bond are then selectively released by reduction of the disulfide following elution with acid of noncovalently attached phage (Fig. 26). Sequencing of 10 of the selected Fabs revealed 2 with unpaired cysteines. One of these was examined for its ability to hydrolyze a thiol ester substrate designed to place the electrophilic center of the carbonyl in the position of original active disulfide of the affinity label. The Fab was shown

Fig. 23. (a) Interaction between the synthetic Fab SD1 and DNA in an electrophoretic mobility shift assay (EMSA). Lanes 1–6 contain 0.08 pmol 32P-labeled double-stranded probe (5'--AAT--GTA--TGC--CGG--CGC--GCT--TTA--GGG--GCC--CC--3') with 0.2 pmol Fab SD1 (lanes 2, 3, and 4). Lanes 3 and 6 included a preincubation with an anti-Fab reagent (α-Fab IgG). Lanes 4 and 5 included 0.2 pmol of a control Fab reacting with HIV-1 surface glycoprotein gp120 (HIV Fab). The thick arrow indicates the
tively charged polyelectrolyte of one of the Fabs to perform a protein was investigated in the ability to form nucleoprotein (Fig. 23). The two Fabs isolated idues in length. Interestingly, the amino- and carboxy-ends (Fig. 24). The clone with the clear preference for poly(dG)-ne bound different oligonucleotids observation and the fact that a portion of HCDR3 suggest preference displayed by one of for the design and selection of fic recognition.

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be selected from the aforementioned strategy the direct covalent selection of is proposed in the original Fab . The strategy proposed was to affinity labels to select for appropriate the combining site of an anti-cid side chains with the appropriate geometries, would then catalyze a ate substrate. To this end, Landa disulfide affinity label to trap an active site of an synthetic anti-the support via a disulfide bonding action of the disulfide following ached phage (Fig. 26). Sequenc-2 with unpaired cysteines. Olylolyze a thiol ester substrate ter of the carbonyl in the pos-itunity label. The Fab was shown

atic Fab SD1 and DNA in an electropho-contain 0.08 pmol 32P-labeled double-CGC-CCT-TTA-GCC-GCC-CC-3') lanes 3 and 6 included a preincubation and 5 included 0.2 pmol of a control Fab ) (HIV Fab). The thick arrow indicates the position of the specific Fab SD1 nucleoprotein complex and the a, arrow indicates the position of the supershifted Fab SD1 nucleoprotein complex. 0 marks the origin of migration and FP the position of the free probe. (b) Specificity of binding of Fab SD1 to DNA. Lanes 1-22 contain 0.08 pmol 32P-labeled probe and 0.2 pmol Fab SD1 in lanes 2-11 and 13-22, respectively. In addition increasing concentrations of competitor oligonucleotide are included as shown. Numbers refer to molar excess of competitor oligonucleotide relative to the probe. Arrows indicate the position of the Fab nucleoprotein complex. From S. M. Barbas et al. (1994).
to be catalytic in its hydrolysis of the ester and was further characterized to proceed through a covalent intermediate. Thus, the catalytic activity had been directly selected to proceed through the mechanism dictated by the design of the affinity label/substrate pair. This is the first report of the selection of a catalytic protein, in this case a Fab, from a random protein library and will likely have a major impact on the field of catalytic antibodies.

Garrard and Henner (1993) have constructed a synthetic antibody library with the introduction of limited diversity over four CDRs of a humanized anti-HER-2 Fab. The doping strategy was designed to incorporate mostly amino acids which are naturally found in the CDRs. The library of Fab fragments was selected to bind rsCD4, insulin-like growth factor 1 (IGF-1), and tissue plasminogen activator. A single Fab was isolated which bound IGF-1 with an affinity of $3 \times 10^5 M^{-1}$.

Two other reports, Hoogenboom and Winter (1992) and Akamatsu et al. (1993), have utilized combinations of genomic V segments with synthetic CDR3 segments as had been previously suggested (Barbas et al., 1992a). The report of Hoogenboom and Winter utilized a defined collection of 49 $\text{V}_\text{H}$ genes and a single light chain. Both groups constructed scFv libraries as opposed to Fab as discussed above. The former report combined a HCDR3 segment randomized over five amino acids with $\text{V}_\text{H}$ genes and selected the library to bind phOX-BSA, 3-iodo-4-hydroxy-5-nitrophenylacetate-BSA (NIP-BSA), BSA, turkey egg white lysozyme, TNF-α, and human thyroglobulin. Several scFv's were shown to bind phOX and NIP while only a single clone was found to bind weakly to one of the protein antigens, TNF-α. The affinities of the phOX and NIP binding scFv's were reported to be $10^5$–$10^6 M^{-1}$.

Akamatsu et al. introduced a biased set of amino acids over portions
ster and was further characterized by the loss of catalytic activity. Thus, the catalytic activity through the mechanism dictated by strate pair. This is the first report in this case a Fab, from a random major impact on the field of constructed a synthetic antibody med diversity over four CDRs of a phoping strategy was designed to are naturally found in the CDRs.ected to bind rsCD4, insulin-like plasminogen activator. A single l with an affinity of $3 \times 10^5 \text{M}^{-1}$.nd Winter (1992) and Akamatsu ons of genomic V segments with en previously suggested (Barbas om and Winter utilized a defined le light chain. Both groups con-o Fab as discussed above. The segment randomized over five o the library to bind phOX-BSA, e-BSA (NIP-BSA), BSA, turkey an thyroglobulin. Several scFv's while only a single clone was protein antigens, TNF-$\alpha$. The long scFv's were reported to be l set of amino acids over portions

![Antibodies from Combinatorial Libraries](image-url)

**Fig. 25.** ELISA profiles of the reactivity of synthetic Fabs SD1 (a) and SD2 (b) with DNA and selected double-stranded oligonucleotides. The plots show the binding of Fabs monitored colorimetrically as a function of Fab concentration. From S. M. Barbas et al. (1994).
of both HCDR3 and LCDR3 to a set of genomic V genes. The library was selected to bind ConA. Six scFv’s were characterized to bind ConA with affinities of $5 \times 10^4$–$10^5 \text{M}^{-1}$. The ConA scFv’s demonstrated good specificity in binding the target antigen.

Targeted CDR mutagenesis can also be utilized to make very modest specificity changes in an antibody. An example of altering the reactivity of an antibody to its anti-Ids has been reported by Glaser et al. (1992). In this report LCDR 1 and 2 were targeted for mutagenesis using a minimal doping scheme called codon-based mutagenesis. This restricted doping scheme employs oligonucleotide synthesis using two columns and the opening, mixing an exchange of resin between the columns during synthesis. This synthesis scheme was necessitated by the use of screening rather than selection in the examination of the library. The system utilized for library screening has been discussed (vida supra, Huse et al., 1992). Screening required that a larger number of positive clones would be represented in the library as only 1000 clones would be screened for reactivity. The goal was to moderate the binding of a number of anti-Ids which had been previously characterized as reacting with the LCDRs and the experiment was successful in this regard.

C. COMBINING DESIGN AND SELECTION IN SYNTHETIC HUMAN ANTIBODIES

An alternative to a random search of binding sites for the desired specificity is a directed search that incorporates information known to be relevant to a given binding problem. For anti-receptor antibodies
information relevant to the binding of the ligand to the receptor may be utilized to construct specialized libraries directed to bind a given receptor or receptor family. The minimal binding sequences of a number of ligands for their receptors have been characterized. Barbas et al. (1993c) described a methodology which could allow for the direct design and selection of human antibodies reactive with human receptors. The overall strategy is outlined in Fig. 27. A minimal sequence known to be important for the binding of the ligand to the receptor is transplanted into an antibody CDR. Since the correct conformational display of the sequence will, in almost all cases, be critical for interaction with the receptor, the transplanted sequence is flanked by randomized segments which form the elements of diversity within the library. The randomized elements also allow for the selection of additional contact residues. As a demonstration of the validity of this approach a member of the Asp–Gly–Arg (RGD) binding integrin receptor family, \( \alpha_\text{v} \beta_3 \), was chosen as a target receptor. The simple tripeptide RGD formed the basis for the construction of the library. In this case, the RGD sequence was placed near the apex of the extended hairpin loop of the HCDR3 of a human anti-HIV Fab. The HCDR3 had a moderately long sequence, 18 residues. Residues at the N- and C-terminal of the CDR were conserved to retain the stem of the loop. On each flank of the RGD were three randomized residues. Following selection of the \( 3 \times 10^7 \) member library for binding to \( \alpha_\text{v} \beta_3 \) 5 Fab clones were characterized. The selected flanking regions showed some homology with known integrin binding peptides; however, some elements were quite unique. The affinities of the Fabs were astonishingly high, \( 10^{10} \text{M}^{-1} \) (Table VI). Specificity characterization revealed the Fabs also bound \( \alpha_\text{v} \beta_3 \) but not \( \alpha_\text{v} \beta_5 \), two integrins highly related to \( \alpha_\text{v} \beta_3 \). These three integrins can share the same high-affinity ligand vitronectin and all bind RGD containing peptides. Furthermore, \( \alpha_\text{v} \beta_3 \) and \( \alpha_\text{v} \beta_5 \) bind with the same affinity to some RGD containing peptides. Fabs were shown to compete with RGD peptides for the integrin ligand binding site as designed. Further functional characterization demonstrated the Fabs to be potent in adhesion assays and subsequently (Smith et al., 1994) in inhibiting platelet aggregation (Fig. 28). Collectively, antagonism of these integrins could have potential in the treatment of osteoporosis and as anti-metastatic and anti-thrombotic agents.

Synthetic antibodies can also be utilized to derive novel minimal ligands for receptors. Smith et al. (1994) demonstrate that randomization of the RGDX sequence within the context of the optimized Fab-9 (Barbas et al., 1993c) architecture and reselection for \( \alpha_\text{v} \beta_3 \) binding leads to the identification of a number of novel non-RGD ligand sequences within the HCDR3. This experiment led to the sug-
Fig. 27. The design and selection of human anti-receptor antibodies is aided by a knowledge of residues within the ligand that are involved in binding to the receptor. The first step involves the characterization of this interaction by mapping regions of the ligand involved in binding or by selecting peptides from linear or constrained peptide libraries to bind the receptor. The minimal ligand is then transplanted into a semisynthetic antibody library. The random sequence is provided to optimize the conformational display of the transplanted sequence which will be dependent on constraints imposed by the antibody. Random sequence also allows for selection of additional contact residues. Selection from a vast library of variants provides the optimal anti-receptor antibody. For integrins which bind RGD containing peptides, the simple RGD sequence is transplanted. For this case we have chosen the heavy-chain CDR3 for insertion of the sequence and optimization. The sequence of the starting anti-gp120 antibody HCDR3 was VGYSPWDDSPQDNMYMDV. Following transplantation into a synthetic version of HCDR3 the library consisted of variants where the HCDR3 sequences are VGCXXXRGDXXXXCYMDV, where X represents a mixture of all 20 amino acids. Following selection for binding to the integrin α5β1, the sequence of the HCDR3 of the highest affinity antibody, Fab-9, was VGCSPGRGDIRNCYMDV. From Barbas et al. (1993c).

gestion of a new minimal sequence consensus for α1β2, namely, R/K-X-D, where X is virtually any amino acid except proline. Several of these novel non-RGD Fabs demonstrated the capacity to discriminate between α5β3 and α1β2 with a maximal difference of 100-fold preference (affinity) for α1β2. Prospects for the use of these antibodies as lead compounds were examined through the characterization of HCDR3 peptides. Peptides were shown to maintain receptor specificity albeit at reduced affinity.

D. Improving the Affinity of Human Antibodies

The systems and mutagenesis strategies described for producing new antibodies can also be utilized to improve the properties of ex-
TABLE VI
INHIBITION CONSTANTS AND AMINO ACID SEQUENCES OF RGD CONTAINING SYNTHETIC HUMAN ANTIBODIES

<table>
<thead>
<tr>
<th>Antibody No.</th>
<th>$\alpha\beta_3$</th>
<th>$\alpha\beta_3$</th>
<th>$\alpha\beta_3$</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>$2.5 \times 10^{10}$</td>
<td>$2.5 \times 10^{10}$</td>
<td>$5 \times 10^{7}$</td>
<td>TQG-RGD-WRS</td>
</tr>
<tr>
<td>7</td>
<td>$2.0 \times 10^{10}$</td>
<td>$5.0 \times 10^{10}$</td>
<td>NI</td>
<td>TYG-RGD-TRN</td>
</tr>
<tr>
<td>8</td>
<td>$2.0 \times 10^{10}$</td>
<td>$3.5 \times 10^{10}$</td>
<td>NI</td>
<td>PIP-RGD-WRE</td>
</tr>
<tr>
<td>9</td>
<td>$1.0 \times 10^{10}$</td>
<td>$1.0 \times 10^{10}$</td>
<td>NI</td>
<td>SFG-RGD-TRN</td>
</tr>
<tr>
<td>10</td>
<td>$2.5 \times 10^{10}$</td>
<td>$2.5 \times 10^{10}$</td>
<td>NI</td>
<td>TWG-RGD-TRN</td>
</tr>
</tbody>
</table>

Note. The ability of the recombinant antibodies to block vitronectin binding to vWb3 and vWb5 and fibronogen binding to $\alpha\beta_3$ was determined with a purified receptor binding assay. The sequences flanking the RGD motif of each antibody are shown. NI, no inhibition at concentrations of up to $5 \times 10^{7}$ M. From Barbas et al. (1993c).

Anti-receptor antibodies is aided by a number of factors involved in binding to the receptor. The interaction by mapping regions of peptides from linear or constrained active ligand is then transplanted into sequence is provided to optimize the sequence which will be dependent on concept also allows for selection of additional variants providing the optimal anti-β containing peptides, the simple RGD we chosen the heavy-chain CDR3 for the sequence of the starting anti-gp120 ADV. Following transplantation into a tandem of variants where the HCDR3 sequence represents a mixture of all 20 amino acids the sequence of the HCDR3 SFGRCDIRNCYYMDV. From Barbas consensus for $\alpha\beta_3$, namely, the capacity to discriminate a difference of 100-fold preference for the use of these antibodies through the characterization of in to maintain receptor specific-

ANTI-BODY ANTIBODIES

Figure 28. The synthetic human antibody fragment Fab-9 blocks platelet aggregation. Human platelets ($1 \times 10^9$) were mixed with 100 μg/ml of purified fibrinogen and 2.0 mM Ca$^{2+}$ in Tyrodes buffer. These were placed in a glass aggregation tube. The indicated concentration of Fab was added and the platelets were stimulated with 20 μM ADP. Aggregation was measured as light transmission through the platelet suspension using an aggregometer. From Smith et al. (1994).
Gram et al. (1992) have utilized error-prone PCR conditions to construct libraries of point mutations. This strategy is interesting since it mimics the random point mutations generated in natural somatic mutation. This approach was utilized to improve a low-affinity mouse scFv selected from a naïve library to bind progesterone. The monovalent display system pComb3 allowed for the selection of an scFv with 30-fold improved affinity. The final scFv had an affinity of ca. $10^5 M^{-1}$.

Hawkins et al. (1992) have utilized a similar strategy to improve a mouse anti-NIP scFv. In this case the multivalent display system fctet-DOG1 was utilized. In order to select for higher affinity variants from this multivalent display system, the antigen was biotinylated and the selection performed in solution. Following several rounds of selection a clone was isolated which was improved fourfold in NIP binding to a final affinity of $10^8 M^{-1}$. In a subsequent report, Hawkins et al. (1993) have utilized the same strategy and vector with a high-affinity mouse anti-lysozyme scFv the structure of which had been previously determined. The library was selected in two different fashions. The first experiment entailed 13 rounds of biotin capture panning. The second experiment was performed in a competitive selection strategy where biotin-captured lysozyme was used with nonlabeled lysozyme as a competitor for five rounds of selection. The second selection was performed to bias the selection for the capture of clones with slower off-rates. These two experiments yielded two clones which were improved threefold in affinity. The two sets of mutations found in these clones were combined by site-directed mutagenesis to yield an scFv with an affinity of $2 \times 10^9 M^{-1}$, a fivefold overall improvement in affinity. One of the potential problems with random mutagenesis of the entire gene is that many of the mutations occur outside the CDRs and thus might more easily generate an antigenic antibody.

Chain shuffling as proposed by Huse et al. (1989) may be used with great success when starting with a low-affinity clone (Marks et al., 1992). Starting with low-affinity clone and shuffling against a library of light chains allowed for the selection of a clone with 20-fold improved affinity. This clone was then combined with a $V_{\text{H}}$ library and selected for an additional improvement of 15-fold. The final clone had an affinity of $10^9 M^{-1}$ for phOX. This approach may be of lesser utility for the improvement of the high-affinity antibodies (Barbas et al., 1993b) as the sequence changes are too great.

A final approach termed “CDR walking” (C. F. Barbas et al., 1994) may prove to be a general method for the improvement of antibody affinity. This approach is a variant of the synthetic antibody approach with one important difference. In this case library completeness is stressed over structural diversity and randomization is limited to six
r-prone PCR conditions to cons.
sider strategy is interesting since 
generated in natural somatic 
DNA improve a low-affinity mouse 
monovariant or the selection of an scFv with 
IV had an affinity of ca. 10^8 M^-1.
a similar strategy to improve a 
multivalent display system f 
lect for higher affinity variants 
were selected in two different f 
mounds of biotin capture panning. 
in a competitive selection strat 
vias used with nonlabeled lys 
selection. The second selection 
for the capture of clones with 
ants yielded two clones which 
he two sets of mutations found 
rected mutagenesis to yield 
vefold overall improvement 
mns with random mutagenesis 
e mutations occur outside the 
berate an antigenic antibody. 
 et al. (1989) may be used with 
H- affinity clone (Marks et al., 
ding against a library of 
a clone with 20-fold improved 
Ve library and selected 
final clone had an affinity 
y be of lesser utility for the 
odies (Barbas et al., 1993b) as 
ning" (C. F. Barbas et al., 1994) 
the improvement of antibody 
he synthetic antibody approach 
 case library completeness is 
randomization is limited to six 
residues or less. The approach may be applied in a parallel fashion 
where libraries are constructed in different CDRs. Following selec 
tion, the improved CDRs are then assembled to give the best antib 
odies, assuming additivity. The approach may also be applied in a sequential 
strategy. C. F. Barbas et al. (1994) applied the sequential approach 
for the improvement of a human anti-HIV-1 gp120 Fab. The Fab chosen 
(designed here as HIV-4 has an identical sequence to Fab b12 
discussed above) had already been shown to potently neutralize HIV- 
1. In this case a library of HCDR1 variants with five residues random 
ized was selected by four rounds of panning against rgp120. The collection 
of clones which resulted were then used in the construction of a 
HCDR3 library where four additional residues were randomized. The 
resulting library was selected by an additional six rounds of panning. 
A clone was isolated with an 8-fold improvement of affinity for a final 
affinity of 1.4 x 10^9 M^-1. The epitope recognized by this antibody is 
the CD4-binding site on gp120. As this functional feature, which is 
not formed by a linear sequence, is retained by all variants of HIV-1, 
it was proposed that affinity should also be increased for divergent 
isolates. This was tested by determining the affinities of several clones 

![Figure 29](image-url) 

**Fig. 29.** The affinity increases of evolving Fabs for binding the divergent envelope 
proteins gp120 IIIB (LA1) and gp120 MN are well-correlated. Affinities were determined 
using the surface plasmon resonance technique. The sequences of evolved clones are 
ranked as compared to the parent and changes in the amino acid sequence from the 
parent are shown as ΔAA. The complete V_{H} sequence of HIV-4 is given in Fig. 15 as 
Fab b4 and is identical with Fab b12. From C. F. Barbas et al. (1994).
for gp120 derived from MN and IIIB strains. These proteins differ in over 80 amino acids and are highly divergent. As shown in Fig. 29 the affinity increases for both gp120's are well correlated. Functional improvement was assayed in virus neutralization studies with laboratory isolates and a 54-fold improvement was determined for the highest affinity clone. Furthermore, neutralization studies with primary clinical isolates demonstrated that the improved Fab acquired the ability to neutralize additional variants not neutralized by the parent.

VI. Conclusions

Since its conception 5 years ago, the combinatorial approach has allowed unprecedented access to the human antibody response. The cloning of antibodies from preimmune, immune, and memory compartments of the human immune system has been demonstrated. Combinatorial antibodies have been shown to provide an accurate functional reflection of the natural response as demonstrated by the ability of cloned antibodies to compete with serum antibodies for binding antigens. Combinatorial antibodies also provide a useful (if somewhat incomplete) guide to the molecular biology of the response.

The ability to select large numbers of antibodies against the same antigen and even the same epitope has led to a greater understanding of the molecular and structural biology of the immune response. Examination of natural libraries has highlighted the functional significance of HCDR3 and its prominent role in recognition. This observation coupled with the observation of light-chain plasticity characterizes an immune response which weights HCDR3 diversity over other elements. The ability to select antibodies to a multiplicity of antigens from synthetic libraries which differ only in the HCDR3 supports this notion. Furthermore competitive selections with synthetic libraries demonstrate that limited structural diversity within the rest of the binding site can be compensated for by increased structural diversity within the HCDR3. This observation may be important in rationalizing the differences between murine and human repertoires as reflected in HCDR3 length and the number of expressed V genes. These studies complement the recent increased appreciation of the role of HCDR3 gained through structural studies (Wilson and Stanfield, 1993).

With its ability to provide large numbers of human antibodies directed against a single antigen, the combinatorial approach allows for the rapid assessment of immunodominant as well as neutralizing epitopes in the context of the human response. This information should be utilized in the future to guide the design of more effective vaccines. Antibodies neutralize viruses by mechanisms which in most cases
strains. These proteins differ in divergent. As shown in Fig. 29 are well correlated. Functional utralization studies with labora-
it was determined for the highest ation studies with primary cli-
proved Fab acquired the ability neutralized by the parent.

sions
the combinatorial approach has human antibody response. The n, immune, and memory compart-
as been demonstrated. Combinato-
provide an accurate functional demonstrated by the ability of rum antibodies for binding anti-
provide a useful (if somewhat oligo of the response.
3 of antibodies against the same is led to a greater understanding of the immune response. Exam-
ited the functional significance a recognition. This observation chain plasticity characterizes an CDR3 diversity over other ele-
ty to a multiplicity of antigens only in the HCDR3 supports this sections with synthetic libraries diversity within the rest of the by increased structural diversity may be important in rationalizing human repertoires as reflected xpressed V genes. These studies preciation of the role of HCDR3 ilson and Stanfield, 1993).

umber of human antibodies dicombinatorial approach allows dominant as well as neutralizing response. This information should assign of more effective vaccines. mechanisms which in most cases are not yet defined. Mechanistic investigation of anti-viral antibodies should allow for the elucidation of novel pathways which might be targeted by small-molecule pharmaceuticals. Thus we suggest that combinatorial antibodies will play a significant role in the design of vaccines and new anti-viral agents. Antibodies may also provide a way of determining receptor function in vivo and serve as templates for the design of small molecules.

One hundred years ago von Behring suggested, “Considering that antitoxin is an inanimate chemical substance, the possibility cannot be discounted that it may, at a later date, be able to be produced without the aid of an animal body,” (as translated in Gronski et al., 1991). Indeed this has now been realized. Our new-found ability to generate human antibodies and to evolve their specificities and affini-
ties ex vivo promises increased use of this class of molecules in the service of human health.

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