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Selection and Rapid Purification of Murine Antibody Fragments That Bind a Transition-State Analog by Phage Display

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ABSTRACT

Functional antibody fragments may be displayed on the surface of filamentous bacteriophage by introducing variable region genes into the viral genome at a gene encoding a viral coat protein. "Phage display" enables the isolation of antibody genes from large libraries according to the binding specificities they encode. We have constructed a new phage-display vector encoding a polyhistidine tag that has been used for rapid purification of soluble antibody fragments. An antibody library derived from immunized mice was cloned into this vector. This library was panned against the transition state analog RT3, and a high proportion of binders isolated after two rounds of panning. PCR analysis revealed that there were 24 different pattern groups. Sequencing of 15 clones within the major pattern group revealed 10 related clones with a range of point mutations. Thus, phage display can provide a large diverse repertoire of candidate catalytic antibodies based on TSA selection and screening.

Index Entries: Immobilized metal affinity chromatography; polyhistidine; immunization.

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INTRODUCTION

There have been a number of advances in the field of antibody engineering in recent years that have greatly enhanced our ability to manipulate and isolate antibody genes. Orlandi et al. (*1*) demonstrated that it was possible to build repertoires of heavy- and light-chain variables region genes using the polymerase chain reaction. It was also demonstrated that functional antibody binding fragments could be expressed in bacteria by secreting antibody heavy- and light-chain variable regions into the periplasmic space of *E. coli,* where they could associate to generate a binding antibody fragment (*2,3*).

Subsequently, it was shown that specific binding fragments could be expressed as a fusion with a minor coat protein encoded by gene 3 of filamentous phage leading to expression in a functinal form on the surface of the phage (*4*). In this way, the functional antibody is closely coupled to its gene within the phage particle, permitting isolation of the gene from an irrelevant background according to the binding characteristics it encodes. Specific binders have now been isolated from a diverse sources of repertoire, including immunized mice, nonimmunized human, and semisynthetic sources (*5–7*). In this article, we describe the use of an immunized mouse repertoire to derive a large diversity of binders to RT3, a transition-state analog (TSA).

TSAs are devised and synthesized in an attempt to make a stable mimic of the expected transition state occurring during cleavage of a chemical bond. Catalytic antibodies are usually generated by immunization of mice followed by preparation of monoclonal antibodies (mAbs) by hybridoma technology, which are screening for TSA binding. Clones identified as binders are then screened for catalytic activity. The above approach selects for binding during immunization and uses binding as the initial screen prior to catalytic screening. The central power of the phage system lies in its ability to couple binding of a protein to isolation of the gene encoding that protein, so the phage system has a great potential to generate a large diversity of binders to TSAs in a readily manipulated form to provide a starting point for catalytic screening.

METHODS

Sequencing was carried out using a sequenase kit on single-stranded phagemid DNA and was analyzed using the MacVector program (IBI). ELISA and PCR analysis of positive clones were as described previously (*5,6*). The double-stranded oligonucleotide *his*6 3/4 for introduction of a histidine tag was synthesized on an ABI 391 DNA synthesizer and is shown below. This was cloned into the *Not*1 site of pHEN-OX16. A *Not*1/*Bam*H1

fragment from the resultant clone pOX16*his1*-11 was transferred into pCANTAB3 and pCANTAB5 to give the cloning vectors pCANTAB3his_6 and pCANTAB5his_6, respectively. These vectors have an extra *Xho*1 site in plasmid sequences, so this was removed to give pCANTAB6 (Hennie Hoogenboom).

HIS6 3/4

	ala	*ala*	*his*	*his*	*his*	*his*	*his*	*his*	*gly*		
5′G	GCC	GCA	CAT	CAT	CAT	CAC	CAT	CAC	GG		3′
3′		CGT	GTA	GTA	GTA	GTG	GTA	GTG	CCC	CGG	5′

All cloning manipulations were carried out in TG1, and the correct clones introduced into the nonsuppressor strain HB2151 for expression as single-chain Fvs.

Measurement of Affinity

Conditions for measurement of K_d by equilibrium ELISA and Klotz plot were as described (*15,16*). Two microliters of supernatant from an overnight induction of the clone mo64 were incubated in duplicate in 100 μL with a range of concentrations of RT3 and allowed to equilibrate for 2 h. Sample was added for 20 min to an ELISA well coated with 1 μg/mL RT3:BSA. Binding was detected with the mouse antibody 9E10 and anti-mouse phosphatase. Substrate used was nitrophenyl phosphate. (A concentration of mo64 single-chain Fv SCFv) requiring overnight incubation with substrate was selected).

Preparation of a Phage-Display Library from Immunized Mice

Generation of a mouse phage library was carried out essentially as described previously (ref. *5* and Pharmacia Recombinant Phage Antibody System) with the following modifications. Priming of cDNAs was carried out using random hexamers (Pharmacia) at a concentration of 400 ng/50 μL cDNA reaction. Primary PCR products were generated using the primers from the Recombinant Phage Antibody System (Pharmacia) and with *Taq* polymerase (Cetus). Following gel purification and purification with Magic clean up (Promega), equal amounts of the heavy-chain, light-chain, and linker fragments were mixed. A range of linkage reactions was set up using different inputs of the fragment (either 20, 5, or 1.25 ng of each fragment) in 20 μL mock PCR reactions, lacking primers. Linkage was effected during 25 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min followed by a final incubation of 10 min at 72°C. One microliter from each linked sample was introduced directly into a 50 μL PCR reaction with the external pull-through primers VH1BACK-ApaL1 and VK4FORNot (*5*), and the reactions subjected to 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min followed by a final incubation of 10 min at 72°C.

The sample giving best yield from the various inputs of primary PCR product was selected and scaled up, and the product prepared for digestion using Magic-PCR prep (Promega). Sufficient PCRs are set up to generate at least 1 μg of product. The Sephacryl S-400 step previously recommended (Pharmacia) is not required. The linked sample from the "mock" PCR can be kept to generate more product as required.

Following digestion with *Apa*L1 and *Not*1 (NEB), the fragment was gel-purified and prepared for cloning with Magic Clean up (Promega). The vector pCANTAB3*his*$_6$ was prepared by digestion with *Apa*L1/*Not*1, Magic Clean up, and Chromaspin 1000 centrifugation (Clontech). Ligations were carried out using a ligation kit (Amersham), and following clean up were introduced into electrocomponent TG1 cells using electroporation (Bio-Rad).

Immobilized Metal Affinity Chromatography

The method for periplasmic preparation was based on a previously described method (*10*). All volumes are for an initial culture vol of 50 mL and all bacterial growth were at 30°C in *E. coli* HB2151 cells. Cells carrying the plasmid of interest were grown to 0.7–1.0 OD/mL in 2x TY medium supplemented with 2% glucose 100 μg/mL ampicillin. The culture was centrifuged at room temperature for 10 min, resuspended in 2x TY/100 μg/mL ampicillin/1 m*M* IPTG, and grown for 3 h. The culture was centrifuged for 15 min at a temperature of 4°C, resuspended in 1 mL of cold buffer 1 (PBS/1*M* NaCl) with 1 m*M* EDTA added, and left on ice for 15 min. The sample was centrifuged 2 × 10 min, the supernatant carrying the periplasmic contents collected, and MgCl added to 1–2 m*M*.

Four hundred microliters of a 1:1 slurry of Ni-NTA agarose (Qiagen), which had been pre-equilibrated with buffer 1 (no EDTA), were added to the periplasmic preparation and incubated with agitation for 10 min. The mixture was centrifuged at low speed on a microfuge for 10–15 s, and the pellet washed by resuspending in 1 mL of buffer 1. This wash process was repeated another two times before eluting in 100 μL of either buffer 1 carrying 250 m*M* imidazole or PBS with 250 m*M* imidazole. After 10 min, the supernatant, carrying the purified antibody, was collected, the pellet re-extracted with another 100 μL of the same buffer, and the eluted antibody pooled. As an alternative to this batch method, antibody can be purified on a column.

RESULTS

Preparation of the Phagemid Vector pCANTAB3*his*$_6$ and pCANTAB5*his*$_6$

Antibodies can be incorporated onto the surface of phage particles by fusing their genes to gene 3 within phagemid display vectors and rescuing with a helper virus. The phagemid display vectors pCANTAB3 and

pCANTAB5 (Pharmacia) were modified to introduce a detectable peptide tag (c-*myc*) and an amber codon between the antibody and gene 3 (Fig. 1) to facilitate expression and detection of nonfused antibody fragments (Fig. 1).

In order to facilitate initial screening for catalysis, a polyhistidine tag was also included in the construct. This permits rapid concentration and partial purification of the encoded antibody fragments by immobilized metal affinity purification (IMAC, ref [*8*]). The ability to concentrate is valuable for catalytic antibodies, which are typified by relatively low levels of activity in relation to most enzymes and so require the use of greater amounts of protein in assays. A simple and rapid purification is also useful for initial screening, since assays of catalytic antibodies are complicated by false positives resulting from low levels of reactive contaminating enzymes.

The construct pOX16*his*-11 was prepared by cloning the double-stranded oligonucleotide HIS6 3/4 (Methods) into the *Not*1 site of pHEN-OX16, which consists of the high-affinity oxazalone binding clone described in Clackson et al. (*5*) cloned into the *Pst*1/*Not*1 site of pHEN-1(9). This construct will give rise to a product consisting of (αOX antibody-his_6-*myc* tag), which can be detected with the 9E10 antibody (*9*). The αOX16 antibody fragment expressed in the form of a single-chain Fv (ScFv) with a polyhistidine tail retains its ability to express and bind oxazalone (not shown). This clone was used to work out the periplasmic preparation and IMAC purification regime described in Methods.

A *Not/Bam* fragment encoding the various features was isolated and cloned into the *Not/Bam* sites of the vectors pCANTAB3 and pCANTAB5 to give pCANTAB3his_6 and pCANTAB5his_6/pCANTAB6, respectively (Fig. 1). pCANTAB6 is the same as pCANTAB5his_6, except an extra *Xho*1 site present in pCANTAB5his_6 has been removed in pCANTAB6.

Rapid Purification of Antibody Fragments Using Immobilized Metal Affinity Chromatography (IMAC)

pOX16*his*-11 was used to test the functionality of the polyhistidine tail for IMAC. For this clone, expression of scFv was observed in the periplasm, but not the culture supernatant after 3 h induction. Antibody was only found in the culture supernatant after overnight incubation (not shown). Isolation of antibody from the periplasm not only has the advantage that it can be prepared after a shorter induction, but also the initial centrifugation of the culture itself effectively concentrates the antibody in the pellet.

Periplasmic extract was prepared from 50 mL of induced culture; 200 μL IMAC matrix was added, incubated at room temperature for 15 min, washed with buffer 1, and eluted with 250 m*M* imidazole. Under these conditions, 20% of the scFv was recovered (Fig. 2). There are two additional major bands as previously described, one of which is apparently

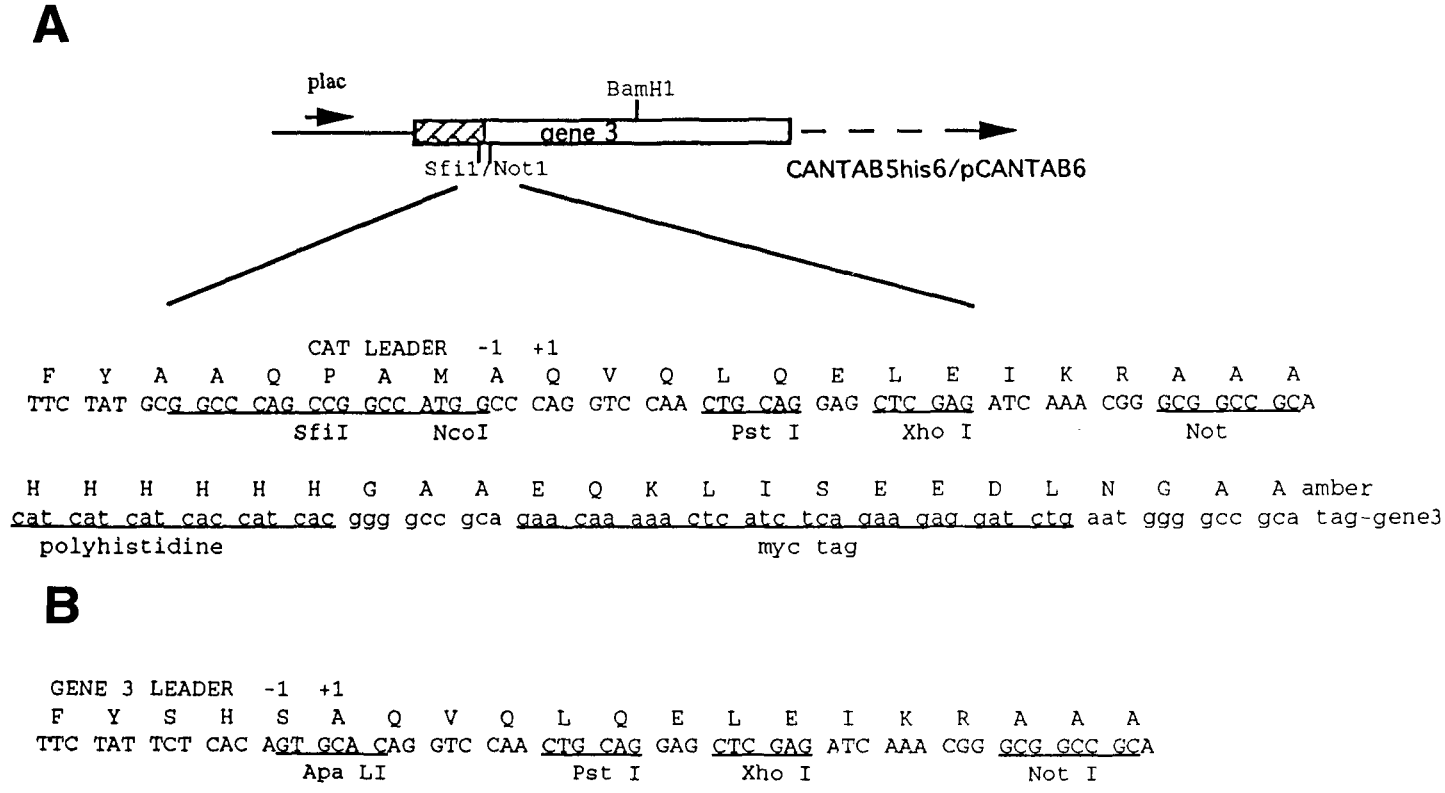

Fig. 1. Construction of pCANTAB3*his*$_6$ and pCANTAB5*his*$_6$/pCANTAB6. (**A**)pCANTAB5*his*$_6$/pCANTAB6. (**B**) pCANTAB3*his*$_6$. The sequence around the cloning site of pCANTAB5*his*$_6$ is shown. The sequence of pCANTAB3*his*$_6$ is identical apart from the 3′-end of the signal sequence as shown in (**B**). Codons in upper case are from the original vector pCANTAB3 or 5. The polyhistidine tag, the c-*myc* tag, and the amber codons were introduced as a *Not*1/*Bam*H1 fragment and are shown in lower case.

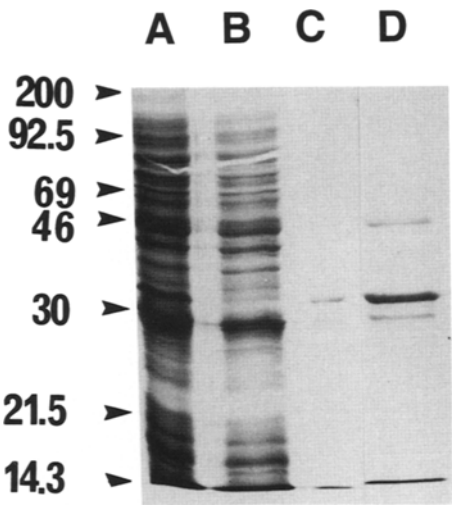

Fig. 2. Coomassie-stained gels showing IMAC purification of antibody from phagemid display vector encoding polyhistidine tails. Cells expressing ScFv from pOX16*his*$_6$ were induced for 3 h with 1 m*M* IPTG and a periplasmic extract prepared and subjected to IMAC. Samples were run on a 12% polyacrylamide gel and stained with Coomassie blue. (**A**) Total periplasmic proteins from 1 mL of cells. (**B**) Unbound fraction from 1 mL of cells after addition of metal chelate matrix. (**C**) Fraction bound and eluted from matrix, equivalent to 1 mL of cells. (**D**) Fraction bound and eluted from matrix, equivalent to 5 mL of cells.

superoxide dismutase (Dobeli, unpublished, cited in Qiagen handbook), which is known to bind divalent metal ions. This batch procedure is a very simple means of concentrating/partially purifying antibodies and will facilitate the preparation of multiple samples simultaneously as required for screening for catalytic activity.

Preparation, Panning, and Analysis of Binders from a Mouse Anti-RT3 Phage Display Library

A phagemid display library was prepared in pCANTAB3*his*$_6$ from the spleens of hyper-immunized mice essentially as described (*5*) with the modifications described in Methods. A library of 1.2×10^6 clones with 90% having insert was generated. This library was rescued with M13 KO7 and panned against the transition-state analog RT3 (Fig. 3), which is coupled to BSA (RT3-BSA) and coated onto Immunosorb tubes (Nunc) at a concentration of 100 μg/mL RT3-BSA. Panning was essentially as described (*6*), but using MOPS buffered saline rather than PBS. Eluates from the first round (PAN1) and second round (PAN2) were infected into HB2151 cells, and individual colonies grown and induced with 1 m*M* IPTG.

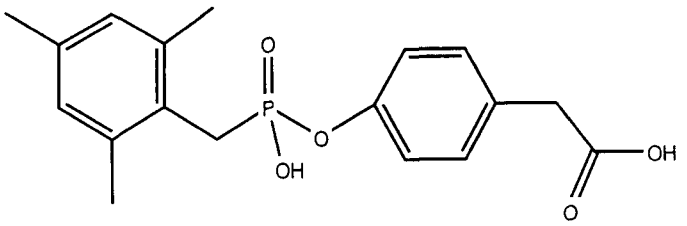

Fig. 3. Chemical structure of RT3 phosphonate transition state analog.

Table 1
Grouping of Mouse RT3 Binders
According to PCR Pattern

PCR pattern	No. of clones in PAN1, 48 positives from 364	No. of clones in PAN2, 114 positives from 148
A	18	78
B	2	24
C	2	3
D	3	3
a	2	
e	2	
q	2	
i	3	
j	3	
E		2
Unique patterns from PAN1	10	
Unique patterns from PAN2		4
Unknown	1	

ELISAs were carried out by coating plates with 2 μg/mL RT3-BSA, incubating with culture supernatant, and detecting with the mouse anti-*myc* antibody, 9E10.

Forty-eight of 364 clones were positive from PAN 1, and 114 of 184 clones were positive from PAN2 (Table 1). PCR was carried out on each clone using primers that flank the insert and the product digested with the frequent cutter, *Bst*N1 (*5*). All clones were grouped according to the digest pattern generated (summarized in Table 1). All clones were negative for BSA binding with the exception of clones in group D.

Seventeen of 48 binders analyzed from PAN1 (35%) had pattern A. In PAN2, a total of 78 binding clones from 115 (68%) had PCR pattern A. Pattern B was found in 4.1% of clones from PAN1 and 21% from PAN2; 4.1% from PAN1 had pattern C, whereas 2.6% from PAN 2 had this pattern. Pattern D was found in 62% clones from PAN1 and 2.6% from PAN 2. Thus, the proportion of positives from each group appears to alter from PAN 1 to PAN 2. This could result in the loss of potentially catalytic clones after several rounds of panning if selection is based solely on strength of binding to TSA.

At least 15 other patterns were found in PAN1 with many appearing only once. Many of the patterns present in PAN1 were not identified in PAN2. In addition, some patterns appeared in PAN 2 that had not been identified in PAN 1. This argues that we are seeing "the tip of the iceberg," and there is much greater diversity in the library than have been identified by this analysis.

Determining Diversity Within Groups by Sequencing

A large proportion of clones from PAN1 and PAN2 fall into the PCR A pattern group. To determine the degree of diversity within this group, a number of these clones were sequenced. Furthermore, in an attempt to determine whether the same heavy and light chains were being used within other major pattern groups, some representative clones from the other major groups were sequenced.

Eight closely related light chains were found in the 15 different clones from pattern A (Fig. 4). The chain associated with clones mR6 and mR8 differs from the germline V gene by a single silent nucleotide change. The chain used in mR9 and mR27 differs from mR6 and mR8 by an additional single silent mutation. Thus, these four clones share the same protein sequence as the germline. Clones mR9 and mR27 have used different primers to derive the same sequence, indicating that they are independent isolates of this same sequence. This is also true of clones mR3 and mR25, which have two amino acid changes from germline. Most changes in these and other clones are clustered in FR3, CDR3, and FR4. The light chain associated with pattern C (clone mR80) appears to be derived from the same germline as used in pattern A. Clones representing the other PCR patterns appear to use different germline-derived sequences (not shown).

Analysis of the heavy-chain sequences associated with PCR pattern A reveals that, as for the light chain, they are all closely related, but in most cases are different from each other (Fig. 4). The alignment to germline is less clear in these samples. The closest germline belongs to subgroup VH-II, but there are numerous differences from this germline in the isolated clones. In addition, there appears to be a greater number of amino acid

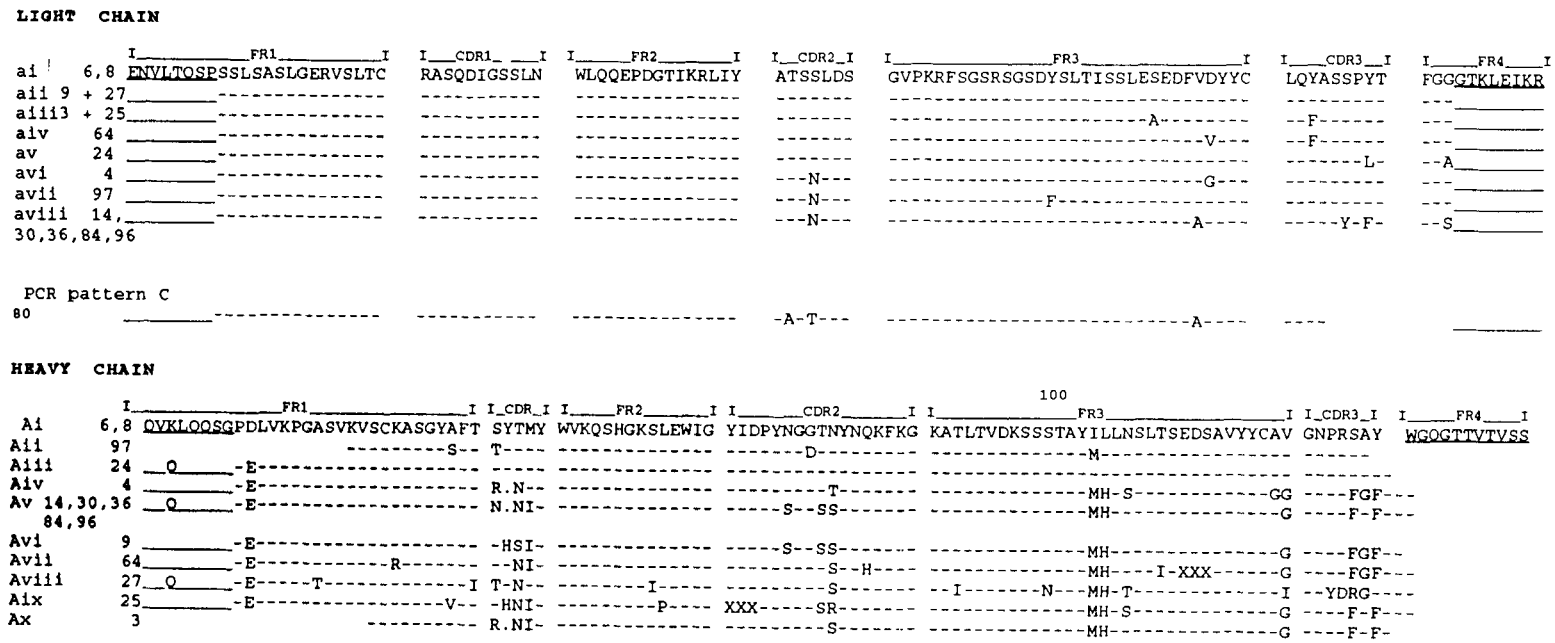

Fig. 4. Sequence comparison of mouse RT3 binders from PCR pattern group A. The underlined sequence represents the primer encoded sequence. The full amino acid sequence of clone 6 (and 8) is shown on the top line and any changes from this sequence are indicated for each clone. 2. Clones 6 and 8 are identical to each other using the same primers, and may represent multiple isolations of the same clone. The same is true for clones 14, 30, 36, 84, and 96. 3. Clones 9 and 27 have the same antibody sequence, but use different primers and so represent independent isolates of the same mouse-derived sequence. The same is true for clones 3 and 25.

Table 2
Chain Usage of Mouse RT3 Binders from PCR Pattern A*

Heavy chains	Light chains							
	ai	aii	aiii	aiv	av	avi	avii	aviii
Ai	6, 8							
Aii							97	
Aiii					24			
Aiv						4		
Av								14, 30, 36, 84 96
Avi		9						
Avii				64				
Aviii		27						
Aix			25					
Ax			3					

*The results of Fig. 4 are summarized here. The identifier number of each clone is shown in the boxes.

changes between clones. As expected, the changes are clustered in the CDRs.

The heavy chains of pattern B align to a different germline and again show numerous changes from this. All four clones in this group appear to be identical (not shown). Thus, it appears that the clones sequenced from pattern B are multiple isolates of the same antibody. The heavy chains of pattern C (80) and pattern I (95) are closely related to that of pattern B (not shown).

Table 2 collates the information derived from sequencing the clones in PCR pattern A. Each different light-chain sequence in the group is given a label ai-aviii. Each different heavy-chain sequence in the group is given a label Ai-Ax. Clones 14, 30, 36 (PAN1) 84, 96 (PAN2) are identical and probably represent duplicate isolates of the same initial clone. Clones 6 and 8 are also identical to each other. Otherwise, every clone is different. There are two cases where the same light chain has been used with two different heavy chains. (As described earlier, the light chains in each pairing used different primers.) Apart from the duplicate isolates, there are no cases here of the same heavy chain being used in different clones. Thus, PCR and sequencing experiments suggest that there is indeed a great diversity in the mouse library both at the gross scale, as judged by PCR analysis, and at a more subtle level, as judged by sequencing.

Determination of Affinity

The affinities of a number of clones were determined using an indirect ELISA method as described by Friguet et al. (*11*). Essentially, antibody and antigen were incubated in solution until equilibrium was reached.

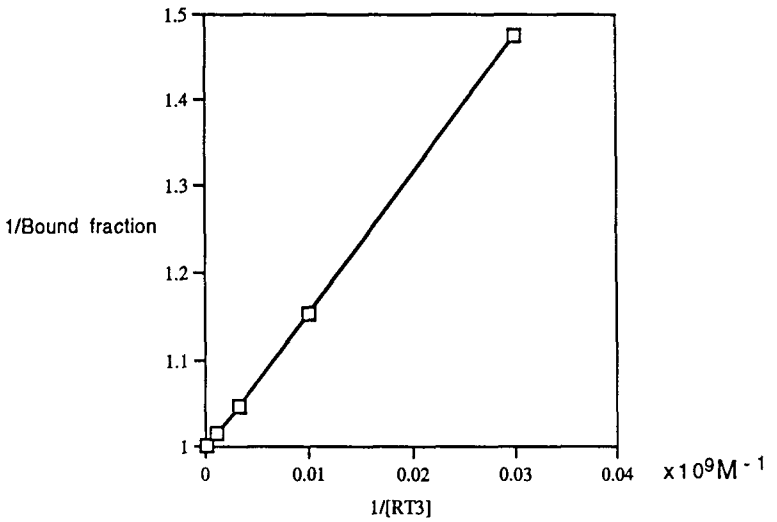

Fig. 5. Determination of K_d by equilibrium assay. Measurement of K_d of mo64 by equilibrium ELISA and Klotz plot is as described in Methods and in refs. *15* and *16*.

ELISA was then used to measure the amount of free antibody present at equilibrium with various concentrations of antigen. It is important to ensure that binding to the ELISA plate itself does not itself disturb the equilibrium being measured. As recommended by Friguet et al., antibody concentrations were used that were at least 10-fold below the affinities being measured. Under these conditions, the relationship between the proportion of antibody bound (x), antigen concentration (a_o), and dissociation constant (K_d) is represented by:

$$1/x = 1 + K_d/a_o$$

Plotting $1/x$ against $1/a_o$ enables K_d to be calculated from the gradient. A representative plot is shown in Fig. 5, where a dissociation constant of 15 n*M* was calculated for the clone number 64 (mo64).

DISCUSSION

We have constructed a phage display library from antibody V genes derived from the spleens of mice hyperimmunized with the transition-state analog RT3. This library was constructed in a vector designed to facilitate expression, detection, and purification of soluble antibody fragments without subcloning. A high proportion of binders (13%) were identified after only one round of panning. The diversity of binders from one and two rounds of panning was analyzed by carrying out PCR on each clone, digesting with a common cutting enzyme, and analyzing on an

agarose gel (5). This analysis revealed that at least 24 groups of clones had been isolated. As well as the diversity revealed at this gross level by PCR analysis, there is a great deal of heterogeneity in the actual sequences of heavy and light chains within the major PCR pattern group (pattern A). Ten different heavy chains and eight different light chains were identified from only 15 clones.

Clackson et al. (5) have previously shown that a range of binders can be isolated from a mouse immune repertoire. The diversity of the range of binders was increased by a chain shuffling procedure, where heavy and light chains from primary binders were recombined with a repertoire of complementary chains to give a large group of different, but closely related chains. The presence of the larger group of closely related combinations in the initial repertoire of this study may be a reflection of the larger size of the initial library or a greater bias for selection of clones containing the heavy and light chains typified by this group. Alternatively, it may arise owing to differences in the mouse immune response to the RT3 antigen.

An affinity of 15 n*M* was measured for a typical clone from the major PCR pattern group. This again confirms the earlier observation (5) that the affinities achieved from immunized repertoires displayed on phage are of the same order as affinities from hybridoma clones. These clones are, however, accessed more easily and in a form that permits rapid characterization by PCR and sequencing. Furthermore, these clones are in a form that can readily be mutagenized by chain shuffling, PCR, and in vitro mutagenesis prior to reselection for improved binders. For example, Marks et al. (*14*) improved the affinity of a clone derived from a non-immunized human source from $3.2 \times 10^{-7}M$ to $1.5 \times 10^{-8}M$ using a light-chain shuffling procedure as described above. A further level of improvement was achieved by shuffling complementary determining regions from within the V genes to give an affinity of $1.1 \times 10^{-9}M$. A similar approach has successfully been followed to isolate and affinity mature human binders to RT3 (unpublished).

The above observations and discussions show that the phage system will provide huge numbers of TSA binders as candidates for catalytic screening, and suggests that problems will not be in isolating different binders, but in actually sorting out catalysts from noncatalysts. Within its present *modus operandi,* the phage system will not isolate according to catalytic activity.

Catalytic selection of phage will be effected if catalysis by a displayed enzyme or catalytic antibody either leads to retention on a matrix or leads to specific elution from a matrix under controlled conditions. Fastrez et al. (*see* pp. 175–190) have demonstrated the first of these options. They report that β lactamase on the phage surface can react with a biotinylated suicide substrate to label the displayed active site. This approach can be used for the enrichment of phage-enzyme particles displaying an active form of enzyme from an inactive variant.

In conclusion, the power of the phage system lies with its ability to isolate antibody or enzyme genes (*4,12*) according to the binding specificity and affinities they encode. The main deficiency of this approach for the isolation of catalytic antibodies is that the characteristic being sought, namely catalysis, does not form the basis of the selection. Thus, the phage-display system will suffer the same limitation as hybridoma technology in the generation of catalytic antibodies. Coupling of activity to selection will be required if the phage system is to help bring catalytic antibodies to the point where useful, high-level activities are generated.

Note Added in Proof

Higher affinity clones have now been isolated directly from very large libraries (6×10^{10} clones) derived from nonimmunized sources (*17*).

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DISCUSSION

J. McCafferty

Stollar: When you make the libraries from animals immunized with phenyloxazolone, what fraction of the clones have the V_H-V_L combinations that have shown up in hybridomas? Are some of the combinations that occur in vivo reproduced, or are they all new combinations?

McCafferty: Have the hybridomas been sequenced, Jeff?

Foote: Yes, they were sequenced, but I do not know the answer to the question.

Stollar: Perhaps you could comment on what your expectation might be on that important question?

McCafferty: If the antibodies present in the hybridoma contain the best combinations available in the system, then my expectation would be that those combinations will be found again, if not in the initial library, then upon shuffling of the subunits. Another question, of course, is whether the pairs of heavy and light chains present in the hybridoma are indeed the best pairs for binding oxazolone. If they are not, then they may well be lost. (Note added in proof: It appears that the major antioxazalone V_H and V_L genes found in hybridomas were isolated in the phage library used by Clackson et al. [*1*], but they were not found in the same combinations [2]. This may partly be explained by the fact that a library of only 200,000 clones was used in that particular phage library.)

Zouali: For human antibodies, you have used primers directed toward the 5′-end of FR1. Do you not think that this may introduce a bias in the selection of the repertoire?

McCafferty: In the mouse library, we use essentially a mixture of primers by introducing wobbles at bases. The human libraries were made by using family-specific primers.

Zouali: Why do not you use the anchored PCR method?

McCafferty: The primers were designed by Jim Marks. Essentially, we just want to produce a diverse population of human heavy- and light-chain combinations. Loss of certain chains is perhaps even less of a worry in these nonimmunized libraries than it is in the immunized libraries, or during attempts to derive recombinant antibodies from hybridomas. We have not used anchored PCR as a way of deriving the genes. Jim originally made these libraries with FR1 primers, and we continue to use these.

Zouali: About 50% of the V genes cannot be amplified using the family-specific primers.

McCafferty: This is untrue. Analysis of the germline V genes (3) shows that the family-specific primers of Marks et al. (4) match at least 90% of the heavy-chain V genes sufficiently well for PCR. In addition, Hoogenboom and Winter (1992) (5) have actually used the primers of Marks et al. 1991 (6) to PCR amplify all 50 germline V genes successfully. Your assertion is therefore untrue on both theoretical and practical grounds as far as the heavy chains are concerned. All we are trying to do is just generate a source of heavy and light chains. Using this approach, we have been able to produce binding antibodies from a nonimmunized library to most of the antigens that we have tried.

Paul: In your answer to Professor Stollar's question, were you implying that natural pairs of heavy and light chains may not be the best binders? Are you suggesting that the chain shuffling may produce better binders?

McCafferty: I do not have any information to say the shuffled libraries will or will not yield better combinations. If the natural combination is the best combination, I would suspect that this combination will be reproduced in the library. On the other hand, if there are better combinations to be made, those may come through.

Paul: Stollar's and Zouali's questions relate to the level of diversity of the library. Another point at which diversity may be lost is during introduction of the linker, when you join the V_H and the V_L by splicing by overlap extension. What is the efficiency of that reaction?

McCafferty: We do a mock PCR with the three fragments, the V_H, V_L, and the linker, and run the material from the PCR on a gel. If I can detect any linkage at all, even 5 ng of DNA, that tells me that the diversity will be very large. Then, we use that linked material as template in secondary PCR to produce the bulk DNA that is actually used to make the library. I am not worried about losing diversity by chain shuffling. The only worry would be if only a handful of clones have been successfully linked. I do not think that happens with our libraries.

Hansen: I was wondering whether you have enough information to generalize about the affinities you could expect if you start with a naive library vs one from an immunized animal.

McCafferty: The example from the Marks' paper is that the starting antibody from a naive library has a K_d $3.2 \times 10^{-7} M$. After a round of chain shuffling and CDR shuffling, they had an antibody of 1 nM K_d. Perhaps at that point, one could look at the sequence to try to identify hot spots for mutagenesis to try to take the affinity a little higher. The bottom end of the affinities we are dealing with is in the μM range. The Marks paper is a good example of getting up to higher affinities.

Hansen: Then, antibodies from naive libraries are about two to three orders of magnitude lower in affinity?

McCafferty: Yes.

DISCUSSION REFERENCES

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