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Peptide phage libraries can be an efficient tool for identifying antibody ligands for polyclonal antisera

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SUMMARY

We have examined the potential of isolating ligands for polyclonal antibodies from a nanopeptide phage library. The library was screened with a rabbit polyclonal antiserum raised against a synthetic peptide (ALWFRNHFVFGGGTKVT). Following screening, the positive phages were tested in an ELISA for their reactivity with the antiserum. Phages that showed positive reactivity with the antiserum compared with a normal rabbit serum were selected and their displayed peptides were determined. Among the 36 random positive clones, 31 clones carried the sequence AVFGGGTKL, PFFGGGSRA or APTGGSKRT that have a significant homology to the immunizing peptide. Five positive phages displayed the ATNIFIEGT sequence, which has no obvious linear homology with either the other selected peptides or with the peptide used for immunization. In contrast to the control peptide, the immunizing peptide inhibited binding of the antiserum to the peptide-displaying phages in a dose-dependent manner, thus demonstrating the specificity of the interaction. Furthermore, the rabbit B cell response to the peptide was found to be limited and focused on its C-terminal. Taken together, our data demonstrate the potential of random peptide phage libraries for defining epitopes for polyclonal antisera as well as for investigation of the nature of B cell responses to any given antigen.

Keywords phage library immune serum mapping

INTRODUCTION

Random peptide phage libraries have become a powerful tool for identifying determinants recognized by homogeneous proteins such as MoAbs [1]. This strategy is based upon the ability of filamentous bacteriophages to display foreign peptides on their surfaces. The technique involves specific screening and affinity selection of phages displaying peptides that are ligands for particular proteins [2]. The potential of the random peptide phage libraries for defining the specificities of MoAbs has been demonstrated with great success [3,4]. We have been interested in investigating the possibility of selecting ligands from the epitope libraries for antibodies in polyclonal antisera, whether or not the antigen is known [5]. Such an approach would allow us to understand the specificity of a humoral response without having to establish B cell hybridomas. In addition, extending the technique to polyclonal antibodies would also allow a comparison of the antibody repertoires of individuals infected by the same or related pathogens. In contrast to MoAbs, the sera from infected or immunized individuals will, in addition to antibodies specific for the antigen, contain a vast majority of other antibodies of unknown or irrelevant specificities. Thus, during phage selection with antiserum, phages with irrelevant ligands could also be enriched. Such undesirable phages could be subtracted by the use of normal sera in an affinity selection step [5]. However, such a subtraction is dependent on both the affinity and the concentration of each antibody within the mixture. These factors have made the removal of undesirable phages incomplete [5,6]. Furthermore, it has in many cases been difficult to find significant linear homology between the short linear peptides that were selected by the use of polyclonal sera from rheumatoid arthritis (RA) patients, and potential autoantigens ([5,7], Dybwad *et al.*, unpublished results). These observations have raised the question of whether a peptide phage library really can be used as a tool for defining the antigens for polyclonal sera. To answer this question, we have constructed a random nanopeptide phage library and screened it with a rabbit antiserum raised against a synthetic peptide. The simplicity of this system would allow us to compare the peptides displayed by the selected phages with the peptide used for immunization.

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MATERIALS AND METHODS

Construction of a nanopeptide phage library

A collection of oligonucleotides encoding nanopeptides were synthesized with the sequence 5′-AAAGAATTC(NNK)$_8$-NNGGATCCAGCCCGCGAATAACGCGGGCTGGATC-3′ (Biotechnology Centre, Oslo, Norway), where N is equimolar amounts of A, T, C or G, and K is G or T in equimolar amounts. This oligonucleotide is self-annealing and self-priming, thereby avoiding the usual hybridization step [3,8–10]. The complementary strand was synthesized by extension of the 3′ end with T7 DNA polymerase. The double-stranded degenerate oligonucleotide was digested with *Eco*RI and *Bam*HI, purified from a 20% (w/v) polyacrylamide gel and then ligated into *Eco*RI, *Bam*HI-linearized phagemid vector PC89 [8]. A total of 15 μg of vector was then ligated with 300 ng purified double-stranded degenerated oligonucleotide. The ligation mixture (0·5 ml) was phenol extracted and ethanol precipitated at −70°C for 12 h. Following sedimentation at 10 000 ***g*** at 4°C for 30 min, the pellet was rinsed with cold 70% ethanol, air-dried, and then redissolved in 10 μl water. Transformation of 1 μl precipitated ligation mix into each of 50-μl aliquots of electrocompetent XL1 cells was performed by electroporation using a Gene Pulser apparatus (BioRad, Hercules, CA) at 2·5 kV, 200 Ω, 25 μF. Immediately after electroporation, 1 ml TY medium was added followed by incubation with shaking at 37°C for 1 h. The library was amplified by superinfection of M13 helper phages, and then cultured in LB medium containing ampicillin and kanamycin. The phage particles were purified by polyethylene glycol (PEG) precipitation [5]. The library was found to have a complexity of approximately 2×10^7 different epitopes, and the percentage of clones forming blue colonies, representing inserts which are in-frame with the α-peptide, was about 50%

Synthetic peptides

Synthetic peptides were synthesized and high performance liquid chromatography (HPLC)-purified by Med Probe, (Oslo, Norway), the Public Health Research Institute (New York, NY) and Basel Institute for Immunology (Basel, Switzerland).

Selection of phages by biopanning

A rabbit polyclonal antiserum was raised against the 91–107 peptide (ALWFRNHFVFGGGTKVT) of the mouse $\lambda2^{315}$ immunoglobulin light chain [11], by repetitions of injection of the peptide, first in Freund's complete adjuvant (FCA), then in incomplete adjuvant. The antiserum reacted with complete $\lambda2^{315}$ free light chain in ELISA (data not shown). Furthermore, serum antibodies could be eluted from a $\lambda2^{315}$–Sepharose column; such affinity-purified antibodies reacted to both 91–107 peptide and the $\lambda2^{315}$ light chain in ELISA (data not shown). Thus, at least parts of the 91–107 peptide within the $\lambda2^{315}$ light chain seem to be exposed to B cells, as would be expected of a peptide corresponding to a CDR3 loop. A pool of post-immunization serum was used in the present experiments for an affinity selection essentially as described by Dybwad *et al.* [5]. Briefly, 10 μl of undiluted antiserum were preabsorbed overnight at 4°C with 10^{10} transducing units (TU) of wild type phage, and then incubated with 10^{11} TU of the constructed library. Following incubation at 4°C overnight, biotinylated secondary anti-rabbit IgG antibodies (preabsorbed overnight with the wild type phage) were added. Phages bound to antibodies were then captured on a streptavidin-coated plate. After extensive washing the phages were eluted from the plate with acid, neutralized and transfected into competent XL1 cells.

Screening for antibody-binding phages in ELISA

Following three rounds of bipanning, clones were picked at random and amplified. Phage particles were prepared and then tested for binding to rabbit anti-$\lambda2^{315}$ antibodies and normal rabbit immunoglobulins, by ELISA as described previously [5]. Reactivity was detected by an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, St Louis, MO). A phage was considered to be specifically bound by the anti-λ^{315} antibodies if the $A_a - A_n > 0{\cdot}2$, where A_a and A_n are the absorbence of antiserum and normal serum binding to phage-coated wells, respectively. Thus, phages binding antibodies present in normal rabbit serum were eliminated.

Determination of peptide sequences

Phages from the supernatants of positive colonies were precipitated with PEG, and their single-strand DNA were prepared by phenol extraction and sequenced with the use of the Sequenase version II Kit (United States Biochemical, Cleveland, OH).

Inhibition experiments

Increasing concentrations of the synthetic peptides or a control peptide (SAAPGQKVTISCSG) were incubated with the antiserum (diluted 1:1000). After incubation overnight at 4°C, mixtures were dispensed into plates coated with phages displaying C1 or C4 peptide (10^{10} TU/ml). The inhibition of the antiserum binding to the phages by the peptides was expressed as percentage of antiserum binding to the phages. In the case of inhibition with the peptides 91–107, 91–101 and 99–107, the plates were coated with 100 μl of a 0·45-μM solution of 91–107 peptide, and the results were expressed as percentage of the antiserum binding to 91–107 peptide without competitor.

RESULTS

Complexity of the peptide phage library

Peptide diversity displayed on the cell surface of the phages is dependent on the diversity of the inserted oligonucleotides, chemically synthesized to contain degenerate codons of the structure $(NNK)_n$, where N is A, C, G or T and K is G or T. This codon motif encodes all 20 amino acids and one amber stop codon. In the present study we have constructed a nanopeptide phage library using the above combination. In addition, we have employed self-priming oligonucleotides which increase the efficiency of both annealing and priming reactions (Fig. 1). The complexity of the nanopeptide phage library was analysed by sequencing random individual clones. The data presented in Table 1 indicate that all the amino acids were present, and that the frequency of each amino acid was in accordance with the frequency predicted from the number of possible codons for each amino acid [4].

Screening of the peptide phage library with the rabbit polyclonal antiserum

In order to see whether the peptide phage library could identify

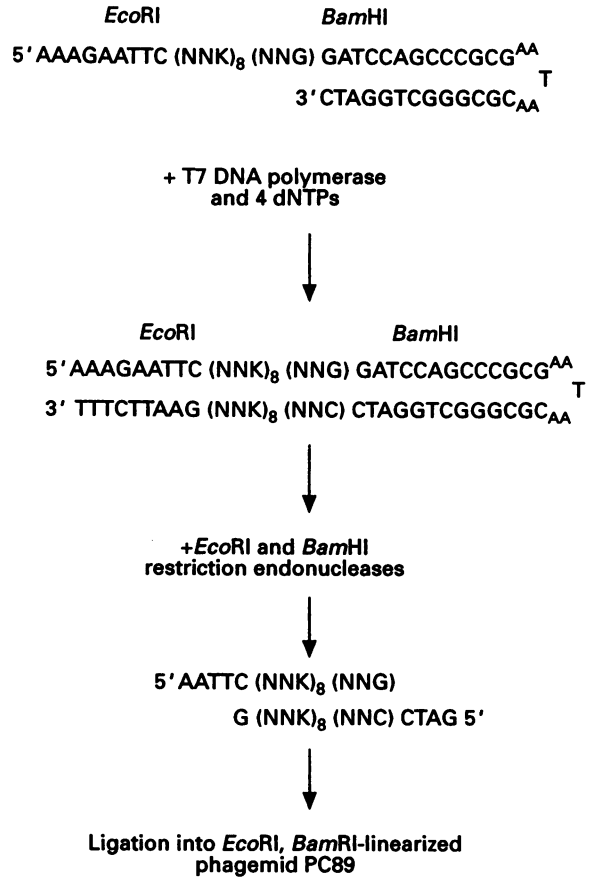

Fig. 1. The strategy used to construct the nanopeptide phage library. Self-annealing and self-priming partially degenerate oligonucleotides were inserted into the PC89 vector as described in Materials and Methods.

ligands for polyclonal antibodies, the nanopeptide phage library was screened with a rabbit antiserum directed against a synthetic peptide comprising residues 91–107 of the mouse $\lambda 2^{315}$ immunoglobulin light chain [11]. Following three rounds of selection with the nanopeptide phage library, random phages were amplified and their reactivity with the antiserum and normal serum was investigated by ELISA. Thirty-six phages

Table 1. Peptide sequences displayed by 20 random phages derived from the unselected library

ARACRVSMS	ASTAVEIPW
FRIGAQLWG	RRLDAKGQL
QSPGADGYL	QSWVGMPSW
YMRRGLCVG	LCLRRTYAT
VGERRVVNW	GRSVIEKAP
RGEGVTDLG	KTERPVGER
GGRGSTHNG	GVPLLAESA
QRSRTIQNV	VERRAYFAM
KAHRPRGIL	QRLVILMVV
CVLASGSLV	FHRGWGPSM

Table 2. Peptide sequences displayed by the selected phages that bind to 91–107 peptide-specific antiserum (a). The number of times each sequence occurred in the 36 phages is given in the frequency column. (b) The peptide sequences are aligned with the 91–107 peptide

a.	Clone	Sequence of the insert	Frequency
	C1	AVFGGGTKL	24
	C2	PFFGGGSRA	4
	C3	APTGGSKRT	3
	C4	ATNIFIEGT	5

b.	Sequence
91–107	ALWFRNHF**VFGGGTK**VT
C1	A**VFGGGTK**L
C2	PF**FGGGSR**A
C3	APT**GGSK**RT
C4	ATNI**F**I**E**G**T**

The replacement of T to S and K to R are conserved substitutions.

that reacted preferentially with the antiserum were propagated and their DNA were sequenced (Table 2a). The insert sequences encoded highly related nanopeptides that were homologous with the immunizing peptide. Thirty-one peptide sequences shared a common GGTK tetrapeptide core sequence (Table 2b), suggesting the involvement of GGTK in the binding of the antibodies to the phages (the replacements of T to S and K to R are conservative substitutions), while the C4 peptide showed no obvious linear homology with the 91–107 peptide.

Inhibition experiments

To confirm that the selection of the phages was due to the binding of 91–107 peptide-specific antibodies to the peptide displayed by these phages, inhibition experiments were performed. Figure 2 shows that the 91–107 peptide inhibited in a

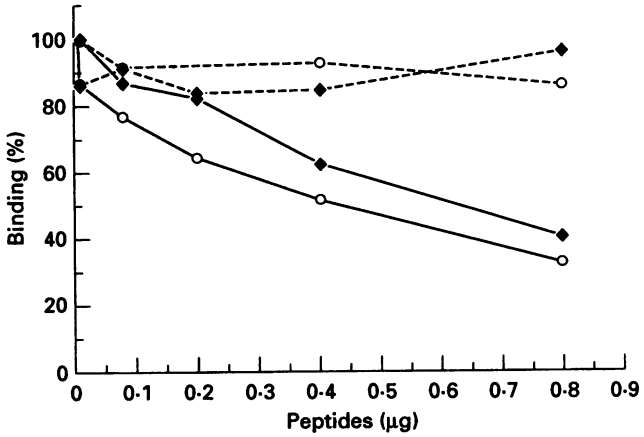

Fig. 2. Inhibition of the binding of antiserum of phages displaying C1 peptide (○) or C4 peptide (- - - -). With the 91–107 peptide (——) and control peptide (SAAPGQKVTISCSG) (◆).

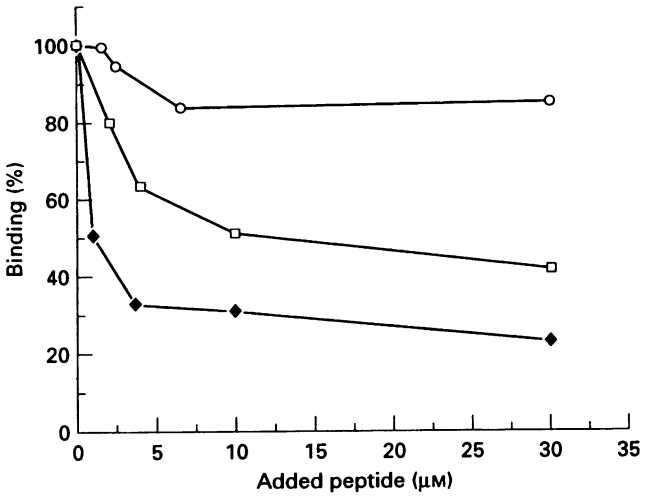

Fig. 3. Inhibition of the binding of antiserum to the 91–107 peptide with the 91–101 peptide (○), 99–107 peptide (□) and 91–107 peptide (◆).

dose-dependent manner the binding of antibodies to phages displaying the C1 peptide or C4 peptide, while the control peptide appeared to have no effect, thus confirming the specificity of the selection.

The rabbit B cell response to the immunizing peptide was found to be limited

Surprisingly, the 36 random sequenced phages displayed only four peptide sequences (Table 2). These sequences were found to be located near the carboxy-terminal end of the 91–107 peptide, suggesting that the B cell response against the peptide was mainly directed to the 99–107 sequence. To verify this observation, two synthetic peptides corresponding to position 91–101 and 99–107 were synthesized and assayed for their ability to inhibit the binding of the antiserum to the 91–107 peptide. The binding of the antiserum to the 91–107 peptide-coated plates was significantly inhibited with the 99–107 peptide, but not as much as with the 91–107 peptide itself, while only weak inhibition was observed with the 91–101 peptide (Fig. 3).

DISCUSSION

By screening a random nanopeptide phage library with an antiserum, we have in the present study demonstrated that the peptide phage library can be used to identify epitopes for polyclonal antibodies. Both linear and possible mimic epitopes were identified. Out of 36 positive phages that we have sequenced, 25 displayed the AVFGGGTKL peptide sequence that is similar to the C-terminal of the 91–107 peptide. Thus, our data indicated that peptide phage libraries could be of general use as a tool to identify linear ligands for polyclonal antiserum, and may suggest that if the epitope is continuous, the homology between the selected phage epitope sequences and the antigen sequence would in general be high. In addition to this observation, our data indicated that the rabbit B cell response to the 91–107 peptide was limited, since 36 random positive clones displayed only four different peptide sequences.

The reactivity of the C4 peptide with the antiserum in ELISA experiments, as well as the inhibition of its binding to the antiserum by the 91–107 peptide (Fig. 2) suggested that the C4 epitope is a mimic epitope. In contrast to the 91–101 peptide, the 99–107 peptide competed with the phage displaying C1 and to lesser extent the phage displaying the C4 peptide (data not shown), suggesting that if the C4 peptide is a mimotope, it would probably mimic the C-terminal of the 91–107 peptide. Clearly from the present study and other studies it seems that some selected ligands do not necessarily resemble the natural ones, but could rather mimic their binding properties [6]. The peptide phage libraries can therefore be used as a source of selecting peptides that show little sequence homology to the natural antigen, such as the C4 peptide, but nevertheless fit the antigen combining site of the antibodies (for review see [1]). These 'novel' epitopes could be useful candidates in vaccine development, where the natural antigens themselves are unable to induce a significant protective immunity. In this regard, it was recently demonstrated that mimotopes displayed on phages elicited a strong response to hepatitis B virus surface antigen (HBsAg) in a strain of mouse reported to show a low response to a recombinant HBsAg vaccine [12]. In addition to the demonstration of the utility of the phage library for isolation of ligands for polyclonal antiserum, the peptide sequences displayed by the positive clones indicated that the B cell response was focused on the C-terminal of the 91–107 peptide, at least in this particular rabbit. However, it is possible that other B cell specificities were present in the antiserum, but these were not selected. As can be seen from Fig. 3, the 91–107 peptide competed with itself much better than the 99–107 peptide. These results indicated that the N terminus of the peptide may be acting as a real scaffold for shaping the B cell epitopes within its C terminus, and/or there is presence of conformation-dependent epitopes that were not selected during the three rounds of biopanning. Nevertheless, our data encourage the use of peptide phage libaries in studying the nature of B cell responses to known antigens, as an alternative to B cell hybridomas. Recently, we have found that the human B cell response against the tumour necrosis factor-alpha (TNF-α) protein is limited, and that the naturally occurring autoantibodies against TNF-α protein may recognize discontinuous epitopes rather than linear epitopes [13].

Taken together, our results demonstrate that peptide phage libraries can be a useful tool in the identification of B cell ligands for polyclonal antisera. This novel strategy can give clues to the nature of microorganisms that may be responsible for the etiology of autoimmune diseases.

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