Puzzling peptides from a phage display library

Gholam Reza Hashemi Tabar[1] and Patrick Carnegie[2]

[1]*Pathobiology Department, School of Veterinary Medicine, Ferdowsi University of Mashad, Mashhad, Iran.*
[2]*Biotechnology Research Group, Murdoch University, Perth, Western Australia.*

Received 22 February 2003; accepted 16 July 2003

Abstract
The commercial availability of random peptide libraries displayed on the M13 phage is increasing their use for studies on epitope identification, enzyme inhibitors, receptor ligands, etc. In this study two experiments where planned for selection of peptides. First with sheep antibodies, the positive selector was IgG, prepared on Protein G column from a pool of 11 sheeps immunized, with a vaccine prepared from larvae of Lucilia cuprina, against blowfly strike. The negative selector was IgG from the same sheeps before vaccination and IgG from vaccinated non-immunized sheeps. Four rounds of positive and negative selections with IgG from sheep were done respectively. In the second experiment using human IgG prepared on a fresh Protein G column, again four rounds of positive selection with either IgG from MS and schizophrenia patients were performed. This assay was alternated with two negative selections of schizophrenia IgG on MS-associated peptides and vice versa and two negative selections on each with a control IgG. After the fourth negative selection in each experiment, the phage was amplified and random clones were picked for nucleotide sequencing. A total of 44 peptides were sorted with the PILEUP program and there was a large number of 12-mer peptides containing either 4 or 5 methionine residues.
Keywords*: Random peptide libraries, Methionine, M13 phage, Sheep, Human.*

INTRODUCTION

Screening of random peptide libraries (RPLs) displayed on phage is a new and powerful technique that, in contrast to the molecular biology approach to obtain useful antigens, is relatively low cost to apply and can quite rapidly produce useful products from mimics of epitopes. These mimotopes are not necessarily identical to the epitopes in the natural antibodies. During routine screening of RPLs displayed on phage, clones can be isolated that bound to the polystyrene surface used to immobilize the target protein (Adey *et al.*, 1995). The RPLs are constructed by inserting 21 to 36 random nucleotides into the gene for an appropriate coat protein in a phage which is propagated in *E. coli* (Parmley and Smith, 1988). Two viral coat proteins have been frequently used to display epitopes, a minor coat protein III (pIII) and the major coat protein pVIII (Scott, 1992). P III, located at one end of the phage, is required for infectivity and stability of the particle (Rakonjac and Model, 1998). These libraries include chemical synthesized peptides (Lam *et al*., 1991; Houghten *et al*., 1991) and biological forms which have become effective tools for research in immunology, pharmacology and physiology (Scott and Smith, 1990). Peptides containing the consensus amino acid sequence motif EPDW were identified using phage display library (Caparon *et al*., 1996). These RPLs typically provide over a billion peptides at a fraction of the cost of chemically synthesized RPL (Devlin *et al*., 1990; Cwirla *et al*., 1990). The use of pathogen or antigen derived from that can be avoided, whenever this technique is applied for identification of potential vaccines (Meola *et al*., 1995). Phage display has proven to be a robust and convenient technology for the selection of high-quality human antibodies from diverse libraries and for the isolation of an antibody that binds to a specific subunit of a macromolecular assembly (Kretzschmar and von Ruden, 2002;

Correspondence to: **Gholam Reza Hashemi Tabar, Ph.D**
Tel: +98 511 6620127, Fax: +98 511 6620166
E-mail: hashemitabar@yahoo.com

Rubinstein *et al*., 2003). It is possible to identify 'disease specific' mimotopes without previous knowledge of the pathogenic antigen (Folgori *et al.*, 1994; Delmastro *et al*., 1997). This technology has become very popular means of finding ligands with high affinities to given targets. Despite their success, they suffer from numerous sources of error and bias, such as very low initial concentrations of species, non-specific binding, and the sampling of only a tiny fraction of the library at the end of an experiment (Levitan, 1998). Purification of the peptides binding to the ligand is achieved by an *in vitro* selection process called biopanning. Enrichment of reactive phage relative to unreactive phage occurs with alternate rounds of affinity selection to the desired molecular target and amplification of the specifically bound phage. This allows the isolation of rare binding species contained in the phage peptide libraries (Daniels and Lane, 1996). The aim of this study was to select peptides by Ph.D™ library useful in vaccine development.

MATERIALS AND METHODS

Phage selection: In our experiments with sheep antibodies, the positive selector was IgG, prepared on protein G column (Amersham Pharmacia Biotech, Sydney, NSW, Australia) from a pool of 11 sheep protected with a vaccine prepared from larvae of *Lucilia cuprina*, against blowfly strike. *Lucilia cuprina* is the most important ectoparasite of sheep and the larvae of this parasite feed on sheep skin over a period of 3-4 days, creating an edematous lesion which attracts further secondary and tertiary flies. The negative selectors were the IgG from the same sheep before vaccination and IgG from vaccinated sheep which were not protected (supplied by Dr. VM Bowles and Professor MR Brandon, University of Melbourne). Four positive selections with IgG from the protected sheep were alternated with negative selections, two with the pre-immune IgG and two with the IgG from the unprotected sheep. In the experiments with human IgG, prepared on a fresh Protein G column, it was obtained from sera from 39 patients with multiple sclerosis (MS) which is a common demyelinating disease which results in death, 7 to 30 years after the first symptoms appear, 39 patients with schizophrenia (a disease of the brain, is one of the most disabling and emotionally devastating illnesses known to man. But because it has been misunderstood for so long, it has received relatively less attention and its victims have been undeservingly stigmatized) and 12 samples (control IgG) from people who had died from non-nervous system diseases (supplied by Professors W.W. Tourtellotte, University of Los Angeles and A.V. Jablensky, University of Western Australia). Four rounds of positive selection with IgG from both MS and schizophrenia patients were done respectively and were alternated with two negative selections of schizophrenia IgG on the MS-associated peptides and *vice versa* and two negative selections on each with a control IgG. Biopanning was carried out according to the recipe of the Ph.D.12™ kit (New England Biolabs, USA). Two culture flasks (Corning Costar Corporation, tissue culture flask, canted neck, plug lid, Cat. No. 430168-2510025) were coated with 200 µg of either sheep or human IgG in 2 ml of 0.1 M $NaHCO_3$, pH 8.6 and incubated overnight at 4°C. The following day, solutions were poured off from each flask and the flasks were washed 3 times with TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). The flasks were blocked with 50ml of blocking buffer (0.1 M $NaHCO_3$, 5 mg/ml BSA and 0.02% NaN_3, pH 8.6 and incubated for 1-2 h at 4°C. The blocking buffer was discarded and the flasks were washed 6 times with TBST (TBS + 0.1% Tween-20 v/v). With each biopanning the library of 1.9×10^9 different peptides displayed on the pIII protein of the M13 phage was added to the flasks containing sheep or human IgG used as the positive selector. The library was diluted in 2 ml of TBST and pipetted into coated flasks and rocked gently at room temperature for 60 min. Non-binding phage were discarded by pouring off and slapping flasks neck down onto a clean paper towel. The flasks were washed with 10 times TBST followed by 2 times by TBS using a clean section of paper towel each time to prevent cross-contamination. The bound peptides were eluted with 2 ml of acidic buffer (0.2 M glycine, containing 1 mg/ml BSA, pH 2.2) and the eluates were decanted and neutralized with 1M Tris-HCl (pH 9.3).

Amplification of phage: A single colony of *E. coli* ER2537 was inoculated into 20 ml of LB medium (10 gr Bacto-Tryptone, 5 gr yeast extract and 5 gr NaCl per litre) in a 100 ml flask and incubated at 37°C with shaking until mid-log phase (OD_{600} ~0.5-1.0). Two ml of each eluate was added separately to 10 ml of above culture and incubated for 4.5 h at 37°C with vigorous shaking. The cultures were transferred to a centrifuge tube and centrifuged for 10 min at 800 ×g at 4°C. The supernatant was recentrifuged and the upper 80% of

the supernatant was transferred to a centrifuge tube. To precipitate the phage 1/6 volume of PEG/NaCl (20% w/v polyethylene glycol-8000, 2.5 M NaCl) was added to the supernatant and left overnight at 4°C. PEG precipitated phage was centrifuged for 15 min at 8000 ×g at 4°C, the supernatant was decanted and the pellets re-centrifuged for 2 min and then residual supernatant was with a pipette. The pellet was suspended in 1 ml of TBS, the suspensions transferred to a microcentrifuge tube and then centrifuged in a microcentrifuge for 5 min at 20700 ×g at 4°C. The supernatants were transferred to a fresh tube, re-precipitated with 1/6 volume of PEG/NaCl and then kept on ice for 60 min. The tubes were centrifuged for 10 min at 4°C, the supernatant was decanted, re-centrifuged for 30 sec and then the total supernatant was removed carefully. The pellet was suspended in 200 µl of TBS, 0.02 % NaN_3, centrifuged for 1 min at 20700 ×g to pellet any remaining insoluble matter, the supernatants transferred to fresh tubes and then stored at 4°C as amplified eluates.

Negative selection of phage: Negative selection, to remove antigenic peptides which would react with commonly occurring antibodies, was carried out with 200 µg of either sheep or human IgG in two flasks and then blocked with blocking buffer as described previously. The amplified eluates phage were added to the blocked flasks and incubated for 1 h at room temperature on rocker shaker. The phage which did not bind to these IgG were removed from the flasks and were directly added to flasks as a second positive selector. Four rounds of positive and negative selection of sheep and human IgG were done before clones were selected for sequencing. Thus, any peptides which bound to the blocker on the plastic, non-antigen binding regions of IgG, etc., should have been removed in the negative selections.

PCR sequencing: After the fourth negative selection of sheep and human IgG, the phage were amplified and titrated as described in the Ph.D.12™ instruction manual. Random clones were picked from plates with no more than 100 plaques for nucleotide sequencing.

Cycle sequencing was carried out using the Dye Terminator Cycle Sequencing Reaction (Perkin-Elmer). For 20 µl of each reaction, 3.2 pico-mole. of the primer (5´-CCC TCA TAG TTA GCG TAA CG-3´) which was supplied with the Ph.D.12™ kit, 8 µl of Terminator Ready Reaction Mix and 3.8 of sterile distilled water were mixed and then 5 µl (~100 ng) of phage DNA, was used as template. The amplification proceeded for 25 cycles in a DNA thermal cycler (Perkin-Elmer). Each cycle involved a 96°C denaturing step for 10 sec followed by 5 sec annealing temperature at 50°C and finally the polymerzation step at 60°C for 4 min. Polymerization time for the last cycle was carried out for 10 min to obtain the full length of amplified products. Each extension product was run on a 1% (w/v) agarose gel to ensure that there was sufficient DNA for sequencing. Following cycle sequencing, the samples were electrophoresed on a 3073A automatic DNA sequencer.

RESULTS

In an attempt to ensure that the only difference between the positive and negative selection steps was the presence of the disease associated IgG, we planned a strategy to maintain the conditions the same in biopanning with the Ph.D.-12™ random peptide library (New England BioLabs, Beverly, MA, USA), except for the IgG samples used to select the peptides. With each biopanning the library of 1.9×10^9 different peptides displayed on the pIII protein of the M13 phage, was added to the sheep and human IgG used as the positive selector. The bound peptides were eluted with acid and the phage were amplified in *E. coli* and then added to the IgG used as the negative selector. The phage which did not bind to this IgG were directly added to a second flask again containing the positive selector. Four rounds of positive and negative selection were carried out and then clones were sequenced. A total of 44 peptides (15, 12 and 17 from the sheep, schizophrenia and MS selections, respectively) were sorted with the PILEUP program (Genetics Computing Group, Madison, WS, USA) using the default matrix. From analysis of the peptides, it was found that many of the peptides from the different selections were not segregating with the diseases and there was a large number of 12-mer peptides with either 4 or 5 methionine residues (4, 5 and 6 from the sheep, schizophrenia and MS selections respectively). Regions of the PILEUP output were analysed with the PRETTY program to generate consensus sequences which had either a cluster of methionines or a scattered motif (Table 1). In addition to the methionine rich peptides there were 7 peptides with 3 methionines and 11 with an MLMK motif but these were not uniformly distributed between the groups selected with three different types of IgG. The Ph.D.-12™ library had a calculated

IgG	Clone	Peptide
HumMS	12	RRRKNRMMML MM…
Sheep	39	PKRKRTMMMT MN…
Sheep	47	…RNNTMRMM MRLS
HumSz	04	…RPPMMMMP RQLR
Sheep	40	…RPPMMMMP RQLR
HumMS	09	HIRRIMMMMP MH…
Consensus	(a)	--R--MMMMP MQLR

IgG	Clone	Peptide
HumSz	09	...MRRMI MLPMLMT...
HumSz	15	...MRRMI MLPMLMT...
Sheep	43	...MRRMI MLPMLMT...
HumMS	15	...RTRMR. MHMRLMR...
HumMS	08	… RPRMRL MNMRQM…
HumSz	05	PLTMKMRMKL MI…
HumSz	03	...NPHL MSMTMMRQ..
HumMS	14	...SRL LRMMPMKNM
HumMS	19	...ML MPSKMRKLQM
Consensus	(b)	RMR-ML MLMMLMT---

Table 1: Methionine rich peptides in phage clones obtained during biopanning of a Ph.D.-12™ random peptide library with different types of IgG. IgG was prepared from pools of sera from patients with MS (HumMS), schizophrenia (HumSz) and sheep protected against blowfly strike (Sheep). The consensus sequence for the clustered (a) and scattered (b) sub-types were obtained with the PRETTY program (Genetics Computing Group, Madison, WS, USA).

frequency of methionine of 3.1% and an observed of 3.4% in 66 randomly selected peptides (Ph.D.-12™ instruction manual). In our 44 peptides the frequency of 21.6% methionine was abnormally high. In contrast when three different preparations of sheep IgG were used to select peptides from the Ph.D.-7™ library only one methionine rich peptide was detected in a total of 67 peptides which were analysed in our laboratory (Hashemi Tabar and Carnegie, 2002). Moreover, 11 out of 14 peptides selected from the Ph.D.-7™ library with the same sheep IgG preparation used in biopanning for the Ph.D.-12™ library, all clustered together in the PILEUP analysis which indicated that quite different peptides were selected by this IgG when compared to the selections with the other sheep IgGs.

DISCUSSION

Screening RPLs with immune serum to find peptides that bind to protective antibodies is a potentially useful approach for vaccine development (Scott, 1992). Affinity purification with sera from patients infected with bacteria, viruses, or parasites may identify peptides that might serve as vaccines. The occurrence of similar sequences among independent phage clones indicates selective enrichment of ligand-binding phage from the library (Scott and Smith, 1990). Selected human sera can be used to identify disease-related peptide epitopes (mimotopes) displayed on phage (Delmastro *et al*., 1997). We recently reported that peptides selected from random peptide library can be used for immunization in sheep (Hashemi Tabar and Carnegie, 2002). In this study, we identified a large number of peptide epitopes with methionine. Because we were aware of “nuisance” peptides (Caparon *et al*., 1996) containing the sequence WHWWXW and others (Adey *et al*., 1995; Gebhardt *et al*., 1996), which bind readily to plastic, we planned a strategy to eliminate this problem. Despite these precautions, although we did not isolate tryptophan-rich peptides we found puzzling peptides were frequently selected from three biopannings with totally unrelated IgG associated with different sheep and human diseases. These peptides had an abnormally high content of methionine.

On the assumption that the only difference in biopanning was the IgGs used for positive and negative selection, what might have driven the preponderance of methionine rich peptides? The possibility that phage with a competitive advantage over other phage becoming the dominant population has been discussed in a theoretical analysis of biopanning (Levitan, 1998) and observed in experiments (Daniels and Lane, 1996; Stern and Gershoni, 1998) but no information on the peptide sequences were reported. The pIII protein is involved in infection of *E. coli*. If the methionine-rich peptides promoted interaction of the phage with its receptor then increased infectivity could have lead to the phage becoming dominant. Infectivity of phage with no insert was compared with phage with a peptide with no methionine, with the methionine rich peptides and with peptides containing the MLMK motif. No significant difference could be detected in the rate of infection of the host *E. coli* used in the experiments (Riechmann and Holliger, 1998). As the pIII protein is also essential for the assembly of the phage and its release from *E. coli* perhaps the methionine rich pep-

tide could give the phage a competitive advantage within the cell. The presence of these peptides in selections from the Ph.D.-12™ but not from the Ph.D.-7™ random peptide library suggests that the larger sized peptide at the N-terminal end of the pIII protein might facilitate folding and better interaction of pIII with pV1 and other key components involved in assembly and release (Rakonjac and Model, 1998). The N-terminal region of pIII is actually increased by an extra 16 amino acids, as each 12 amino acid peptide also has the spacer GGGS. Therefore, there would be an opportunity for the peptides from the Ph.D.-12™ library to fold more easily than those from the Ph.D.-7™ library. Perhaps phage with a methionine rich spacer could be used to prepare more efficient vehicles for the presentation of peptides for use in studies of protein ligand interactions. It is obvious from the above results that others working with phage displayed peptides should treat peptides with the motifs shown in table 1 as potentially irrelevant artifacts.

Acknowledgments

The authors thanks Dr V.M. Bowles and Professor M.R. Brandon, University of Melbourne for supplying sheep IgG and also Professors W.W. Tourtellotte, University of Los Angeles and A.V. Jablensky, University of Western Australia for supplying human IgG. Ferdowsi University of Mashhad is thanked for financial support.

References

Adey NB, Mataragnon AH, Rider JE, Carter JM, Kay BK. (1995) Characterization of phage that bind plastic from phage-displayed random peptide libraries, *Gene*, 156: 27-31.

Caparon MD, De Ciechi PA, Devine CS, Olins PO. Lee SC. (1996) Analysis of novel streptavidin-binding peptides, identified using a phage display library, shows that amino acids external to a perfectly conserved consensus sequence and to the presented peptides contribute to binding, *Mol. Divers*, 1: 241-246.

Cwirla SE, Peters EA, Barrett RW, Dower WJ. (1990) Peptides on phage: a vast library of peptides for identifying ligands, *Proceedings of the National Academy of Sciences of the United States of America*, 87: 6378-6382.

Daniels DA, Lane DP. (1996) Phage Peptide Libraries. Methods: A Companion to *Methods in Enzymology*, 9: 494-507.

Delmastro P, Meola A, Monaci P, Cortese R, Galfre G. (1997) Immunogenicity of filamentous phage displaying peptide mimotopes after oral administration, *Vaccine*, 15: 1276-1285.

Devlin JJ, Panganiban LC, Devlin PE. (1990) Random peptide libraries: a source of specific protein binding molecules, *Science*, 249: 404-406.

Folgori A, Tafi R, Meola A, Felici F, Galfre G, Cortese R, Monaci P, Nicosia A. (1994) A general strategy to identify mimotopes of pathological antigen using only random peptide libraries and human sera, *EMBO Journal*, 13: 2236-2243.

Gebhardt K, Lauvrak V, Babaie E, Eijsink V, Lindqvist BH. (1996) Adhesive peptides selected by phage display: characterization, applications and similarities with fibrinogen, *Pept. Res*. 9: 269-278.

Hashemi Tabar G R, Carnegie P. (2002) Immunization of sheep with phage mimotopes against dermatophilosis, *Iranian Biomedical Journal*, 6: 131-139.

Houghten RA, Pinilla C, Blondelle SE, Appel JR, Dooley CT, Cuervo JH. (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery, *Nature*, 354: 84-86.

Kretzschmar T, von Ruden T. (2002) Antibody discovery: phage display. *Curr Opin Biotechnol*, 13(6): 598-602.

Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. (1991) A new type of synthetic peptide library for identifying ligand-binding activity, *Nature*, 354: 82-84.

Levitan B. (1998) Stochastic modeling and optimization of phage display, *J. Mol. Biol.* 277: 893-916.

Meola A, Delmastro P, Monaci P, Luzzago A, Nicosia A, Felici F, Cortese R, Galfre G. (1995) Derivation of vaccines from mimotopes. Immunologic properties of human hepatitis B virus surface antigen mimotopes displayed on filamentous phage, *Journal of Immunology*, 154: 3162-3172.

Rakonjac J, Model P. (1998) Roles of pIII in filamentous phage assembly, *J. Mol. Biol.* 282: 25-41.

Riechmann L, Holliger P. (1997) The C-terminal domain of ToIA is the coreceptor for filamentous phage infection of *E. coli*, *Cell*, 90: 351-360.

Rubinstein JL, Holt LJ, Walker JE, Tomlinson IM. (2003) Use of phage display and high-density screening for the isolation of an antibody against the 51-kDa subunit of complex I, *Anal Biochem.* 314(2): 294-300.

Parmley SF, Smith GP. (1988) Antibody-selectable filamentous fd phage vectors: Affinity purification of target genes, *Gene*, 73: 305-318.

Scott JK. (1992) Discovering peptide ligands using epitope libraries, *Trends in Biochemical Sciences*, 17: 241-245.

Scott JK, Smith GP. (1990) Searching for peptide ligands with an epitope library, *Science*, 249: 386-390.

Stern B, Gershoni JM. (1998) Construction and use of a 20-mer phage display epitope library, *Methods Mol. Biol.* 87: 137-154.