Immunology 1996 **89** 579–586

Peptides isolated from random peptide libraries on phage elicit a neutralizing anti-HIV-1 response: analysis of immunological mimicry

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SUMMARY

Peptides binding to a murine, human immunodeficiency virus type 1 (HIV-1) neutralizing monoclonal antibody (F58/H3) were isolated from two random peptide libraries expressed on the surface of phage. The antibody was originally elicited by immunization with HIV-1 envelope protein $gp120_{LAI}$, and has previously been shown to interact with the -I–GPGRA- motif of the V3 loop. The peptide libraries consisted of nine or 15 random amino acid residues flanked by two cysteines, and fused to the amino terminal end of the cpIII protein on the filamentous phage. Selection of specific peptides was carried out in three rounds, with decreasing antibody concentration. An expected peptide motif -GPGRA-, a similar segment, -GPAR-, and two unrelated motifs -FRLLG- and $-WR^{M}/_{A}LG-$ were selected. Binding of antibody was tested both to synthetic peptides in solution, and the corresponding peptide on phage. The GPXR motifs bound in both formats, while the FRLLG bound antibody only when present on the phage. The reactivity of peptides on phage was highly dependent on an intact disulphide bond between the cysteines flanking the peptide. The molecular mimicry of the found motifs was tested by immunizing mice and rabbits with conjugated synthetic peptides or peptide on phage. In mice, peptide-specific antisera were raised, but no reactivity to the whole protein (gp120) was detected. In rabbits, however, this was accomplished with the -GPGRA- containing peptide when present on phage. In addition, this antisera precipitated virus particles, and neutralized $HIV\text{-}1_{SF2}$ virus *in vitro*.

INTRODUCTION

The envelope glycoproteins gp120 and gp41 of human immunodeficiency virus type 1 (HIV-1) are major targets for the host immune response. Most virus neutralizing antibodies in sera from HIV-infected individuals are directed towards these proteins.[1–3]

Linear epitopes with the capacity to induce neutralizing antibodies have been identified using synthetic peptides,[3–5] but many broadly neutralizing antibodies are directed towards conformation dependent epitopes within or outside the CD4 binding region.[2,6,7] Even antibodies reactive to linear peptides, e.g. based on V3 loop sequences, exhibit sensitivity to the conformational presentation of these peptides.[8]

Epitope libraries expressed on the surface of filamentous phage offer unique possibilities to identify candidate peptides that may mimic discontinuous or linear epitopes presented in a three-dimensional structure.[9–11] The technology has been used to identify peptide motifs binding to antibodies as well as other proteins: to monoclonal antibodies (mAbs) against the protein myohaemorythrin,[9] the linear amino acid motif for an anti-HIV1 neutralizing mAb,[12] peptides specifically binding to streptavidin,[10] the binding specificity of the immunoglobulin-binding protein, BiP.[13]

We wanted to identify peptides with capacity to sterically mimic the epitopes for virus neutralizing monoclonal antibodies directed against linear or conformational epitopes. We constructed two different libraries with nine or 15 amino acid random peptides linked to cpIII, and flanked by two cysteine residues to constrain the flexibility of the peptides. In this study, we selected peptides specific for a neutralizing monoclonal antibody, F58/H3, previously epitope mapped to the apex of the V3 loop of the $HIV\text{-}1_{LAI}$ gp120.[5] The identified peptides were used to evoke anti-virus antibodies in mice and rabbits to assess the potential immunological mimicry of the selected peptides.

MATERIALS AND METHODS

Library construction

The fUSE 2 vector[14] from filamentous phage vector fd-tet[15] was modified as follows to obtain the vector fAST.

A fragment containing restriction sites for *Sac*I and *Spe*I was cloned into the *Bgl*II site of fUSE 2 (Fig. 1a). The peptide coding DNA was prepared by polymerase chain reaction (PCR). Each

Received 11 January 1996; revised 30 July 1996; accepted 1 August 1996.

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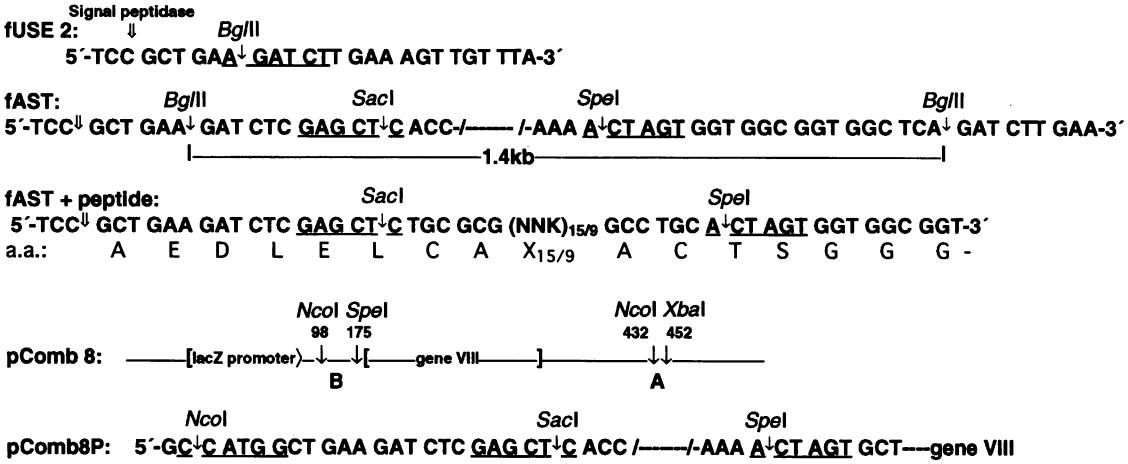

Figure 1. (a) Nucleotide sequence of fUSE 2 and fAST in the cloning region near the 5′ end of gene III. The signal peptidase site is indicated as well as the restriction sites used, and the amino acid sequence for the peptide fused to the phage protein III. (b) Phagemid pComb 8 contains two cloning sites, the most 5′ is associated with gene VIII. By deletion of fragment A between the *Nco*I site at position 432 and the *Xba*I site, the second cloning site was removed. The DNA was treated with Mungbean nuclease and self-ligated. Fragment B was removed by *Nco*I and *Spe*I restriction and the sequence shown in pComb8P was inserted in two steps. pComb8P was then used to express selected peptides at the N-terminal of cpVIII.

reaction contained 40 nM template 5′-GCGCGGGAGATCTCTG-CGCG(NNK)$_{15/9}$ GCCTGCACTAGTCTCGGTAC-3′ (where N were A, C, G and T, K were G and T, all equimolar), 800 nM each of the 5′-biotinylated primers 5′-GCGCGGGAGAGCTCTGCG-CG-3′ and 5′-GTACCGAGACTAGTGCAGGC-3′ (Scandinavian Gene Synthesis, Köping, Sweden), 200 μM dNTP (Pharmacia, Uppsala, Sweden) in 1 × PCR buffer, and 2·5 units AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT) in a total volume of 100 μl. Each amplification was performed by six cycles of 1 min at 94°, 30 seconds at 48° and 3 min at 72° (Omnigene, Hybaid Ltd, Teddington, UK). The PCR products were digested with *Sac*I and *Spe*I (all restriction endonucleases were from Life Technologies, Gaithersburg, MD). Incompletely digested fragments and the cleaved ends were removed by incubation with streptavidin coated paramagnetic beads (Dynal, Oslo, Norway) for 15 min at 37°. DNA in the supernatant was phenol-chloroform extracted and ethanol precipitated.

The fAST vector was cleaved with *Sac*I and *Spe*I and the resulting 8·5 kB fragment was gel purified and recovered using β-agarase I (New England BioLabs, Beverly, MA). After ligation of digested PCR products and vector at 4° overnight (o/n) (T4 ligase, Life Technologies), the reactions were precipitated and the DNA dissolved in distilled H_2O. The DNA was electroporated into the *E. coli* strain MC1061 using a GenePulser at 200 W, 25 μF and 12·5 kV/cm (Bio Rad, Richmond, CA), suspended in SOC medium,[16] and incubated at 37°. After 1 hr shaking, the cultures were diluted to 10 ml using SB medium containing 10 μg tetracycline/ml (SB-tet). Small aliquots were plated on LB -agar-plates containing 10 μg tetracycline/ml (LA-tet). The cultures were propagated in 200 ml SB-tet at 37° overnight. Bacteria and phages were recovered as described.[17]

Monoclonal antibodies (mAbs)

The mouse mAb F58/H3 epitope is contained within the amino acid sequence IQRGPGRA[5] with amino acid I–GPGRA most important according to omission peptide analysis.[18] Control mAb EF7 (γ1/κ) has been described previously.[19] Biotinylation was performed using the NHS–LC–Biotin reagent (Pierce, Rocherford, IL) according to the manufacturer's protocol.

Phage selection

Biotinylated mAbs and adsorption to streptavidin-coated paramagnetic beads were used for selection. To remove unspecific and streptavidin binding phages, 100 μl phage stock was incubated with 100 μl streptavidin-coated beads pre-blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS). After 15 min at 37° the beads were removed using a magnet, and phages remaining in the solution were used for selection. In the first round 50 μl phages were incubated with 10 μl 5% dry milk in PBS and biotinylated mAb at a final concentration of 190 nM. After 2 hr incubation on a rotating wheel at room temperature, the beads were washed 10 times with PBS–0·5% Tween-20 and once with distilled H_2O. Bound phages were eluted at low pH as described[14] and used to infect freshly cultured K91 bacteria (OD_{600} 0·9–1·0). An aliquot was plated on LA-tet plates and the remainder was propagated in 100 ml SB-tet at 37° o/n. Phages were precipitated, and used for another round of selection. The mAb concentration was 190, 19 and 1·9 nM in the three consecutive selections, respectively. After each round of selection, single colonies were picked and analysed for specific mAb binding in a 'micro panning' assay.[14]

Sequencing

The nucleic acid sequences of phage clones were determined using solid phase.[20] Single-stranded DNA for the gene III coat protein was PCR amplified using the primers 1878fuse (5′-GCCTTC-GTAGTGGCATTACG-3′) and SpC263 (5′-CCGCCGCCAGCA-TTGACAGGAG-3′) with either primer biotinylated. Sequencing was performed using a Sequenase T7 sequencing kit (USB, Cleveland, OH)[21] together with the seqfUSE primer,[15] or the primer 2215fuse (5′TTATTATTCGCAATTCCTTTAG-3′). Sequence analysis was performed using MacMolly software (SoftGene, Berlin, Germany).

Peptide synthesis

Peptides were synthesized according to the amino acid sequences derived from the DNA sequencing of the selected phages. Four flanking constant amino acids on each side of the sequences (including the cysteine residues) were included in each peptide. Synthesis was performed using solid phase[22] 9-fluorenyl-methoxy-carbonyl (Fmoc) chemistry[23] in an automated synthesizer (Multi-syntec, Bochum, Germany). At the end of synthesis the peptides were chemically oxidized using iodine. The peptides were 80–85% pure as assessed by reversed phase high pressure liquid chromatography (HPLC).

Construction of pComb8P and production of cp8-peptide phages

The pComb8 phagemid vector[24] was used to express 15 amino acid peptides after modifications: The cloning cassette between the second *Nco*I site at position 432 and the *Xba*I site at position 452 was removed, the remaining vector Mungbean nuclease treated, self-ligated and propagated. The vector was then cleaved with *Nco*I and *Spe*I. The first insert was prepared by PCR using the primers pC8pNcoI (5′TATTCTCCCATGGCTGAAGATCTCGAGC-3′; *Nco*I and *Sac*I sites underlined) and SpC263 (5′CGCCGCCAG-CATTGACAGGAG-3′), and with DNA of one of the fAST peptide phages as template. The insert was cleaved with *Nco*I and *Spe*I and cloned into the phagemid. Phagemid DNA of a single clone was cleaved with *Sac*I and *Spe*I, gel purified, and the 1·4 kb stuffer fragment from fAST was inserted 5′ of the cpVIII gene to complete pComb8P (Fig. 1b).

The peptide clones to be transferred to pComb8P were PCR amplified using primers 1878fuse and SpC263, cleaved with *Sac*I and *Spe*I and ligated into pComb8P. All clones were sequenced to confirm the insertion of a correct DNA sequence. One phagemid clone for each peptide was cultured in SB-tet containing 20 μg ampicillin (amp) and 1% glucose. When the culture reached OD_{600} 0·4–0·5 the medium was changed to SB-tet containing 20 μg amp and 1 mM isopropyl β-D-thiogalactoside (IPTG) (Sigma, St. Louis, MO), and 1×10^{11} plaque-forming units (PFU) of helper phage VCS M13 (Stratagene, La Jolla, CA) were added. After 5 hr incubation at 37° the phages were precipitated. Phages used for immunization were dialysed extensively against PBS.

Immunizations

Selected peptides were used for immunization using four different protocols.

(i) Synthetic 15 amino acid peptides mixed with muramyl dipeptide (MDP) (Sigma) in Freunds incomplete adjuvant (FIA) were used for immunizations; no additonal peptides with T-cell epitopes were added. One hundred micrograms of peptide and 2 μg MDP was injected subcutaneously (s.c.) into Balb/c mice (Bomholtgård, Ry, Denmark); boosts of the same amount were given week 3, 5, and 7.

(ii) For nine amino acid clones, the corresponding synthetic peptides were coupled to keyhole limpet haemocyanine (KLH) by glutaraldehyde; peptide to KLH ratio 1:10 (w/w). Two Balb/c mice were immunized s.c. with each peptide using 100 μg KLH-peptide in Freunds complete adjuvant (FCA), and boosted with 10 μg KLH-peptide in FIA week 2, 4, and 7.

(iii) Mice were immunized with purified bacteriophages expressing the 15 a.a peptides in fusion with cpVIII (cp8-peptide phages). Each mouse received initially $1-5 \times 10^{11}$ colony forming units (CFU) in 250 μl PBS intraperitoneally, and boosted three times with $1-5 \times 10^{11}$ CFU every 2 weeks.

(iv) Two of the cp8-peptide phage clones were used to immunize Lop rabbits (ESF-producter, Norrtälje, Sweden). Each rabbit was injected intramuscularly with 1×10^{12} CFU of cp8-15-1:8 or cp8-15-3:1 phages in FCA. The rabbits were boosted with $0{\cdot}5-1 \times 10^{12}$ CFU in FIA at week 2,5 and 8 and finally with $0{\cdot}1-0{\cdot}4 \times 10^{12}$ CFU in PBS at week 11 and 13. The rabbits were bled 1 week after each boost.

ELISA methods

Enzyme-linked immunosorbent assays (ELISAs) were performed essentially as described[25] with the following modifications.

Inhibition ELISA. Recombinant $gp120_{LAI}$ (Intracell, Cambridge, MA) was coated to 96-well plates (Costar#3690, Cambridge, MA) at 1 μg/ml PBS. Blocking agent was 5% dry milk in PBS. A mixture of phages or peptides and mAb were added and incubated at room temperature for 2 hr. Washings were done in PBS–0·05% Tween-20 (PBS-T) and bound mAb was detected using alkaline phosphatase (ALP) conjugated goat anti-mouse Fab (Pierce) diluted 1:500 in HS buffer (2% goat serum, 0·5% BSA and 0·05% Tween-20 in PBS).

Peptide ELISA. Ten micrograms of peptide/ml was coated in carbonate buffer pH 9·6, 100 μl/well, overnight at room temperature in 96-well plates (Nunc #4-39454, Roskilde, Denmark). Plates were washed three times with PBS-T, sera diluted in HS buffer added and incubated at 37° for 2 hr. Washings and detection were as above.

gp120 ELISA. Plates as for 'inhibition ELISA' above, were antigen coated at 2 μg/ml. After blocking and washings, sera diluted in HS buffer were added and incubated at 37° for 2 hr. Detection of bound antibodies was as described, or, for rabbit antibodies, using ALP-goat anti-rabbit Fab (Pierce). The specificity for gp120 was verified by competition with 2 μg free gp120/ml.

Western blot of phage proteins

Phages ($2{\cdot}5 \times 10^9$ CFU) were boiled for 2 min in 20 μl gel loading buffer with and without 5% β-mercaptoethanol. After separation on precast 8–16% gradient gels in sodium dodecyl sulphate–polymerase gel electrophoresis (SDS–PAGE), the proteins were blotted to a nitro-cellulose filter in Tris–glycine buffer by the use of a Novex electroblotting device (Novel Experimental Technology, San Diego, CA) according to the manufacturer's protocol. After blocking in 5% non-fat dry milk in PBS at 4° overnight, the filters were incubated in antibody solution (10 μg mAb/ml PBS-T with 5% milk) for 2 hr at room temperature, washed four times with PBS-T and incubated for another hour with alkaline phosphatase-labelled goat anti-mouse Fab (Pierce). The filters were developed, after another four washes, with BCIP/NBT (Sigma) in 0·1 M Tris buffer pH 9·5 containing 0·1 M NaCl and 5 mM $MgCl_2$.

Immunoprecipitation of viral particles

$HIV\text{-}1_{LAI}$ (kindly provided by Dr R. C. Gallo, Bethesda, MD) was propagated by infecting 5×10^6 peripheral blood mononuclear cells (PBMC) that had been stimulated with PHA and IL-2. Virus and PBMCs were incubated overnight at 37° in 5% CO_2. Cells were washed twice with RPMI-1640 and further incubated. Cell-free supernatants were collected after 48, 72, and 96 hr, and titrated on PBMCs to determine the 50% tissue culture infectious dose ($TCID_{50}$). One millilitre of virus mixed with 40, 8 or 1·6 μg mAb, or serum at indicated dilutions, was incubated at 37° for 1 hr before

goat anti-human Fab (Pierce), or swine anti-rabbit immunoglobulin (Orto Diagnostika, Espoo, Finland) was added, followed by an additional 1 hr incubation at 37°, and centrifuged at 2500 *g* for 40 min at +4°. The pellet was washed with 5 ml PBS, centrifuged for 40 min as above, and dissolved in 50 μl PBS with 0·05% Triton X-100. The amount of HIV protein p24 in the pellet was determined in an ELISA.[26] When serum was assayed, p24 was measured only after the dissociation of antigen-antibody complexes using 1·0 M glycine–HCl.[27]

Virus neutralization assay

Sera from peptide immunized mice and from rabbits immunized with peptide phages were tested for neutralization of HIV-1_{LAI} and HIV-1_{SF2} virus basically according to a published protocol.[3] Inhibition of syncytia formation was performed as a variation of a published protocol,[28] the modification being that we used C8166 and MT-2 cells.

RESULTS

Library constructions

The 15 amino acid and nine amino acid peptide libraries consisted of $1{\cdot}8 \times 10^6$ and $1{\cdot}6 \times 10^6$ clones, respectively, of which 80% and 85%, respectively, carried peptide-coding inserts of correct length (data not shown). None of 10 clones sequences from each library were found to be identical. In the nine amino acid library, however, three out of 10 clones contained, in addition to the distinct nine amino acid peptide sequences, an identical six amino acid peptide sequence where the C-terminal cysteine should have been (-PALGTE- replaced -AC-), probably because of incomplete removal of the DNA fragment cleaved off from the 3′ end of the PCR product.

Phage peptide selection with mAb

The fraction of specific binders increased in each selection step resulting in 9/10 specific clones after the third selection of the nine amino acid library, and 10/10 specific clones from the 15 amino acid library as determined by micro panning (data not shown). One out of four sequences among the specific 15 amino acid peptides (clone 15-1:8) contained the motif -GPGRA-, and another sequence contained the similar motif -GPAR- (Table 1). Interestingly, one sequence appearing after the last selection and thus indicating a high affinity for the mAb F58/H3 was completely different (clone 15-3:1). A motif from this peptide -FRLLG- also appeared in 1/5 peptide sequences selected from the nine amino acid library. Three of the other peptides selected from that library contained the motif -$WR^M/_A$LG- (Table 1).

Binding to mAb F58/H3 by selected peptides on phage or in solution

Synthetic peptides and peptide carrying phages were used in inhibition ELISA to block the binding of mAb to rgp120. (Fig. 2a, b). Peptide 15-3:1 (amino acid motif -FRLLG-) on phage showed highest activity in blocking the binding of F58/H3 to rgp120. Clone 15-1:4 and 15–1:8 (-GPAR- and -GPGRA-, respectively) were also good inhibitors. Clone 9-2:4 (-WRMLG-) was the most potent inhibitor of the nine amino acid clones. Clone 9-2:2 (VYGPGARFS) did not show any inhibition at the phage concentrations tested ($1{\cdot}0–3{\cdot}3 \times 10^{10}$ CFU/ml) (Fig. 3a). Notably, when using synthetic free peptides, peptide 15-3:1 did not block the antibody binding at all, while peptide 15-1:4, 9-2:2, 9-2:5 and 9-2:7 gave 40–60% reduction in absorbance values at peptide concentrations of 1 μg/ml (Fig. 2b).

Requirement of the disulphide bond for specificity

All phage clones selected from the 15 amino acid library interacted with F58/H3 under non-reducing conditions, while reactivities were markedly reduced or completely abolished after reduction of the phage by β-mercaptoethanol. Binding to clone 15-1:8 with the motif -GPGRA- (present in the reported linear epitope for the mAb), was also highly dependent on the configuration created by the disulphide bridge (Fig. 3 lanes C and I). Among the nine amino acid peptide phages, clone 9-2:5 was the only clone reacting in Western blot. Binding was abolished under reducing conditions (Fig. 3, lanes A–D); no binding was seen to non-peptide carrying

Table 1. Peptide sequences of mAb-specific phages

Specific peptides selected with mAb F58/H3				
Clone		P1*	P2	P3
15-1:4	AEDLELCA**LGSMRGAGPKWV**ACTS-	1/2†	6/8	3/5
15-1:8	AEDLELCA**AWRGRGALLGPGRAW**ACTS	1/2	1/8	0/5
15-2:3	AEDLELCA**QGPRVKWMPGPARGS**ACTS-	0/2	1/8	0/5
15-3:1	AEDLELCA**YLQSQYFRLLGQRWA**ACTS-	0/2	0/8	2/5
9-2:2	AEDLELCA**VYGPGARFS**ACTS-		2/6	3/6
9-2:4	AEDLELCA**EWWRMLGGG**ACTS-		2/6	1/6
9-2:5	AEDLELCA**VCEFFRLLG**PALGTETS-		1/6	1/6
9-2:7	AEDLELCA**PWRALGARP**ACTS-		1/6	0/6
9-3:4	AEDLELCA**PWRALGARS**ACTS-		0/6	1/6

Peptide sequence selected from randomized segment in bold face.

*P1–P3 indicate selection round 1–3 performed with 190, 19 and 1·9 nM mAb F58/H3, respectively.

† Number of peptides with indicated sequence/number of peptides sequenced.

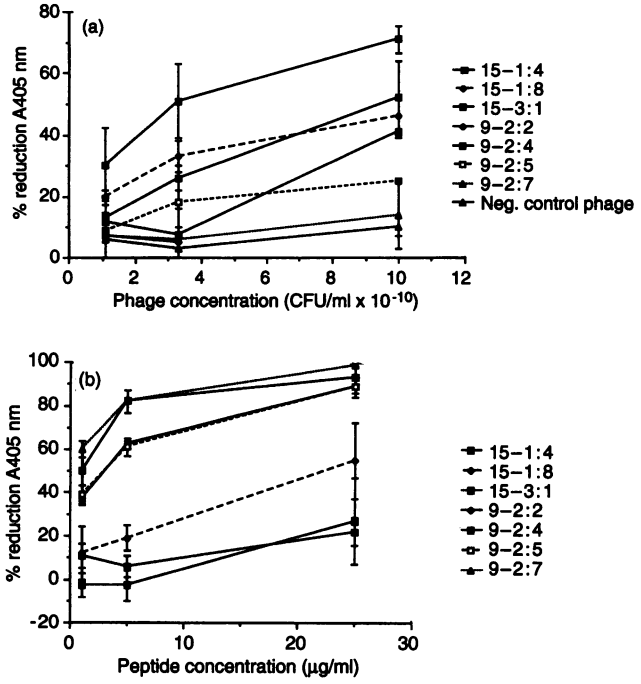

Figure 2. Inhibition of binding of mAb F58/H3 to recombinant gp120 in ELISA: mAb, 0·5 μg/ml, was mixed with different concentrations of (a) peptide phages or (b) synthetic peptides, and incubated on gp120 coated plates. Percentage reduction in absorbance values compared to mAb incubated with PBS alone are indicated. Error bar indicates SEM of three different experiments.

phage (Fig. 3, lane F), and control mAb was negative against all phages (data not shown).

Peptide phages elicit anti-gp120 antibodies in rabbits but not in mice

Phages expressing peptides 15-1:8 (cp8-1:8) or 15-3:1 (cp8-3:1) in

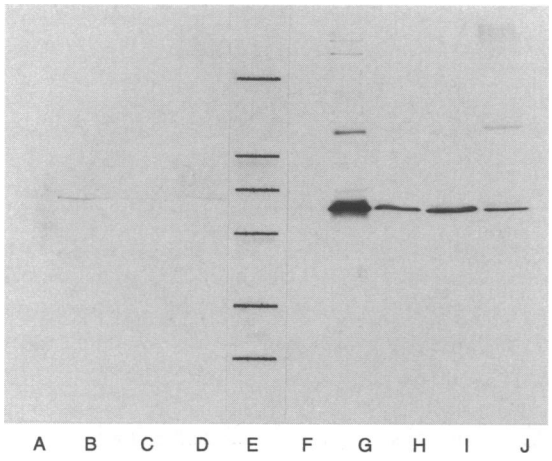

Figure 3. Western blot of peptide phages with the peptide expressed on phage cpIII stained with mAb F58/H3. Whole phage particles were separated in SDS–PAGE under reduced (lanes A–D) and non-reduced (lanes F–J) conditions. Clone 15-3:1 lanes A and G, clone 15-2:3 lanes B and H, clone 15-1:8 lanes C and I, clone 15-1:4 lanes D and J, and control phage (non-peptide carrying phage), clone B-15:3, in lane F. Size of MW markers (lane E) from top 220, 97, 66, 46, 30 and 14 × 10^3 D.

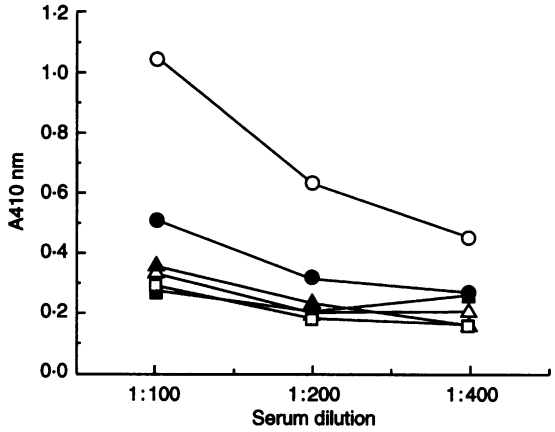

Figure 4. Rabbit sera reactivity to rgp120 in ELISA after immunization with (○) cp8-1:8 and (□) cp8-3:1 peptide phages. (Δ) Preimmune serum. Filled symbols indicate serum in presence of 2 μg rgp120/ml.

fusion with cpVIII were used for immunizations of rabbits. After four boosts, the animal immunized with phage cp8-1:8 was producing antibodies reactive to gp120 in ELISA (Fig. 4). Even though the titre was low, a 50% reduction in absorbance values in the presence of 17 nM free antigen indicated a mean avidity in the order of 10^7 M^{-1}. The rabbit immunized with cp8-3:1 did not show any gp120 reactivity after five boosts.

The anti-gp120 positive serum neutralized HIV-1_{SF-2} *in vitro*, shown as reduction in p24 production (infection of PBMCs), and as inhibition of syncytia formation (MT2 cells) (Table 2), while HIV-1_{LAI} was not neutralized. This serum precipitated whole HIV-1_{LAI} virus, while the antiserum against cp8-3:1 did not. The positive serum even precipitated more virus at dilution 1:100 than 8 μg of mAb F58/H3 (Table 3).

Mice immunized with synthetic peptides and MDP as adjuvant had high anti-peptides titres and cross-reactivity to the other peptides to various degrees (Table 4). Peptides coupled to KLH gave generally weaker responses with less cross-reactivity to the other peptides (data not shown).

None of these, and none of the phage-immunized mice, gave sera that were positive for gp120 in ELISA, nor did they show any virus neutralization *in vitro* (data not shown).

DISCUSSION

Our major finding was that a phage peptide selected to bind to a HIV-1 neutralizing monoclonal antibody, originally raised against whole envelope glycoprotein, was able to elicit antibodies with similar capacity, i.e. antiserum that could precipitate and neutralize virus. We further explored this by immunizing with other selected motifs. They did not result in anti-viral antisera despite the fact that some peptides bound better to the mAb than the one first mentioned. The binding of the latter, -GPGR-, was highly dependent on a configuration that is forced on the peptide by the disulphide bridge, despite the fact that the mAb bound to linear peptides in a peptide ELISA used for epitope mapping.[5,18] Most likely the flanking amino acids, that are different in the phage peptide *vis-à-vis* those found in the synthetic peptide derived from the gp120 sequence, might influence the epitope in distinctly different ways.[29] In gp120 the GPGR sequence is predicted to create a β-turn.[30]

The completely different amino acid motif -FRLLG-, found

Table 2. Virus neutralization and syncytia inhibition of HIV-1_{SF2} with rabbit sera

	Serum dilution	NT* PBMC (8 TCID50)	NT Jurkat tatIII cells (16 TCID50)	NT Jurkat tatIII cells (60 TCID50)	Reduction of syncytia formation† in MT2 cells day 3
Prebleed	1/7	0	7	<0	26
	1/14	17	15	25	5
	1/28	<0	14	21	42
	1/56	10	14	17	NT
Anti-cp8-1:8	1/7	**79**	**95**	16	79
	1/14	**50**	**75**	<0	79
	1/28	**62**	39	18	47
	1/56	39	19	17	42
Anti cp8-3:1	1/7	**73**	16	20	0
	1/14	**63**	28	22	26
	1/28	<0	17	21	0
	1/56	<0	29	20	5

* % inhibition in p24 ELISA after 7 days. Figures in bold indicate values ≤50% of the result in cultures with virus preincubated with culture medium only.

† % reduction in number of syncytia compared to the number formed in the medium control.

after repeated selection and in both the 15 and the nine amino acid library, showed higher affinity to F58/H3 when presented on phage. This interaction was very sensitive to reduction of the peptide. The lack of gp120 specific response by the -FRLLG- motif, despite tight binding to the monoclonal antibody, differs from that of Zhong *et al.* who found positive correlation between affinity and immunological mimicry.[31] Still, only the (-GPGR-) motif elicited anti-gp120 antibodies in rabbits. Why HIV-1_{SF2} was neutralized but not HIV-1_{LAI} is not clear. The approximate avidity of this serum to gp120 was $10^7\,M^{-1}$, which most likely should be sufficient for neutralization if directed to the correct epitope.[32] Interestingly, HIV-1_{LAI} was precipitated but not neutralized, indicating a subtle mechanism for neutralization since binding to presumably the same epitopes on HIV-1_{SF2}, but not on HIV-1_{LAI}, conferred neutralization. Keller *et al.* were also able to elicit antibodies that neutralized the SF2 strain, but not HIV-1_{MN} or HIV-1_{LAI}, in a similar study.[12]

Table 3. Precipitation of HIV-1_{LAI} particles by rabbit antisera

Antibody	Serum dilution	Precipitated p24 ng/ml	Ratio Ab/NC
anti-cp8-1:8	1/50	625	9·9
	1/100	200	3·2
	1/500	162	2·6
prebleed	1/50	92	1·5
	1/100	58	0·9
anti-cp8-3:1	1/50	82	1·3
	1/100	110	1·7
	1/500	72	1·2
prebleed	1/50	100	1·6
	1/100	61	1·0
F58/H3	40 μg	1250	19·8
	8 μg	138	2·2
	1·6 μg	62	1·0
mAb 2D1*	10 μg	65	1·0
mAb 6A12*	50 μg	60	1·0
No antibody		63	1·0

* Negative control mouse monoclonal antibodies.

The total lack of gp120 response in peptide-immunized mice was surprising. Whether this is because of difference in species immunized or caused by different presentations of the immunogen cannot be deduced from our studies: synthetic peptides were not tried in rabbits. In addition, adding peptides representing T-cell epitopes would likely have been of benefit; this, and immunizations of mice of different strains, should be included in future experiments.[33,34]

The libraries used in this study were quite small, but also a thousand times larger library will only cover 0·3% of all possible peptides in a nine amino acid random peptide library, and for a 15 amino acid library the fraction would be 10^{-11}. However, larger peptides have the capacity to adopt additional structural motifs compared to shorter peptides and may contain several epitopes within each peptide, further enlarging the epitope repertoire. Considering the design of the present study, the weak neutralization should not reduce the importance of the fact that one selected peptide was indeed able to elicit neutralizing antibodies in the rabbit. This epitope thus proved useful to assess the epitope mapping potential of our phage displayed peptide libraries, emphasizing the possibilities to identify relevant epitopes for vaccine production. For vaccine use, a wealth of information has been presented on the use of synthetic peptides as immunogens to elicit immune responses to epitopes representing linear epiotpes.[35] By functional selection from combinatorial peptide libraries, the potential of peptides to mimic conformational epitopes has started to be explored.[36] As we learn more about the particular properties of truly protective antibodies, approaches such as these to ascertain

Table 4. ELISA reactivity of mouse sera against synthetic peptides

	antisera reactivity to							
Immunogen	9-2:2	9-2:4	9-2:5	9-2:7	15-1:4	15-1:8	15-3:1	JB4*
9-2:2 VYGPGARFS†	–	–	–	–	–	–	–	–
9-2:4 EWWRMLGGG	–	–	–	–	–	–	–	–
9-2:5 VCEFFRLLG	++	++	++	–	+/++	–/++	–/++	–
9-2:7 PWRALGARP	–	–	–	++	–	–	–	–
15-1:4 LGSMRGAGPKWV	++/–	++/–	++/–	–	++/–	++/–	++/–	–
15-1:8 AWRGRGALLGPGRAW	–	–	–	–	+	++	–	–
15-3:1 YLQSQYFRLLGQRWA	–	–	–	–	–	–	–	–
NS	–	–	–	–	–	–	–	–
MDP	0·081	0·131	0·152	0·175	0·086	0·078	0·072	0·151

Mice were immunized with soluble peptides and MDP. Sera were assayed at dilution 1 : 500. NS, normal mouse sera; MDP, sera from mice immunized with MDP alone. –, <4 times background reactivity by MDP antisera. +, 4–10 times background, and ++, >10 times background. Results from two mice, separately reported if different within the pair.

* Disulphide constrained control peptide from gp41.

† Only the 'random' portion of the selected peptide motif is indicated.

a limited but directed immune response are likely to be of increased value in vaccine strategies.

ACKNOWLEDGMENTS

We thank Dr George P. Smith for valuable discussions and for providing the fUSE vectors and the accompanying manual. The enthusiastic technical assistance by Bartek Zuber is gratefully acknowledged. This work was supported by the Karolinska Institute, the Swedish Ministry of Health, the Swedish Cancer Society, the Swedish Research Council for Technological Sciences, the Swedish Medical Research Council, the Sven and Dagmar Salén Foundation, and the Nanna Svartz Foundation.

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