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Immunogenicity of HIV-1 gp120 CD4 Binding Site Phage Mimotopes

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ABSTRACT

The conserved domain of the CD4 binding site (CD4bs) on the human immunodeficiency virus type 1 (HIV-1) envelope represents a potential target for vaccine development. Here we describe selection of peptide mi-motopes by panning a phage peptide library on the HIV-1 CD4bs-specific, broadly neutralizing anti-HIV-1 monoclonal antibody, IgG1 b12. We identified an initial consensus sequence for IgG1 b12 binding (M/V Θ SD, where Θ represents an aromatic amino acid). A molecular evolution approach, using second- and third-generation libraries, led us to identify a refined consensus sequence (GLLVWSDEL). The resulting IgG1 b12 phage mimotopes compete with gp160 for the IgG1 b12 antigen-binding site, but the phage coat protein (pIII) may play an important structural role, since both free peptides and KLH-conjugated peptides have no detectable binding activity. Mice immunized with IgG1 b12 phage mimotopes elicited a weak but persistent humoral response directed against the HIV-1 envelope. An antibody fragment was isolated from the antibody repertoires of these animals. It is noteworthy that while it has a relatively low affinity for HIV-1 gp160, the antibody targets an epitope that overlaps with that of IgG1 b12. Our data therefore suggest that engineered IgG1 b12 mimotopes share immunogenic features with the CD4bs. However, these peptidic structures will require further improvement in order to generate broad specificity neutralizing antibodies like IgG 1 b12.

Abbreviations used: CD4bs, CD4 binding site ; ScFv, Single-chain variable fragment ; PBSM, Phosphate Buffer Saline with 3% (w/v) milk powder ; PBST, PBS with 0,1% Tween 20 ; CFA, Complete Freund Adjuvant ; IFA, Incomplete Freund Adjuvant.

INTRODUCTION

The Human Immunodeficiency Virus type 1 (HIV-1) interacts with target cells by virtue of a high-affinity interaction between host cell CD4 and the viral envelope glycoprotein (Env), which is formed by cleavage of the glycoprotein (gp)160 precursor into two non-covalently associated polypeptide chains, gp120 and gp41.¹ The HIV-1 envelope is currently the major focus in the design of HIV-1 humoral vaccines and antigen-based immunotherapeutic strategies.

Two functionally and temporally distinct classes of neutralizing antibodies (Abs) are generated against HIV-1 in infected humans. Highly strain-specific antibodies are elicited early after initial infection. Later in the course of infection, antibodies capable of neutralizing a wider range of HIV-1 isolates appear, but cross-neutralizing titers remain typically low.²⁻⁴ Attempts to induce a broadly protective antibody response against genetically diverse HIV-1 isolates have not been successful.⁵⁻¹⁰ This can be explained in part by continuous *in vivo* evolution of the virus (viral escape, interclade recombinations, and emergence of new viral isolates), which leads to a high degree of antigenic polymorphism. Moreover, potential neutralizing epitopes on gp120 are occluded by genetically variable loops,^{11,12} extensive glycosylation,¹³⁻¹⁵ and subunit interactions within the trimeric structure of the mature HIV -1 envelope.¹⁶⁻¹⁸

The poor accessibility of broadly neutralizing epitopes probably explains why human monoclonal antibodies (mAbs) that neutralize primary viral isolates in a broad crossclade manner have proven difficult to isolate.¹⁵ Only five such human mAbs have currently been identified. The epitope of one such antibody, IgG1 b12, has previously been studied using different approaches including gp120 mutagenesis,¹⁹ crystallography,^{20,21} or phage display technology.^{22,23} It has been revealed to be a discontinuous epitope that overlaps the CD4 binding site (CD4bs) on gp120.^{24,25} 2G12 mAb recognizes a glycan discontinuous epitope involving the C3-V4 region of gp120.²⁶⁻²⁸ The antibody fragment Fab X5, which binds to a CD4-induced (CD4i) epitope, was obtained, like IgG 1 b12, after selection of a phage

display library against a CD4-gp120-CCR5 complex.²⁹ Finally, 2F5³⁰⁻³² and z13/4e10^{30,33} mAbs bind epitopes on the ectodomain of gp41.

Passive immunization studies in hu-PBL-SCID mice^{34,35} and nonhuman primate models³⁶⁻³⁹ have shown that high doses of Env-targeted neutralizing antibodies can prevent infection. However, no immunogen capable of eliciting broadly neutralizing Abs has been engineered so far. Elucidation of neutralizing epitope structures may offer valuable insights into the design of such potentially important compounds.

The goal of this study was to identify immunogenic structures that elicit a broad specificity, neutralizing humoral anti-HIV-1 response by screening a phage-displayed peptide library on the anti-CD4bs mAb, IgG1 b12. Our results confirm and extend previously published data defining consensus sequences for the epitope of this antibody.²³ Most importantly, we show that immunization of mice with phage displaying IgG1 b12 mimotopes induces gp120-specific antibodies.

MATERIALS AND METHODS

Antibodies and purified proteins

MAbs IgG1 b12, F105,⁴⁰ and recombinant proteins HIV-1SF2 gp120, HIV-IMN gp120, HIV-ICM gp120, HIV-IIIB (LAV) gp120, HIV-IMN gp160 MN, and soluble CD4 (sCD4) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Control proteins ferritin, KLH, and BSA were obtained from Sigma.

Phage-peptides

A peptide library displayed on minor coat protein (pIII) was kindly provided by Igor Fisch and Greg Winter, MRC, Centre for Protein Engineering, Cambridge, UK. This library consists of two recombined repertoires of random decamers, joined together through a central five-residue spacer (ALLRY) and fused to the pIII coat protein of the

bacteriophage.⁴¹ A control phage displaying tag HA1.1 (YPYDVPDYA)-pIII fusion was constructed by cloning an appropriate DNA insert into the phage vector fdMED1⁴² (kindly provided by I. Fisch) between restriction sites *Sfi*I and *Not*I. FdMED1 is derived from the fdDOG1 vector.⁴³ For alanine scanning, DNA inserts bearing mutations were introduced into FdMED1 clones. Phage dsDNA vectors were purified with QIAprep Spin Miniprep Kit (Qiagen, Courtaboeuf, France) from overnight bacterial cultures. DNA vectors were electroporated into *Escherichia coli* XLI blue (Stratagene, La Jolla, CA). DNA sequencing was performed using an ABI Prism 377 automatic sequencer (Perkin Elmer, Norwalk, CT).

Biopanning

For phage selection, 5-ml polystyrene tubes (Becton Dickinson, Lincoln Park, NJ) were coated overnight at 4°C with 50 µg of protein in 2 ml phosphate-buffered saline (PBS). Tubes were washed twice with PBS and blocked with PBS-3% low-fat milk (PBST) for 2 hr at room temperature. Input phage, 10¹² colony-forming units (cfu) that had previously been blocked in 2 ml of PBST were added to the tubes and incubated for 4 hr at room temperature. Unbound phage were removed by washing 10 times with PBS 0.1 % Tween 20 (PBST) and 10 times with PBS. *E. coli* TGI suspension at mid-log phase (OD₆₀₀ = 0.6) was added directly into each tube and incubated for 1 hr at 37°C with vigorous shaking. The bacteria were then plated onto LB petri dishes containing tetracycline (15 µg/ml) and incubated overnight at 37°C. Infected bacteria were scraped from plates and collected in 50 ml of 2YT broth, incubated for 2 hr at 37°C, and then diluted 1/500 in 2YT broth and cultured overnight. Phage were then purified and titered as previously described.^{41,44} After each round of biopanning, isolated clones were randomly picked from LB agar plates and grown overnight at 30°C in 96- well plates (ATGC Biotechnologie, Noisy le Grand, France) filled with 200 µl of 2YT/tetracycline. Plates were centrifuged (250 X g, 10 min) and the supernatants used directly for phage enzyme-linked immunosorbent assay (ELISA).

Phage ELISA and competition assays

Ninety-six-well Maxisorp immunoplates (NUNC, Roskilde, Denmark) were coated overnight at 4°C with 0.2 µg of mAb in PBS/well. After three washes in PBS, plates were incubated 1 hr at 37°C with PBSM. Dilutions of phage or culture supernatants (100 µl) were blocked with 100 µl of PBSM and incubated in plates for 1 hr at 37°C. For competition assays, CsCl-purified phage were added to each well in a volume of 100 µl of PBSM. Soluble gp120 was then added in a 50 µl volume of PBSM to reach final concentrations of 1, 5, or 10 µg/ml and plates were also incubated for 1 hr at 37°C. Plates were then washed three times with PBST followed by three more with PBS and wells were incubated for 1 hr at 37°C with 100 µl of HRP-conjugated anti-M13 (Amersham, Saclay, France; 1/5000 in PBSM). Plates were again washed (three times PBST, three times PBS) and developed with 100 µl of ImmunoPure TMB Substrate Kit (Perbio Science, Bezon, France) according to the manufacturer's instructions.

Free peptide mimotopes

Mimotope peptides, with N-termini blocked by acetylation and C-termini blocked by amidation, were synthesized by Boc-based solid-phase peptide synthesis on methylbenzhydrylamine resin and purified by reverse-phase high-performance liquid chromatography to reach a purity of at least 95%. Peptide identity was confirmed by mass spectrometry (MALDI/TOF).

Immunization of mice

Five-week-old female C57BL/6 (Iffa Credo, Lyon, France) were used in all experiments. For intragastric administration, eight animals were fed by delivering to the stomach 10^{12} cfu of PEG-purified phage using a blunt steel feeding tube.⁴⁵ Mice received two gavages on day 0 and one at days 7, 14, and 21. Blood samples were collected by retroorbital puncture

before each immunization. For intraperitoneal administration, 10^{11} cfu CsCl-purified⁴⁶ phage that had been dialyzed against PBS at 4°C were emulsified with complete Freund adjuvant (CFA) for the first immunization (week 0) or with incomplete Freund adjuvant (IFA) (boosting, weeks 3 and 6). For conjugation to keyhole limpet hemocyanin (KLH), an additional cysteine residue was added when necessary to the C-terminal end of the peptide. Peptides were then bound to maleimide- KLH (Maleimide- activated KLH Conjugation Kit, Sigma) and purified using gel filtration. Mice were immunized with 50 µg of protein in either CFA or IFA. Blood samples were collected both before and 10 days after each injection and serum was harvested by centrifugation and stored at -20°C. Sera from immunized and control animals were subjected to ELISA. Serum dilutions in PBSM were incubated 1 hr at 37°C in 96-well plates previously coated with 40 µl/well of a 3 µg/ml solution of target proteins [gp160, gp120, ferritin, KLH, bovine serum albumin (BSA)]. After washing, bound IgG were revealed with 1/5000 horse- radish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma).

Antibody library construction

Total RNA was extracted⁴⁷ from 10^8 splenocytes obtained from three mice that had been immunized with 3×10^5 . RNA was reverse transcribed using the Stratagene single-strand synthesis kit according to the manufacturer's instructions. Oligonucleotides (purchased from Eurogentec, Ougree, Belgium) used for library construction are listed in Table 1. Single-chain variable fragments (ScFvs) were constructed from rescued genes, essentially as described previously.⁴⁸ Gene segments encoding the variable domains of the heavy (VH) and of the light chain (Vk) antibodies were amplified separately (94°C 40 sec, 55°C 50 sec, 72°C 2 min 30 times, then 3 min at 72°C) using first round polymerase chain reaction (PCR) primers,^{49,50} and subsequently ligated together by addition of a gene segment encoding a 15-mer amino acid linker (G4S)3. To this end, first round PCR products were purified (PCR purification kit, Qiagen) and reamplified to append DNA sequences encoding portions of

the linker at the 3' end of the VH and the 5' end of the VL genes using JH-link: and VK-link primers together with previously used VHMLn and C kappa primers. VH and Vk segments were then assembled in an overlap extension reaction (10 cycles at 95°C 2 min, 55°C 1 min, and 72°C 3 min) using third round PCR primers encoding appropriate restriction sites for cloning into pHEN1⁴³ (Table 1). ScFv gene repertoires were transfected in *E. coli* and rescued as phagemid particles as previously described.⁴⁸ For selection of gp160-specific antibodies, 10¹⁰ cfu of input phage were incubated for 4 hr at room temperature in 5-ml polystyrene tubes (Becton Dickinson) previously coated overnight with a 2 ml solution of 25 µg/ml of HIV-1 gp160. Washing steps and rescue of eluted phage were performed as described.⁴⁸ After three rounds of biopanning, individual clones were tested by phage ELISA (described above).

RESULTS

Selection of IgG1 b12-specific phage mimotopes

After biopanning using IgG1 b12, 54 clones from the pIII library were individually amplified and tested by phage ELISA. All of these clones (Table 2) bind IgG1 b12, but do not bind irrelevant proteins (BSA, hemocyanin, and ferritin) or control Abs (IgG1k, an isotype-matched human control antibody of unknown specificity, and F105, a human antibody specific for a gp120 epitope that overlaps the CD4bs but distinct from the IgG1 b12 epitope^{40,51}). Figure 1 shows binding analysis of two representative phage clones. Alignment of the sequences of IgG1 b12-specific phage clones enabled us to define an initial consensus sequence M/VΘSD, where Θ represents an aromatic amino acid (Table 2). This motif is also present in a previously reported IgG1 b12-specific peptide.²³ To refine the consensus sequence for binding to IgG1 b12, we chose to conduct further investigations based on the overrepresented clone 3e5 (LGPVWSDLTC, 22 of 54 clones, Table 2).

Identification of a minimal peptide sequence critical for interaction with IgG1 b12

To evaluate the contribution made by each residue in the 3e5 sequence (Table 2) toward IgG1 b12 binding, we constructed a panel of individual phage clones bearing single alanine substitutions at each position in the mimotope sequence. The capacity of these 3e5 mutants to bind to IgG1 b12 was compared to that of unmodified 3e5 phage ELISA. While substitution of amino acids in the initial consensus sequence resulted in a dramatic reduction of specific interaction with IgG1 b12, substitution of residues outside this region had less impact, particularly at positions N-terminal to the consensus sequence (Fig. 2).

Molecular evolution of IgG1 b12 mimotopes

In an attempt to select structures with improved IgG1 b12 binding capacity, we undertook the construction of a second-generation library (L2), in which the consensus sequence M/VWSD was fixed at positions 4, 5, 6, and 7, and diversity was introduced into the flanking regions (Table 3). Biopanning of this library led to the selection of two dominant sequences, VLMVWSDEKG and CLLVWSDEAL. After biopanning,³² clones were picked at random for sequence analysis (Table 3). Comparison of these sequences suggests that a small or hydrophobic residue (preferably leucine) is best accommodated at position 2, while a valine is preferred at position 4 and a glutamic acid at position 8. Based on this information, a third-generation library (L3: XLXVWXDEXX) was designed. We chose to randomize position 6 in this library to verify the importance of serine in the initial consensus sequence, M/V Θ SD, since serine was abundant (27 clones out of 54) but not ubiquitous at this position after selection of the initial library. Most clones selected from this third library included glycine or serine at the first position and a hydrophobic residue (usually leucine or methionine) at position 3. Histidine and leucine were similarly abundant (9 of 28 and 10 of 28, respectively) at position 9. The data also confirm that serine is indeed favored at position 6 (Table 3). We therefore identified G/SLL/MVWSDEL/H as a refined consensus sequence for binding to IgG1 b12 (Table 3), and we opted to carry out further studies using clones 3e5, 3r9, and 19 as representatives from selection on the starting library, L2 and L3,

respectively. We first compared by ELISA the binding of these first-, second-, and third-generation phage mimotopes to IgG1 b12 (Fig. 3). It would appear from these results that the 3r9 and 19 phage mimotopes (second and third generation, respectively) have a higher affinity for IgG1 b12 than 3e5 (first generation).

Phage peptides and gp160 compete for binding to IgG1 b12

As expected, increasing concentrations of gp120 (1-10 µg/ml) inhibited the binding of phage-displayed mimotopes to IgG1 b12 (Fig. 4) while KLH (used as an irrelevant control) had no effect. These results indicate that the selected phage-displayed peptides bind to IgG1 b12, mimicking the structure of the IgG1 b12 epitope on gp120. On the other hand, we did not detect specific inhibition of the interaction of IgG1 b12 with HIV-1 gp160 by the corresponding free peptides, which were also inactive in an envelope-dependent cell fusion assay⁵² (data not shown).

Selected phage mimotopes are weakly immunogenic

KLH-peptide conjugates elicited a strong anti- KLH response but did not elicit any gp160-specific antibodies *in vivo* (data not shown). We therefore focused on the immunogenic potential of phage mimotopes. Anti-HIV-1 gp160 IgG or IgA were not detected in the sera of mice subjected to intragastric administration of phage (not shown). Mice were subjected to three intraperitoneal injections with phage peptides 3e5, 3r9, and 19 at three weekly intervals. Sera were collected every 10 days and tested by ELISA for the presence of anti-phage and anti-HIV-1 gp160 antibodies. At the end of the immunization schedule, all animals had raised substantial amounts of anti-phage antibodies, with end point titers reaching at least 10^4 (data not shown). Notably, anti-gp160 antibodies were also detected in most vaccinated animals, with ELISA signals at least 4-fold higher on gp160 than on BSA (Figs. 5 and 6). Stronger ELISA signals were seen with the second- and third-generation phage peptides, 3r9 and 19, than with 3e5, the phage peptide clone isolated from

the initial library (Fig. 6). Typically, anti-gp160 antibodies were detected 11 days after the first injection; serum titers peaked at day 31, declined thereafter, and became undetectable after 60 days of follow-up (Fig. 5A). As expected, immunization with control phage HA 1.1 elicited an anti-phage response, but anti-gp160 antibodies were not elicited (Fig. 6). The anti-gp160 response elicited by the phage peptides appears to be of broad selectivity, since sera of immunized mice were reactive against envelope from at least five different, unrelated strains of HIV-1 viruses: HIV-1SF2 gp120, HIV-1MN gp120, HIV-1CM gp120, HIV-1IIIB (LAV) gp120, and HIV-1MN gp160 MN (Fig. 5B). Despite the presence of anti-gp160 antibodies in the immune sera, we were unable to detect neutralizing activity on HIV-1 TCLA (data not shown).

IgG1 b12-like antibody recovered from a phage display library

To further characterize the anti-gp160 antibody response elicited by the phage peptides, we set out to rescue the VH and VL genes from three mice immunized with the 3e5 phage and clone them for phage display. The complexity of the resulting ScFv library was estimated to be at least 2×10^5 different clones. After three rounds of biopanning, phage clones were amplified from single colonies picked at random. The amplified phage were then tested by phage ELISA. Clones were considered HIV specific when their phage ELISA reactivity on gp160 MN was at least 5-fold higher than the background signal with BSA in three separate experiments. Only one such clone (phage ScFv5) was identified. A titration assay suggests that ScFv5 has a significantly lower affinity than ScFvb12 for gp160 (Fig. 7). Sequence analysis of phage ScFv5 revealed that the VH and VL CDR3 sequences (GSLDY and QQSNEDPYT, respectively) do not bear any clear homology with the corresponding regions of IgG1 b12.⁵³

Assuming that ScFv5 was elicited in response to immunization with an IgG1 b12 mimotope, we set out to ascertain whether ScFv5 recognizes the same epitope as IgG1 b12, by measuring inhibition of binding of the phage ScFv to gp160 by IgG1 b12. We found that

gp160 binding of phage ScFv5 and phage ScFvb12, a phage clone expressing the VH and VL genes of IgG1 b12 in ScFv format,⁵⁴ was inhibited to a similar extent by IgG 1 b12 (Fig. 8). Hence it is likely that ScFv5 recognizes an epitope similar to that of IgG1 b12.

DISCUSSION

Here we report the use of a phage display approach to identify mimotopes of the neutralizing anti-gp160 antibody IgG 1 b12,^{24,25} and we explore their potential for use as phage peptide vaccines.

Panning a phage peptide library on immobilized IgG1 b12 revealed a strong four-residue consensus motif MN Θ SD (where Θ represents an aromatic residue, preferably W) (Table 2), and the importance of each residue in the motif was confirmed using an alanine-scanning approach (Fig. 2). Interestingly, a previously described peptide that specifically binds IgG1 b12, B2.1 (HERSYM**FSD**LENRCI),²³ which was selected from a phage peptide library displayed on pVIII, also contains the four-residue motif (bold letters). In this study, the authors emphasized the importance of an unpaired cysteine residue for binding activity, showing that it enabled the formation of IgG1 b12-binding peptide homodimers to be presented on the phage coat protein via a disulfide bond. Some of the IgG1 b12-specific mimotopes described in our study (including 3e5) include an unpaired cysteine residue, but since this is not a constant feature of all specific clones, we would suggest that the formation of homodimers is not essential for the binding activity of the phage mimotopes.

To extend and refine the consensus sequence, we constructed second- and third-generation libraries (L2 and L3), in which the amino acids identified in earlier selection experiments were fixed, and diversity was introduced into flanking positions. Analysis of selected sequences from L2 and L3 libraries led us to identify a refined, nine-residue consensus sequence, G/SLL/MV**WSDEL**/H.

Representative clones selected from first-, second-, and third- generation libraries were then compared in terms of immunogenicity. We show in Figure 6 that mice vaccinated with

clone 19 (third generation) exhibited on average a stronger gp160-specific antibody response than mice vaccinated with 3e5 (first generation) or 3r9 (second generation). It should be noted, however, that increased immunogenicity is not necessarily a direct reflection of increased binding affinity for IgG1 b12. It could equally relate to more efficient expression on the surface of phage, i.e., more copies of pIII-peptide fusions per phage particle.

While we were able to detect competition between phage peptides and soluble gp160 for binding to IgG1 b12, the free, soluble peptide mimotopes were unable to compete efficiently with gp160 or phage-borne peptide for binding to IgG1 b12 (data not shown), and they were inactive at blocking envelope-mediated cell fusion when tested up to high micromolar concentrations (data not shown). Together, these results suggest that the environment in which the peptide is presented may have a major impact on its ability to bind IgG1 b12. Binding of unconstrained peptides with their targets invariably leads to entropic loss, which will lower the binding affinity compared to that of the same peptide that is constrained in a binding conformation.⁵⁵ It is plausible that the peptides were presented in a constrained form, owing to interactions with pIII, and that the phage clones that were selected during biopanning displayed peptides that were constrained into an IgG1 b12-binding conformation, thereby enhancing interaction with the antibody. It was shown that a phage-displayed peptide corresponding to the V3 loop of HIV-1 binds anti-V3 loop antibodies and adopts a conformation similar to the antibody-bound free peptide.⁵⁶ If pIII did play an important role in the presentation of mimotope peptides in a binding conformation, it could be predicted that only pIII-fused mimotopes would efficiently induce cognate antibodies *in vivo*. This hypothesis is supported by our observation that while immunization with phage peptide mimotopes elicited gp160-specific antibodies, immunization with peptide mimotopes conjugated to KLH did not. Therefore, it is possible that structural constraints imposed by the phage coat play an important role in the

immunogenicity of the selected peptide, and that the phage particle, or at least the pIII protein, should be considered an important element in vaccine formulation.

In contrast to mice immunized with the B2.1 mimotope peptide,²³ mice vaccinated with IgG1 b12 phage mimotopes 3e5, 3r9, or 19 clearly mounted an HIV-specific humoral response (Figs. 5 and 6). No experimental data or details of the immunization procedure are provided in the article by Zwick et al.²³ and it is possible that differences in the immunization protocol used (phage titer, type of adjuvant) could account for the lack of B2.1-induced antibodies cross-reactive to gp120.

In an attempt to characterize the anti-gp160 antibodies in the immune sera from 3e5-vaccinated mice, we attempted to rescue anti-gp160 antibodies by phage display. We identified one phage antibody clone (ScFv5) that shares a common epitope with IgG1 b12 on gp120 (Fig. 8). This result provides further confirmation that immunization with phage mimotopes elicits the production of antibodies with IgG1 b12-like specificity.

Despite the presence of anti-gp160 immunoreactivity, the immune sera from the immunized mice did not neutralize laboratory-adapted strains of HIV *in vitro* (data not shown). This could be explained, at least in part, by the relatively low titer of gp160 cross-reactive antibodies in the sera (Figs. 5 and 6). Another potential explanation would be that as previously emphasized,²³ 18-residue CDRH3 loops, such as the one borne by IgG1 b12, are not produced in mice.⁵⁷ Therefore, it is plausible that only low-affinity antibodies could be raised in mice against that particular epitope. In any case, our results presented in Figure 7 suggest that ScFv5 has a significantly lower affinity than ScFvb12 for gp160.

The problem of low titer could be addressed by vaccine formulation. A notable consequence of the low number of copies (3-5) of pIII per phage particle is that the amount of phage-displayed peptide that can be injected into an animal is limited, for example, a large dose of phage (10^{14} particles) would represent, at best, a few nanomoles of peptide. A potential solution would be to use purified preparations of peptide fused to soluble variants of pIII protein⁵⁸ instead of phage. Such an approach would enable the dose of peptide injected to

be significantly increased while retaining what we suspect is an essential constrained conformation imposed by the phage protein. The issue of CDRH3 loop length would need to be addressed by vaccination experiments in nonhuman primates, which would be more likely to raise an immune response including antibodies bearing long CDRH3 loops. Of note, protective immune responses have been successfully induced in macaques vaccinated with phage-displayed HIV-1 epitopes.⁵⁹ It would be interesting to test mimotopes of a highly neutralizing epitope such as the one recognized by IgG1 b12 in such a setting.

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FIGURE LEGENDS

FIG. 1. Analysis of binding of IgG1 b12-selected clones by phage ELISA. (A) Binding to indicated (box) plate-adsorbed antibodies. (B) Binding to plate-adsorbed IgG1 b12 and indicated irrelevant proteins (box). In each case (A,B) one representative experiment out of five performed is presented.

FIG. 2. IgG1 b12-binding abilities of 3e5 mutants. Phage clones bearing the indicated mutant peptides were tested by ELISA for their abilities to bind plate-adsorbed IgG1 b12. Maximal effect on binding is seen when the substitutions affect a residue in the VWSD motif.

FIG. 3. Comparison of the capacity of first-, second-, and third-generation phage mimotopes (3e5, 3r9, and 19, respectively) to bind to IgG1 b12. Phage clones bearing the indicated peptides were tested by ELISA for their abilities to bind plate-adsorbed IgG1 b12. Phage-HA is a negative control that does not bind IgG1 b12.

FIG. 4. Competition for phage binding to immobilized IgG1 b12 by in-solution gp120 (1 μ g, 5 μ g, 10 μ g, as indicated) or 10 μ g/ml of control KLH is shown. A greater than 70% decrease in binding is obtained using the highest concentration of gp120. Data shown are from one of three separate experiments, which gave similar results.

FIG. 5. Antibody response induced by vaccinations with phage 3e5. (A) Serum reactivity (dilution 1/100) of three different mice (1, 2, 3) to plate-adsorbed HIV-1 MN gp160 and irrelevant proteins was assayed in ELISA. (B) Serum Abs from animal number 4 were tested for binding to gp120 from four different HIV-1 strains (as indicated) by ELISA.

FIG. 6. Groups of mice were injected with the indicated phage clone mimotopes or control phage displaying the HA 1.1 peptide. Serum reactivities with immobilized BSA (open circles) or gp160 (closed circles) were assayed in ELISA at day 21 after the first injection. Phage displaying the HA peptide was used as control. The number of mice injected in each group is indicated in parentheses. A 3 $\mu\text{g/ml}$ solution of target proteins (gp160 or BSA) was used to coat the plates.

FIG. 7. Analysis by phage ELISA of binding of phage ScFv5, rescued from mice immunized with the 3e5 IgG1 b12 mimotope, phage ScFv-b12, and irrelevant control phage ScFv-OKT3 to plate adsorbed gp160.

FIG. 8. Phage ScFv5 has a similar binding specificity to phage ScFv-b12 itself. Competition for phage ScFv binding to immobilized gp160 by soluble IgG1 b12 (gray boxes) or control IgG1k (black boxes). No binding was observed to plate adsorbed with control protein KLH (open boxes). Phage HA 1.1 was used as an irrelevant control. Data shown are from one of three separate experiments, which gave similar results.

TABLES

Table 1. Oligonucleotides used in construction of murine ScFv library

Round 1	
VHML1	ATGAAATGCAGCTGGGTCAT
VHML2	ATGGGATGGAGCTRTATCAT
VHML3	TGAAGWTGTGGTTAAACTGG
VHML4	ATGRACTTTGGGYTCCAGCT
VHML5	ATGGACTCCAGGCTCAATTT
VHML6	ATGGCTGTCYTRGSGCTRCT
VHML7	ATGGRATGGAGCKGGATCTT
VHML8	ATGAGAGTGCTGATTCTTTT
VHML9	ATGGMTTGGGTGTGGAMCTG
VHML10	ATGGGCAGACTTACATTCTC
VHML11	ATGGATTTTGGGCTGATTTT
VHML12	ATGATGGTGTTAAGTCTTCT
VKML1	ATGAAGTTGCCTGTTAGGCT
VKML2	ATGGAGWCAGACACACTCCT
VKML3	ATGAGTGTGGCTCATTACAGG
VKML4	ATGAGGRCCCCTGCTCAGWT
VKML5	ATGGATTTWCAGGTGCAGAT
VKML6	ATGAGGTKCCYTGYTCAGYT
VKML7	ATGGGCWTC AAGATGGAGTC
VKML8	ATGTGGGGAYCTTKTTYCMMT
VKML9	ATGGTRTCCWCASCTCAGTT
VKML10	ATGTATATATGTTTGTGTC
VKML11	ATGGAAGCCCCATGCTCAGC
C γ	CTCAGGGAARTAGCCYTTGAC
Round 2	
JH1m Link	CCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCC
JH2m Link	CCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACTGTGAGAGTGGTGCC
JH3m Link	CCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGACTGAGGTCC
JH4m Link	CCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGCAGAGACAGTGACCAGAGTCCC
VK1 Link	CAGGCGGAGGTGGCTCTGGAGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC
VK2 Link	CAGGCGGAGGTGGCTCTGGAGGTGGCGGATCGGATATTGTGATGACCCAGACTCCAC
VK3 Link	CAGGCGGAGGTGGCTCTGGAGGTGGCGGATCGGAAATTGTGWTGACRCAGTCTC
VK4 Link	CAGGCGGAGGTGGCTCTGGAGGTGGCGGATCGGACATCGTGATGACCCAGTCTCC
VK5 Link	CAGGCGGAGGTGGCTCTGGAGGTGGCGGATCGGAAACGACACTCACGCAGTCTCC
Round 3	
VH1 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCCAGGTGCAGCTGGTRCARTCTGG
VH2 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCCAGGTACCTTGARGGAGTCTGG
VH3 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG
VH4 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGSAGTSGGG
VH5 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCYGS
VH6 Sfi	GTCCTCGCAACTGCGGCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG
CK Not	GCTCACTTGGATGGGCGGCCGCTGGATACAGTTGGTGCAGCATC

Table 2. Phage clones selected by IgG1 b12

<i>Library</i>	<i>Clone</i>	<i>Peptide sequence</i> ^a	<i>Recovery</i>	<i>ELISA</i> ^b	
pIII		XXXXXXXXXXXX <u>ALLRY</u> XXXXXXXXXXXX	54 ^c	IgG1 b12	Control Ab
	KTa2	<u>ALLRY</u> TTSNPGLKPS	1	0.575	0.010
	KTa12	FLCKCYTLLE <u>ALLRY</u> SLTPWGASPG	1	0.902	0.152
	5f11	<u>ALLRY</u> DDPQASP	1	0.575	0.102
	5a2	MMHDY FFCGD <u>ALLRY</u> DDPQASPV	9	1.290	0.215
	5b11	MMHDY FFCGD <u>ALLRY</u> VPESTRTS	1	0.882	0.217
	5b8	SGRSTW MWSD <u>ALLRY</u> VPDRWSPV	1	0.973	0.026
	5g7	NFSL MWDE F <u>ALLRY</u> VPESTRTSSP	14	1.756	0.030
	5g11	LKGLQL VYSD <u>ALLRY</u>	2	0.946	0.276
	3e5	<u>ALLRY</u> LGP VWSD LTC	22	0.953	0.153
	2.2H5	<u>ALLRY</u> SVPAL PFS FP	1	0.850	0.145
	5c6	<u>ALLRY</u> PPA FSR	1	0.554	0.075
Consensus		M/V Ⓢ SD ^d			

^aUnderlined letters indicate the fixed residues in the libraries; bold letters show redundant residues.

^bAbsorbance at (650–450) nm from ELISA.

^cNumber of total clones sequenced in the biopannings.

^dM/V, M or V; Ⓢ, aromatic residue; X, random residues.

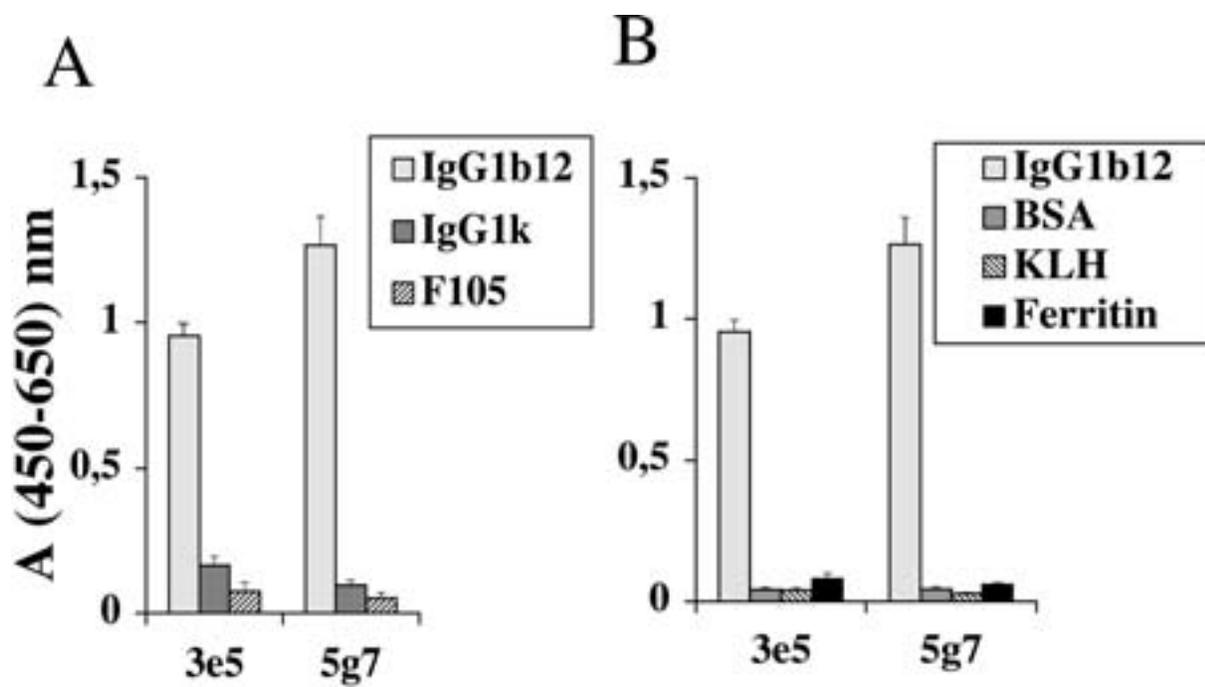
Table 3. Molecular evolution of IgG1 b12 mimotopes

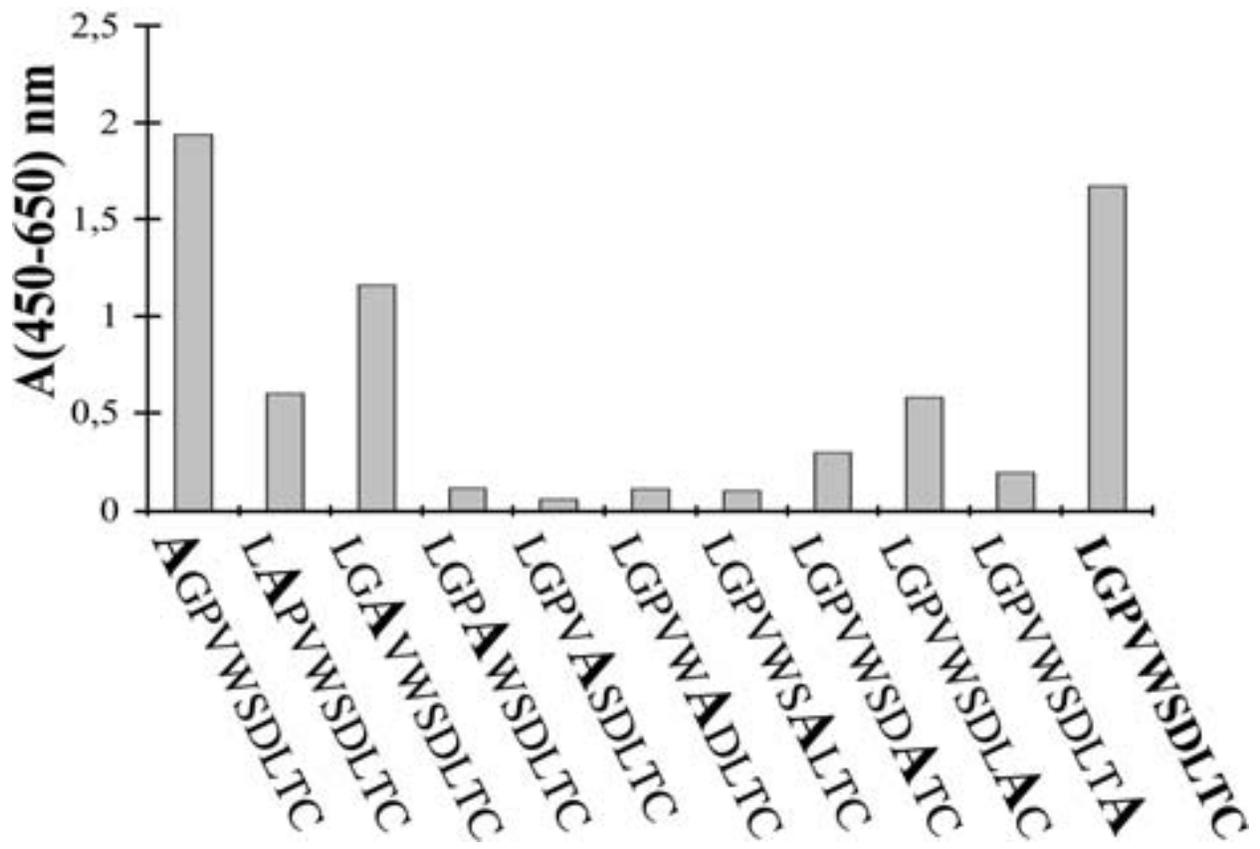
<i>Peptide sequence^a</i>										<i>Clone</i>	<i>Recovery</i>
X	X	X	M/V	W	S	D	X	X	X	(L2)	32 ^b
V	L	M	V	.	.	.	E	K	G	3r9	13
C	L	L	V	.	.	.	E	A	L		4
C	W		V	.	.	.	E	Y	N		1
E	V	V	V	.	.	.	F	Y	Q		1
F	G	M	V	.	.	.	Y	A	P		1
G	A	V	M	.	.	.	Q	A	L		1
G	L	L	M	.	.	.	L	S	P		1
L	D	D	M	.	.	.	E	L	P		1
L	G	F	V	.	.	.	G	D	A		1
L	G	S	V	.	.	.	D	T	P		1
R	G	G	V	.	.	.	F	A	V		1
R	G	V	M	.	.	.	S	I	P		1
R	I	L	M	.	.	.	P	L	P		1
S	S	W	M	.	.	.	L	E	P		1
V	S	V	V	.	.	.	P	T	S		1
Y	V	T	V	.	.	.	E	A	P		1
X	L	X	V	W	X	D	E	X	X	(L3)	28 ^b
G	.	M	.	.	S	.	.	H	G	19	5
S	.	L	.	.	S	.	.	L	D		3
S	.	L	.	.	S	.	.	H	C		3
G	.	M	.	.	S	.	.	L	G		2
S	.	L	.	.	S	.	.	K	M		2
S	.	L	.	.	S	.	.	M	N		2
D	.	L	.	.	S	.	.	L	G		1
E	.	R	.	.	S	.	.	F	D		1
G	.	L	.	.	S	.	.	A	C		1
G	.	L	.	.	S	.	.	L	S		1
G	.	M	.	.	S	.	.	H	G		1
G	.	M	.	.	S	.	.	M	C		1
G	.	T	.	.	S	.	.	L	G		1
Q	.	R	.	.	S	.	.	L	G		1
S	.	L	.	.	S	.	.	F	D		1
Y	.	L	.	.	S	.	.	M	D		1
Y	.	R	.	.	S	.	.	L	N		1
G/S	L	L/M	V	W	S	D	E	L/H			

^aBold characters indicate redundant residues in the second (L2) and third (L3) sublibrary.

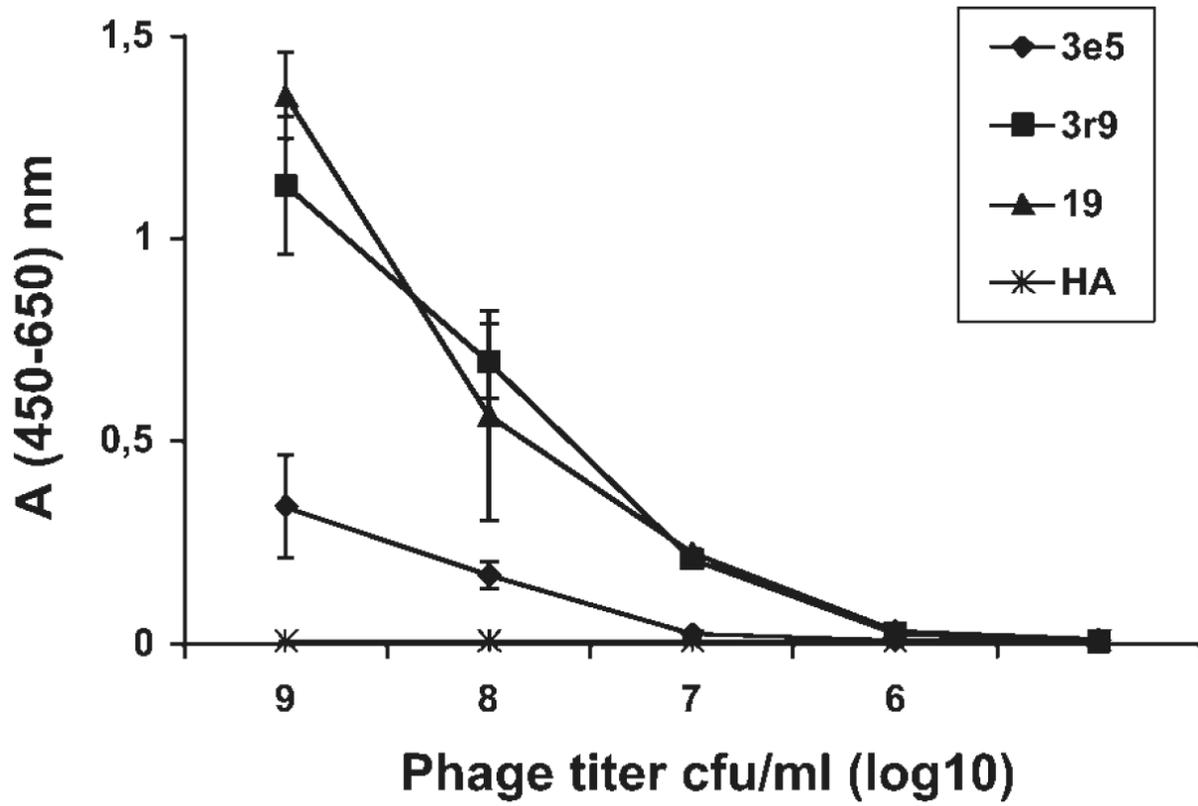
^bNumber of clones sequenced in the biopanning.

FIGURES

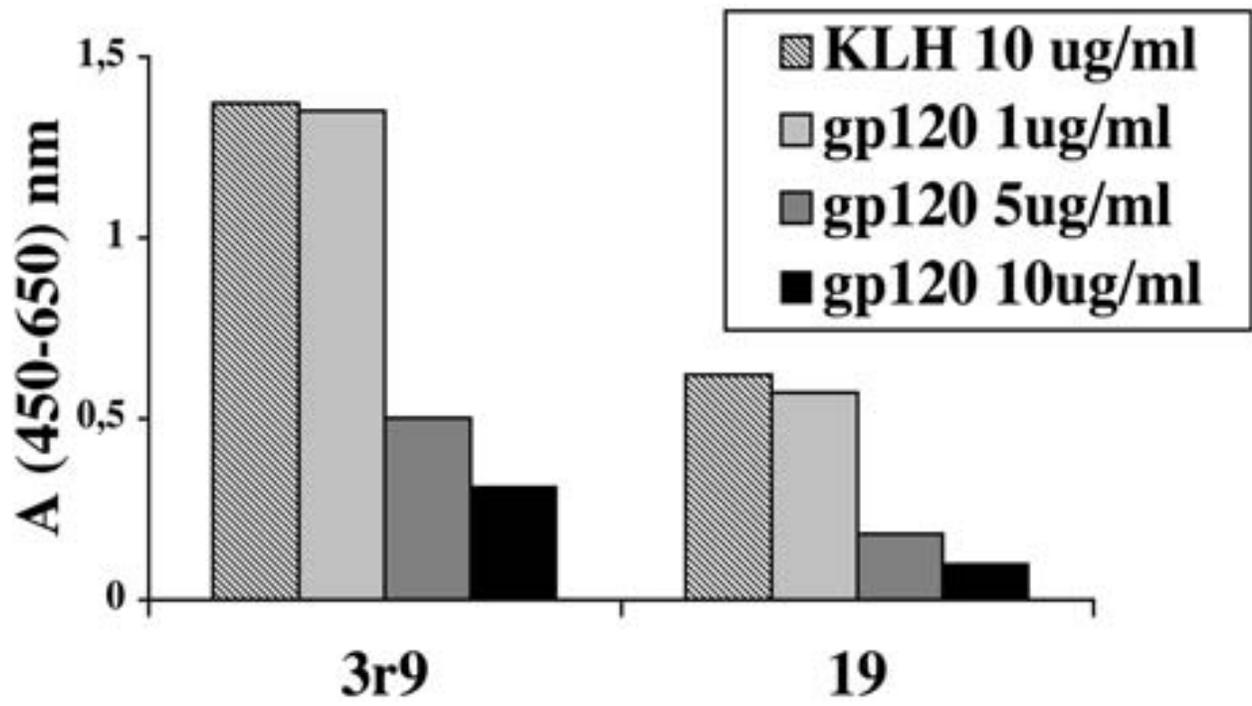


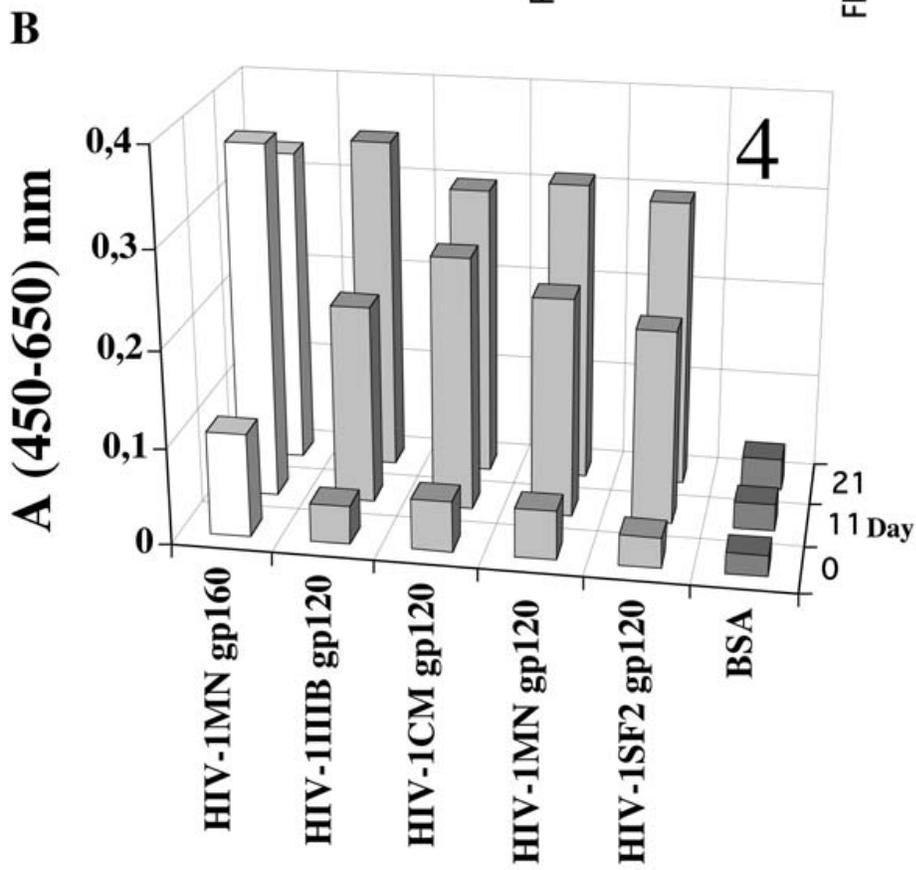
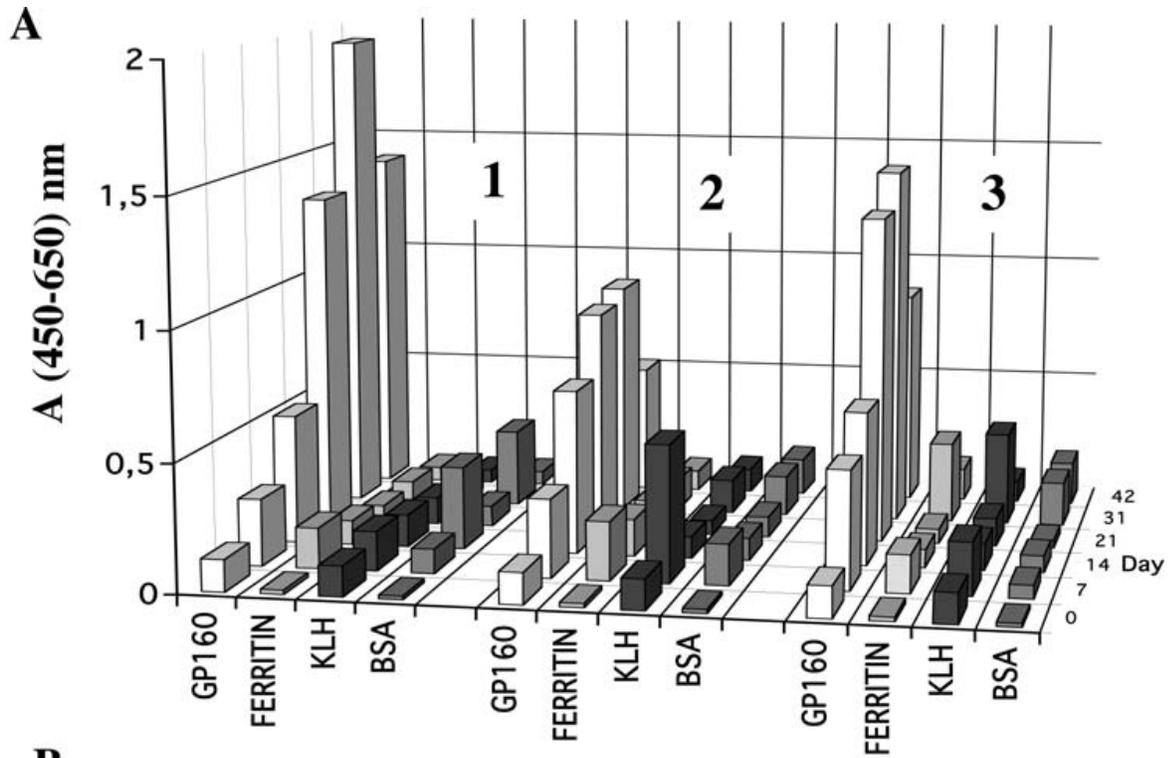


Dorgham et al. Figure 2

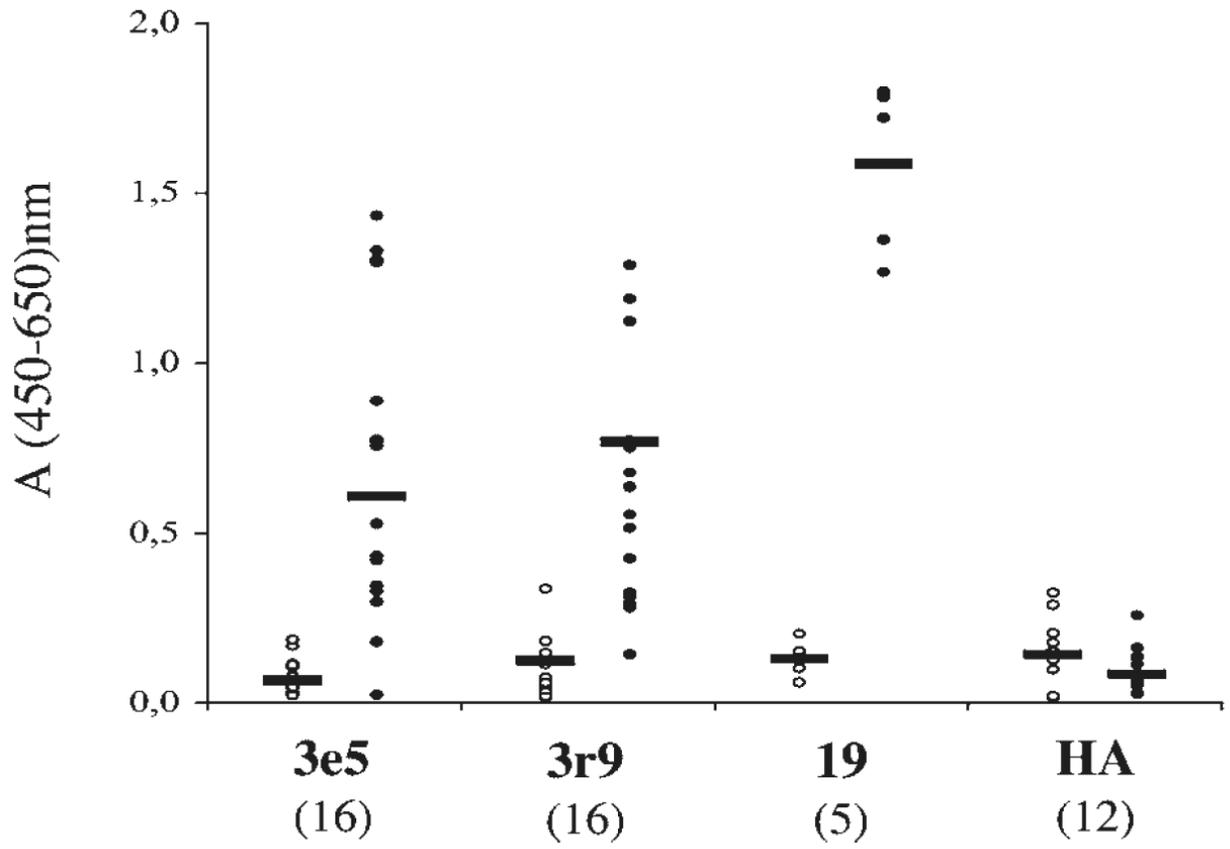


Dorgham et al. Figure 3

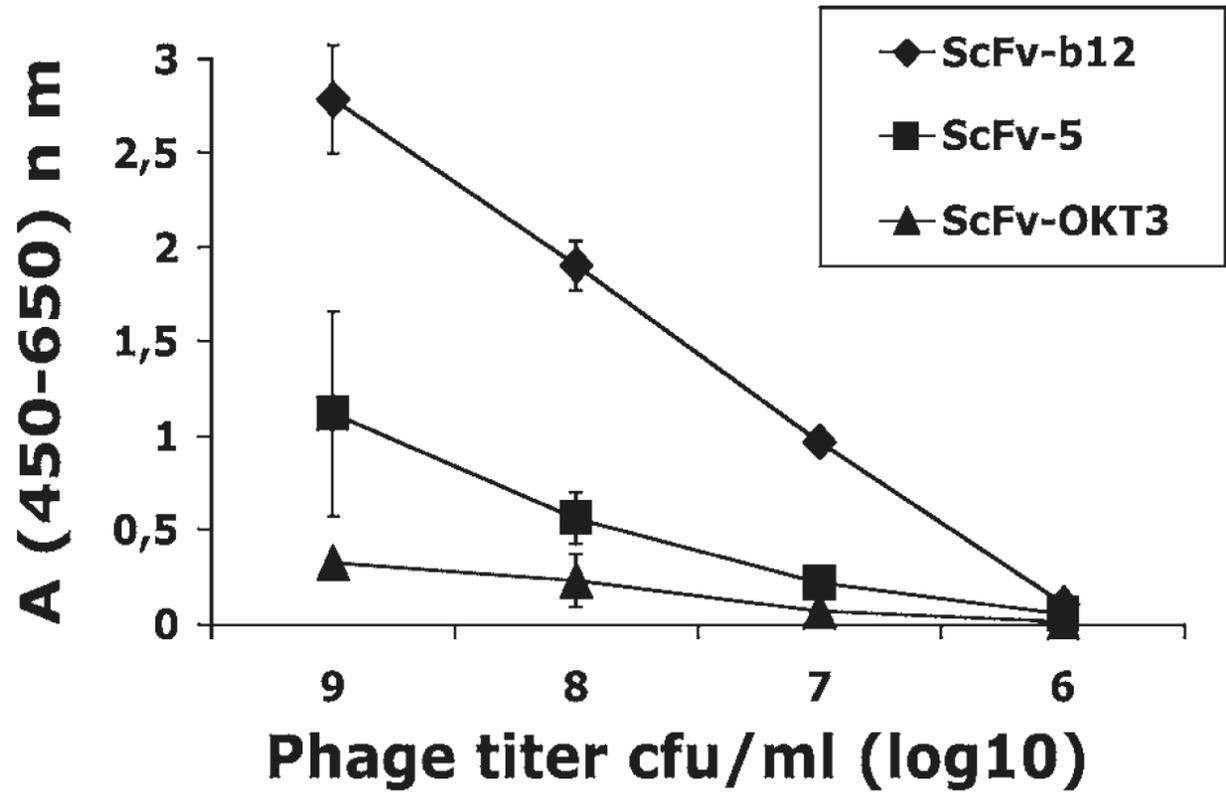




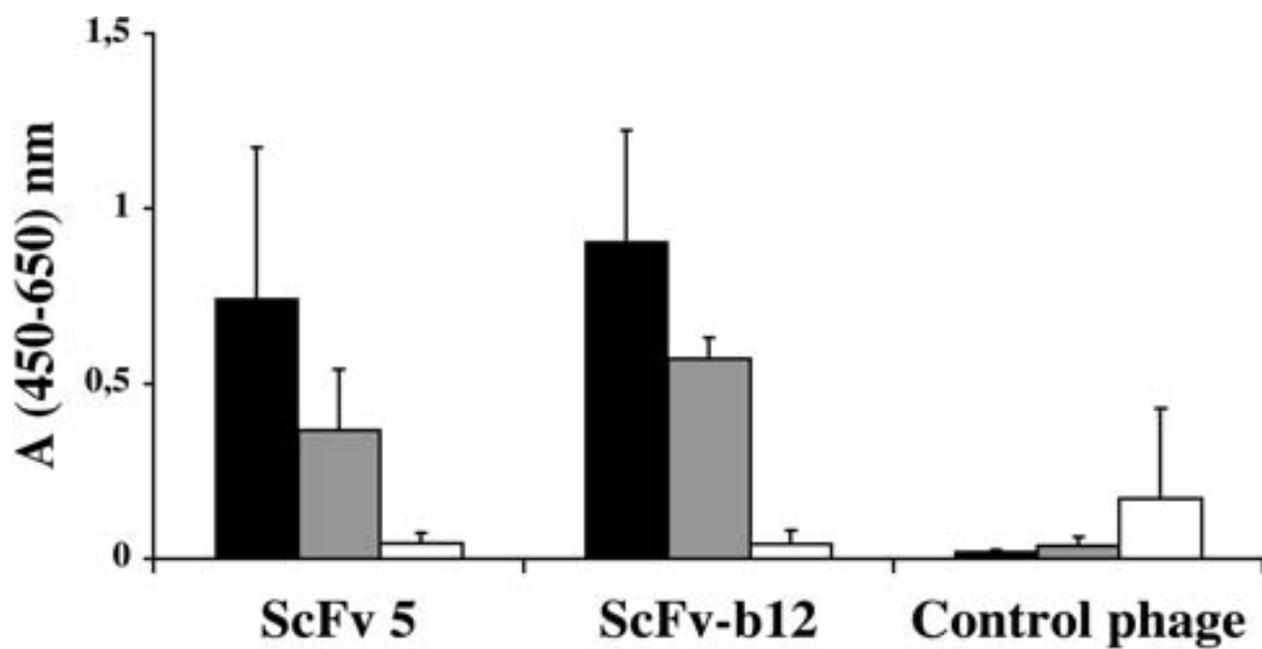
Dorgham et al. Figure 5



Dorgham et al. Figure 6



Dorgham et al. Figure 7



Dorgham et al. Figure 8