Vaccination with peptide mimetics of the gp41 prehairpin fusion intermediate yields neutralizing antisera against HIV-1 isolates

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Eliciting a broadly neutralizing polyclonal antibody response against HIV-1 remains a major challenge. One approach to vaccine development is prevention of HIV-1 entry into cells by blocking the fusion of viral and cell membranes. More specifically, our goal is to elicit neutralizing antibodies that target a transient viral entry intermediate (the prehairpin intermediate) formed by the HIV-1 gp41 protein. Because this intermediate is transient, a stable mimetic is required to elicit an immune response. Previously, a series of engineered peptides was used to select a mAb (denoted D5) that binds to the surface of the gp41 prehairpin intermediate, as demonstrated by x-ray crystallographic studies. D5 inhibits the replication of HIV-1 clinical isolates, providing proof-of-principle for this vaccine approach. Here, we describe a series of peptide mimetics of the gp41 prehairpin intermediate designed to permit a systematic analysis of the immune response generated in animals. To improve the chances of detecting weak neutralizing polyclonal responses, two strategies were employed in the initial screening: use of a neutralization-hypersensitive virus and concentration of the IgG fraction from immunized animal sera. This allowed incremental improvements through iterative cycles of design, which led to vaccine candidates capable of generating a polyclonal antibody response, detectable in unfractionated sera, that neutralize tier 1 HIV-1 and simian HIV primary isolates in vitro. Our findings serve as a starting point for the design of more potent immunogens to elicit a broadly neutralizing response against the gp41 prehairpin intermediate.

membrane fusion | vaccine | coiled coil | mimotope

A variety of approaches have been used across many research groups to develop a vaccine against HIV-1 (1–3). Eliciting broadly neutralizing antibodies against HIV-1 has proven to be a formidable challenge. Nevertheless, promising progress continues to be made (4, 5). We are focusing our efforts on eliciting neutralizing antibodies that target a transient viral entry intermediate. Earlier studies have shown that this transient intermediate, referred to as the prehairpin intermediate, can be targeted by peptide inhibitors that prevent HIV-1 infection in vitro (6) and in human patients (7).

The HIV-1 envelope glycoprotein complex consists of two noncovalently associated subunits, gp120 and gp41. According to the currently accepted model for HIV-1 fusion, following engagement of host cellular receptors, the viral envelope complex undergoes a cascade of conformational changes culminating in the insertion of part of the viral fusogenic subunit gp41 into the host cell membrane. The mechanism of fusion involves two helical regions of gp41, the N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR), which associate to form a fusion-active "trimer-of hairpins," a common structure in the fusion mechanism of many enveloped viruses (8, 9). This structure consists of a bundle of six α-helices, in which three CHR peptides pack in an antiparallel manner against a central three-stranded coiled-coil formed by the NHR regions of the same gp41 monomers (10–14). A significant body of evidence supports the notion that fusion progresses via formation of a prehairpin conformation that places the amino-terminal fusion peptide of gp41 in the target cell membrane, exposing both the NHR and CHR regions (8).

The gp41 prehairpin intermediate is a potential target for vaccine development (15). Importantly, because the prehairpin intermediate is transient, attempts to elicit an immune response against this intermediate require the engineering of a stable mimetic to serve as an immunogen. Eckert et al. (15) designed a soluble trimeric peptide mimetic (denoted IQN17) of the NHR region of the prehairpin intermediate. IQN17 was used to select for NHR-binding inhibitors of HIV-1 infection using mirror image phage display, providing strong evidence that IQN17 presents the NHR region in the same structure as is present in the actual prehairpin intermediate (15).

Using subsequently designed peptide mimetics of the NHR trimer, denoted IZN36 (16) and 5-helix (17), as selection agents with a single-chain antibody variable region (scFv) phage-display library, we previously identified human mAbs that neutralize various HIV-1 isolates in vitro, validating the NHR region as a viable vaccine target (18). The crystal structure of the Fab fragment of one of these mAbs (denoted D5) in complex with 5-helix (19) confirmed that D5 binds to the NHR region. Interestingly, D5 binds the same conserved hydrophobic pocket of the NHR trimer (20) that was previously shown to be the target of D-peptide (15) and peptidomimetic (21, 22) HIV-1 fusion inhibitors. These findings provide proof-of-concept that antibodies can bind to and neutralize the prehairpin intermediate in vitro.

Nonetheless, eliciting a neutralizing polyclonal response against the prehairpin intermediate has proven difficult. In our earlier studies, we used immunogens that were shown to be excellent mimics of the gp41 prehairpin intermediate as judged by x-ray crystallography and the observation that they were subnanomolar inhibitors of HIV-1 infection (16, 23). Other groups have also identified human or rabbit antibodies that can bind to NHR mimetics and inhibit HIV-1 in vitro (24–27). However, in these published reports from other groups, and in our own

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earlier studies, it has not been possible to demonstrate antiviral activity in unfractionated polyclonal sera raised against these mimetics. Obviously, such a demonstration is critical for the development of an antibody-based HIV vaccine.

Here, we use two strategies to increase our ability to detect weak neutralization responses, which could subsequently be used to improve immunogen design. First, we identified a mutant virus of the HIV-1 isolate HXB2, designated HXB2-V570A, which is hypersensitive to neutralization by D5 and other inhibitors that target the HIV-1 prehairpin intermediate, and used this virus in our initial assays. Second, we purified IgG from immune sera to provide more concentrated antibody solutions in our initial assays. These more sensitive approaches allowed us to detect weak neutralizing activity in initial experiments. Subsequently, we modified the peptide mimetics of the prehairpin intermediate in an iterative manner and tracked the antiviral activity that resulted from immunization with these modified immunogens. Ultimately, we were able to detect cross-neutralizing responses in unfractionated immune sera, a critical step toward the development of an HIV-1 vaccine.

Results

Immunogen Design. ***Peptide mimetics of the hydrophobic pocket region.*** Our first set of immunogens (Fig. 1) focused on residues 565–581 of the NHR (numbering according to HXB2 sequence), corresponding to the prominent hydrophobic pocket of the coiled-coil (11, 20), which encompasses the D5 epitope (18, 19). The chimeric mimetic IZN17 consists of an engineered trimerizing and solubilizing coiled-coil (denoted IZ) scaffold fused to aa 565–581 of gp41 (16). The second mimetic is a covalently stabilized trimer of IZN17 called $(CCIZN17)_3$ (23). In this construct, the three peptide chains are covalently stabilized at the N termini by three interchain disulfide bonds between pairs of cysteines located outside the coiled-coil domain. This engineering imparts a very high degree of thermodynamic stability to the trimer, resulting in higher antiviral potency. This peptide is potentially a better immunogen than IZN17, because the trimeric state of the peptide is not dependent on self-association as for IZN17, and the conformational epitope within the hydrophobic pocket is therefore expected to be present even at the highly diluted concentrations resulting from vaccination. Previously, Louis et al. (27) were able to show a neutralizing response in vitro but only with affinity-purified antibodies elicited by trimeric gp41 peptides covalently stabilized by disulfide bonds within the coiled-coil domain. In contrast, our vaccine strategy utilizes peptide immunogens with increased helicity and improved subnanomolar inhibition of HIV infection (23).

Suppression of the immune response to irrelevant epitopes. The third immunogen, $(CCIPN17)_3$, was designed to shield the IZ scaffold from immune recognition in an attempt to minimize generation of antibodies against the nonrelevant portion of the peptide chimera, and subsequently focus the immune response against the NHR region. The strategy is to mask the undesired IZ epitope selectively with suitably located low molecular weight polyethylene glycol (LMWPEG) chains (28). Starting with $(CCIZN17)_3$, we designed $(CCIPN17)_3$ by introducing PEG chains with a nominal length of 15 Å at two exposed positions along the IZ scaffold of each peptide chain (Fig. 1). The LMWPEGs were positioned so as not to perturb accessibility to the 17 NHR residues encompassing the D5 neutralizing epitope. $(CCIPN17)_3$ is fully helical, as determined by CD spectroscopy. Thermal denaturation experiments at neutral pH show that even in the presence of 2 M GuHCl, the peptide has a melting temperature (T_m) >90 °C (Table S1), which compares well with what had previously been reported for the corresponding non-PEGylated $(CCIZN17)_3$ (23). Furthermore, $(CCIPN17)_3$ binds D5 with an efficiency similar to $(CCIZN17)_3$ (28), suggesting that the chosen positioning of the LMWPEG chains does not

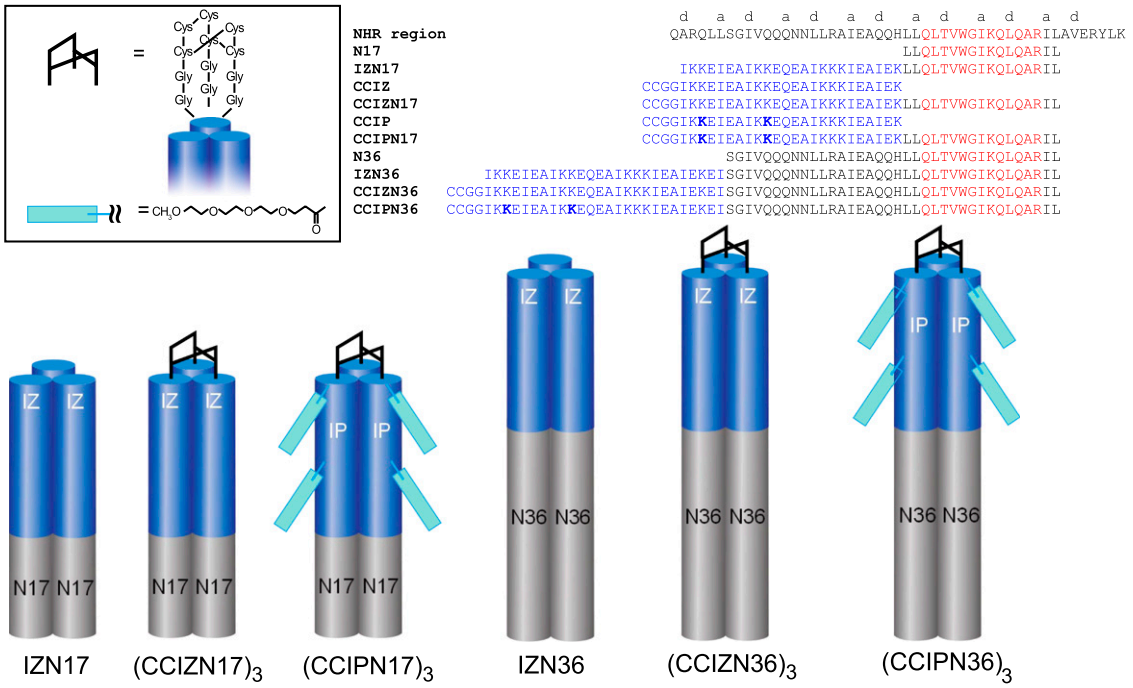

Fig. 1. Schematic model of engineered peptide immunogens. IZN helices forming a homotrimeric coiled-coil are represented by cylinders. Each chimeric N-peptide consist of an N-terminal solubilizing and trimerizing coiled-coil domain (IZ, blue) fused to a sequence from gp41 NHR (gray), designated N17 (residues 565–581 of HXB2) or N36 (residues 546–581 of HXB2). $(CCIZN)_3$ helices are covalently stabilized at the N termini of each peptide chain by three disulfide bonds between cysteine pairs located outside the coiled-coil domain. Only one of the possible combinations of the three disulfides is drawn. (*Left Inset*) "IP" scaffolds were shielded from immune recognition by LMWPEG chains, attached by amide linkage to the ε-amino group of lysine residues and represented by light-blue boxes in the main diagram. (*Right Inset*) Alignment of the sequences of each peptide or fragment to the NHR sequence (residues 540–588) and corresponding positions "a" and "d" of the heptad repeats of the coiled-coil. HIV-1 residues in the hydrophobic pocket are shown in red, non-HIV-1 and scaffold residues are shown in blue, and lysine residues derivatized with LMWPEG are shown as bold.

preclude accessibility to the HIV-1 sequence and is not detrimental to the structural integrity of the D5 epitope.

To test the ability of LMWPEG chains to shield the scaffold from immune recognition, we compared $(CCIZN17)_3$ and $(CCIPN17)_3$ in immunization studies in guinea pigs. Animals were injected three times with either peptide at weeks 0, 4, and 8. Antibody titers to $(CCIZN17)_3$ and $(CCIPN17)_3$ as well as the scaffolds alone, denoted $(CCIZ)_3$ and $(CCIP)_3$, were determined by ELISA using the corresponding biotinylated peptides on streptavidin-coated plates. As shown in Fig. S1, although anti-$(CCIZN17)_3$ and anti-$(CCIPN17)_3$ titers were comparable ($\approx 1.5 \times 10^5$), the majority of the response to $(CCIZN17)_3$ was directed toward the IZ scaffold. In contrast, very little response toward either the $(CCIP)_3$ or $(CCIZ)_3$ scaffold was elicited by immunization with $(CCIPN17)_3$, demonstrating that by shielding the IZ scaffold with LMWPEG, it is possible to suppress the immune response to that region selectively, while retaining the immunogenicity of the epitope of choice.

Peptide mimetics of a longer segment of the NHR. Our previous work showed that by selecting a scFv phage library with antigens spanning the longer N36 region (HXB2 residues 546–581), only antibodies preferentially binding in the smaller hydrophobic pocket region, as defined above, were identified (18). This was the motivation to focus initially on N17-based peptides. However, the possibility exists that antibodies recognizing the trimeric coiled-coil at multiple sites outside the hydrophobic pocket could strengthen the neutralization efficiency of the elicited humoral response. To test this hypothesis, we included as immunogens several chimeric N-peptides consisting of IZ fused to N36. In particular, we synthesized IZN36 (16), which had previously been employed for D5 selection (18); $(CCIZN36)_3$, a covalent trimeric form of IZN36 analogous to $(CCIZN17)_3$; and $(CCIPN36)_3$, a covalent trimeric construct with LMWPEG chains to shield the IZ scaffold (Fig. 1). All three N36 constructs had high helical content, as determined by CD spectroscopy (Table S1). Thermal denaturation experiments at neutral pH and at a concentration of 10 μM peptide demonstrated a high degree of stability with a $T_m > 90$ °C (Table S1). In the presence of 2M GuHCl, IZN36 had a T_m of 72 °C, whereas both of the covalent constructs maintained higher stability with a $T_m > 90$ °C. These results compare favorably with data obtained on the N17-based constructs (16, 23) (Table S1).

Serological Analysis of Animals Immunized with NHR Peptide Mimetics. NHR peptide mimetics were evaluated as immunogens in guinea pigs and rabbits to determine whether a neutralizing antibody response was induced. Antibody titers in matched post-dose 3 (PD3) and preimmune sera from individual animals were determined by end-point ELISA. All immunogens elicited a robust peptide-specific response, with group geometrical mean titers of $\approx 10^5$–10^6 (Table S2).

NHR peptide mimetics elicit hydrophobic pocket-targeted D5-like antibodies. To determine whether our NHR mimetics were capable of eliciting a "D5-like" antibody response (i.e., one directed primarily toward the well-conserved hydrophobic pocket), we employed a D5/5-helix competitive binding assay (DCBA) (*SI Materials and Methods* and Fig. S2). The IgG fraction from corresponding individual preimmune and PD3 sera was isolated by batch protein A affinity chromatography, followed by a buffer exchange and concentration step (final concentration approximately 5-fold higher than in the starting sera). Analysis of these purified IgG fractions in the DCBA shows that the vaccines can elicit D5-like IgGs and, importantly, the proportion of vaccine-induced D5-competitive antibodies increased as a function of both conformational constraint and increasing peptide sequence length (Table S2). In guinea pigs immunized with either $(CCIZN36)_3$ or $(CCIPN36)_3$, the concentration of D5 competitive antibodies is roughly 10- to 100-fold higher than in those immunized with $(CCIZN17)_3$ or IZN36.

Use of a hypersensitive virus to rank-order NHR peptide mimetics. Amino acid substitutions of CHR residues that pack into the NHR hydrophobic pocket have been shown to destabilize CHR binding (20). Based on our previous work (18), we identified a mutant virus containing a single Val-to-Ala substitution at position 570 within the hydrophobic pocket region of the HXB2 NHR (V570A) that was more sensitive to inhibitors that bind to the prehairpin intermediate but not to neutralizing mAbs that bind elsewhere (a detailed characterization is provided in *SI Materials and Methods* and Fig. S3). Fig. 2 summarizes the neutralization potencies of purified IgG from guinea pigs (Fig. 2 *A* and *B*) or rabbits (Fig. 2*C*) immunized with NHR peptide mimetics. As expected, neutralization potencies are higher against HXB2-V570A (Fig. 2*A*) than against HXB2 (Fig. 2*B*). The immunogen rank-order is preserved between viruses: The most effective peptide immunogens against either virus are $(CCIZN36)_3$ and $(CCIPN36)_3$. The advantage of using HXB2-V570A was even more apparent in the analysis of rabbit sera. Consistent with the observation that the total and functional immune responses were lower in rabbits for all immunogens, no neutralization was detected against HXB2. The use of HXB2-V570A confirmed that the rank-order was maintained in rabbits, with $(CCIZN36)_3$ and $(CCIPN36)_3$ being the best immunogens (Fig. 2*C*). Overall, it appears that higher neutralizing activity is elicited with the N36-based peptides compared with their N17 counterparts. Higher neutralization activity is also obtained with covalent tri-

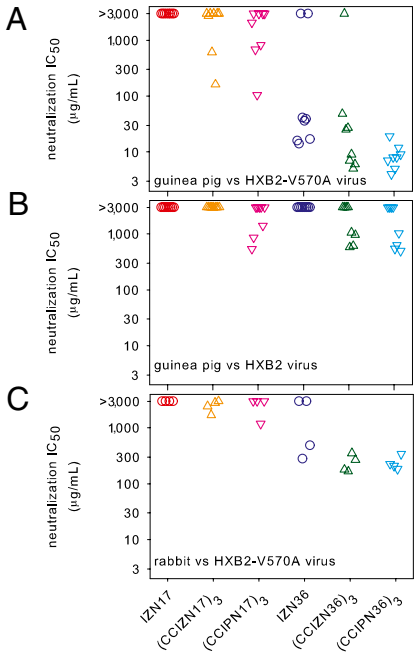

Fig. 2. Neutralization of HIV-1 infection with purified IgG. The IgG fraction from sera of individual guinea pigs (*A* and *B*) or rabbits (*C*) vaccinated with NHR immunogens was purified and tested in the p4-2/R5 single-cycle infectivity assay for its ability to neutralize either D5 hypersensitive HXB2-V570A virus (*A* and *C*) or parental HXB2 virus (*B*). The IC_{50} is plotted on the *y* axis for each sample and grouped by NHR immunogen IZN17 (red ○), $(CCIZN17)_3$ (orange △), $(CCIPN17)_3$ (pink ▽), IZN36 (blue ○), $(CCIZN36)_3$ (green △), and $(CCIPN36)_3$ (turquoise ▽). All preimmune IC_{50}s were >3,000 μg/mL, with the exception of a single sample [1,800 μg/mL vs. HXB2-V570A for one guinea pig in the $(CCIPN36)_3$ group]. All rabbit sera had IC_{50}s >3,000 μg/mL vs. HXB2 virus. For guinea pigs, comparing the group of all N17 peptides vs. the group of all N36 peptides, N17 and N36 were highly statistically different ($P < 0.001$, two-tailed *t* test on ranked data) for HXB2-V570A but were not statistically significant ($P = 0.08$) for HXB2. For HXB2-V570A in guinea pigs, $(CCIZN36)_3$ and $(CCIPN36)_3$ were superior to all N17 immunogens (maximum $P < 0.017$, Dunnett's test with control on ranked neutralization values).

meric immunogens: $(CCIZN17)_3$ and $(CCIPN17)_3$ are more effective immunogens than IZN17, and $(CCIZN36)_3$ and $(CCIPN36)_3$ are more effective than IZN36 (Fig. 2).

Neutralization potency is correlated with the concentration of D5-like antibodies. X-ray crystallographic studies show that D5 binds to the conserved hydrophobic pocket of the prehairpin intermediate (19). The concentration of antibodies capable of competing with D5 for the hydrophobic pocket region was measured by DCBA (Fig. S2). Fig. 3 plots the correlation between the concentration of D5-like antibodies as a function of neutralization potency for guinea pig data (Fig. 3*A*) and rabbit data (Fig. 3*B*). Strikingly, the longer N36-based immunogens are more effective at eliciting D5-like antibodies even though the hydrophobic pocket region is contained within the N17 region alone. One explanation for the observed correlation is that the longer N36 peptides are more accurate mimetics of the hydrophobic pocket, increasing the likelihood of raising specific antibodies targeting this pocket after immunization. However, previous data from our group have shown that D5 binds N17- and N36-based mimetics equally well (18), suggesting that they share the same conformation.

Detecting neutralization activity in unfractionated antisera and with other viral isolates. The higher neutralizing IC_{50} titers obtained with HXB2-V570A in purified IgG from sera of animals immunized with either $(CCIZN36)_3$ or $(CCIPN36)_3$ suggested that neutralization responses should be detectable in unfractionated serum. To gain preliminary insight on the breadth of the neutralization response, we tested individual guinea pig serum samples from these groups against a small panel of HIV-1 isolates as well as simian HIV (SHIV) SF162p3, which has previously been tested in multiple monkey challenge studies (29–31). The HIV-1 viruses used for this panel are representative of tier 1 isolates, defined as being sensitive to antibody-mediated neutralization and mainly comprising T cell-adapted viruses (32). For comparison, the antiviral potency of two broadly neutralizing human mAbs in our P4-2/R5 assay is shown in Fig. S3. The IC_{50} data are plotted in Fig. 4*A* for individual animals for both immunogens. As expected, the most potent neutralization response is observed against HXB2-V570A. Interestingly, isolates 89.6 and 129 appear to be more neutralization-susceptible than parental HXB2. The data indicate that unfractionated antisera that best neutralize HXB2-V570A also tend to neutralize unrelated viral isolates more potently, suggesting that broad cross-reactivity may be possible in some cases.

This qualitative assessment of a correlation between activity against HXB2-V570A and other unrelated viruses was confirmed by statistical analysis. Nonparametric rank correlations of IC_{50} for each viral isolate vs. HXB2-V570A were calculated (Fig. 4*B*). Nonparametric methods have the advantage that no model-specific assumptions are made between the variables, at the loss of some statistical power. Ranks were highly correlated; that is, the best neutralizing sample for HXB2-V570A tended also to be the best neutralizing sample for another virus, the second-best neutralizing sample likewise, and so forth, with high significance ($P < 10^{-4}$ for HXB2, SF162p3, DH012, and 129, and $P = 0.005$ for 89.6). Thus, HXB2-V570A is a sensitive probe of D5-like neutralization that retains correlation with other viral isolates and was a useful tool in the search for antisera and vaccine candidates in this experiment.

Expanded panel of HIV-1 isolates. We next prepared pools of sera from individual animals immunized with $(CCIZN36)_3$ and $(CCIPN36)_3$ and tested these in the TZM-bl pseudovirion entry inhibition assay against an expanded panel of isolates composed of 12 tier 1 and 10 tier 2 viruses, representative of clades A, B, and C. Tier 1 viruses tend to be sensitive to antibody-mediated neutralization, whereas tier 2 viruses predominantly encompass primary isolates that are more neutralization-resistant (32). We included HXB2-V570A, 89.6, and 129 as bridging viruses between the p4-2/R5 and TZM-bl assays.

The data for the tier 1 panel are summarized in Table 1. Both $(CCIZN36)_3$ and $(CCIPN36)_3$ elicited detectable neutralizing antibody titers in guinea pigs against approximately half of the tier

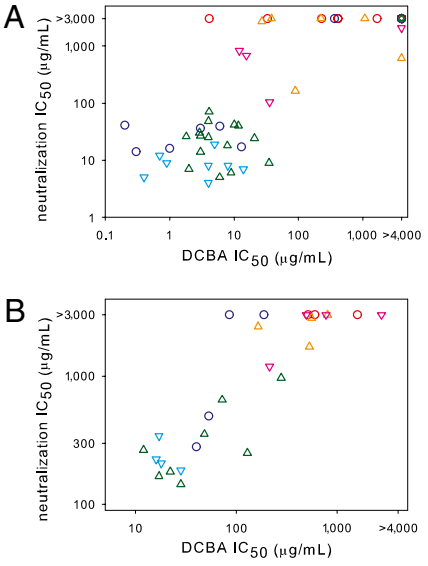

Fig. 3. Neutralization potency correlates with D5-like antibody response. Neutralization IC_{50} titers for purified IgG determined in the p4-2/R5 assay against HXB2-V570A from vaccinated guinea pig (*A*) or rabbit (*B*) antisera were plotted as a function of the antibody concentration that competes for the D5 epitope as determined by DCBA (scheme is shown in Fig. S2). Symbols for individual immunogens correspond to those in Fig. 2. Each data set is rank-correlated ($P < 0.0001$) with Spearman's $\rho = 0.76$ (*A*) and $\rho = 0.85$ (*B*).

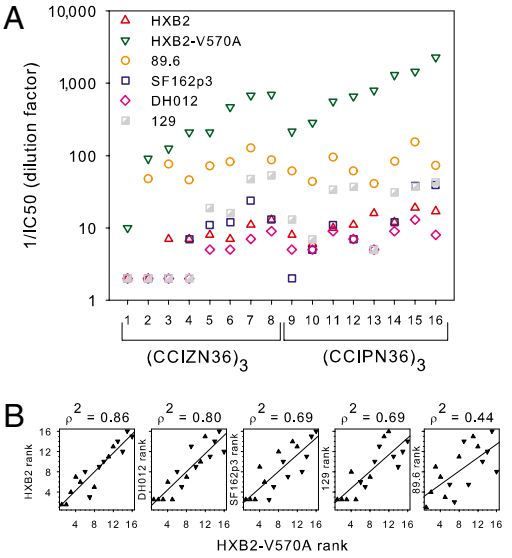

Fig. 4. Neutralization breadth and correlations to HXB2-V570A in unfractionated sera. Antisera from guinea pigs immunized with either $(CCIZN36)_3$ (identification numbers 1–8), or $(CCIPN36)_3$ (identification numbers 9–16) were tested in the p4-2/R5 neutralization assay against a small panel of HIV-1/SHIV viral isolates. (*A*) Reciprocal of IC_{50} is plotted for individual animals. Note that sera with high neutralization of HXB2-V570A tend to higher neutralization of other viral isolates, an indication of broad cross-reactivity. (*B*) These data are presented as individual rank-correlation plots for each viral isolate relative to HXB2-V570A, in descending order of correlation. Rank correlations (Spearman's ρ) title each plot; all correlations were highly significant ($P < 10^{-4}$ for all except $P = 0.005$ for 89.6 vs. HXB2-V570A). $(CCIZN36)_3$ (▲) and $(CCIPN36)_3$ (▼).

Table 1. Neutralization of tier 1 pseudoviruses in the TZM-bl assay with pooled unfractionated sera

Pseudovirus	Clade	Guinea pig IC_{50}, 1 per dilution		Rabbit IC_{50}, 1 per dilution	
		$(CCIZN36)_3$	$(CCIPN36)_3$	$(CCIZN36)_3$	$(CCIPN36)_3$
SF162.LS	B	11	<10	<10	<10
Bal.26	B	<10	<10	<10	<10
SS1196.1	B	20	18	<10	<10
BZ167.12	B	85	62	<10	<10
MN.3	B	88	83	<10	<10
TV1.21	C	30	22	<10	<10
92BR025.9	C	<10	<10	<10	<10
MW965.26	C	50	47	<10	<10
Bx08.16	B	42	28	<10	<10
W61D_TCLA.65	B	<10	14	<10	<10
89.6	B	38	54	<10	<10
129	B	32	55	<10	<10
HXB2_V570A	B	1,009	620	22	15

Pools were prepared from individual PD3 animal sera in each of the corresponding groups shown in Table S2 (n = 8 for guinea pigs, n = 4 for rabbits). Positive results (gray-shaded cells) are defined as ≥3-fold rise above the matched preimmunization pooled sera titer.

1 viruses. The magnitude of the response detected in the independent neutralization assays was comparable between bridging viruses, as evidenced by the IC_{50} titers observed for HXB2-V570A, 89.6, and 129. Rabbit sera showed no neutralization in the TZM-bl assay against any virus other than HXB2-V570A, for which the titers were ≈100-fold lower than those obtained in guinea pigs. Neither the guinea pig nor rabbit sera displayed any activity against the more resistant tier 2 viruses. To ascertain whether this lack of activity against tier 2 viruses resulted from an insufficient titer of neutralizing antibodies or whether this class of viruses was inherently resistant to hydrophobic pocket-mediated neutralization, we tested D5 against the same tier1/tier2 panel. The data indicated that D5 can neutralize tier 2 viruses (4 of 10 strains, geometrical mean IC_{50} = 280 μg/mL), although less frequently than tier 1 (9 of 10 strains, geometrical mean IC_{50} = 120 μg/mL). The coverage and relative susceptibility ranking between tiers for D5 is similar to previously reported results (32), suggesting that there is no bias against neutralization of the hydrophobic pocket in tier 2 strains relative to other neutralization mechanisms.

Discussion

The aim of the current study was to demonstrate that vaccinating animals with optimized peptide mimetics of the gp41 prehairpin fusion intermediate would result in an immune response that neutralizes HIV-1. Stable mimetics of the intermediate were required because this structure is only transiently exposed during fusion of the virus with the host cell. Indeed, for this reason, antibodies targeting this region are not expected to be readily generated during natural infection, and no such naturally occurring antibodies have been identified to date.

We anticipated, even when using highly stabilized mimetics, that the proportion of the total antibody response generated against the desired epitopes might be low. Therefore, we used two tools to aid in the detection of weak neutralizing antibody responses: (*i*) a D5 hypersensitive mutant virus HXB2-V570A and (*ii*) isolation of serum IgG as a means of concentrating the antibody component. There are clear correlations between antisera that neutralize HXB2-V570A and those that neutralize other HIV-1 strains (Fig. 4*B*). These correlations support the relevance of results obtained with this hypersensitive mutant virus to the development of a broader HIV-1 vaccine. To concentrate the antibodies, we chose to use whole IgG purification as opposed to affinity isolation of specific anti-gp41 antibodies to minimize the possibility of false-positive results that might occur as a result of contamination of the purified antibodies with the peptides used for selection, because these molecules are potent antiviral compounds in their own right (16–23).

The first improvement in immunogen design that we investigated was the use of covalent constructs that maintained the trimeric state independent of concentration. Specifically, although IZN17 and IZN36 present the prehairpin conformational epitope in an effective manner (16, 18), this presentation requires self-association of the trimers. Dissociation of the structure is expected to occur as the peptide is diluted in vivo. The use of interchain disulfide bonds outside the coiled-coil domain to stabilize the trimeric structure has been shown to result in highly stable structures that are very potent (subnanomolar) inhibitors of HIV-1 fusion per se (23). In our immunization experiments, a clear trend was observed toward increased potency in antisera elicited by immunization with the covalent trimeric immunogens $(CCIZN17)_3$, $(CCIPN17)_3$, $(CCIZN36)_3$, and $(CCIPN36)_3$ relative to their noncovalent counterparts IZN17 and IZN36 (Fig. 2). These data strongly indicate that covalently stabilized trimers are more potent immunogens for eliciting antibodies against the prehairpin intermediate.

The second improvement in immunogen design followed from the observation that a significant, and presumably irrelevant, immune response is generated toward the peptide region used as a scaffold to ensure a soluble trimeric structure (i.e., the IZ sequence). By using LMWPEG moieties strategically positioned on the scaffold, we were able to reduce the immunoreactivity toward the IZ sequences substantially without adversely affecting the desired immunogenicity of the conformational gp41 epitopes (Fig. S1). However, the current strategy did not improve the functional response; the neutralization potencies (and the levels of D5-like antibodies as quantified by DCBA) were comparable between animals immunized with PEGylated and non-PEGylated immunogens.

Third, the neutralization potency of purified IgGs increased with the length of the gp41 sequence, with higher potency measured from animals immunized with N36 peptide mimetics than with N17 peptide mimetics (Fig. 2), although the reasons for this are not clear. The N17 sequence primarily presents the hydrophobic pocket that comprises the D5 epitope. Thus, the higher neutralizing potency observed with N36-based immunogens could be attributed to additional neutralizing epitopes that are distinct from the D5 epitope. However, serum neutralization potency correlated well with the quantity of D5-like antibodies elicited for all the peptide mimetics (Fig. 3). Specifically, the antisera from the longer N36 constructs are not only more neutralizing but contain a higher concentration of D5-like antibodies.

The two most promising immunogens, $(CCIZN36)_3$ and $(CCIPN36)_3$, elicit neutralizing antibody responses that can be detected in unfractionated serum from guinea pigs. The potency of this response is highest against HXB2-V570A, but reasonable potency is also observed against a number of tier 1 viral isolates (Fig. 4). Although we did not detect neutralization of any tier 2 viruses, which are chosen to be more neutralization-resistant, we did detect moderate neutralizing potency for ≈50% of the tier 1 HIV-1 isolates (Table 1). Serum IC_{50} values ranged from 1:8–1:88 for neutralization-susceptible viruses, with the exception of HXB2-V570A, which was neutralized ≈10–100-fold more readily.

Thus, antiviral activity can be obtained in unfractionated polyclonal sera raised against peptide mimetics of the gp41 fusion intermediate. Although our neutralization titers are relatively low, it is encouraging to note that a previous study on passive transfer of neutralizing antibodies in rhesus macaques reported that a calculated plasma neutralization titer of 1:38 or greater (33) against a broad range of HIV-1 isolates would confer 99% protection against challenge with $SHIV_{DH12}$ (TCID50), whereas a titer of 1:6.5 yielded 50% protection. Neutralization titers in the same dilution range have been shown to confer protection in other nonhuman primate challenge studies (34, 35). These reported protective titers may represent

a conservative estimate, because significantly lower amounts of infectious virus are encountered during natural transmission than in the challenge doses typically used (10–100 ID_{50}) during passive immunization studies (36). This view has been reinforced by recent studies showing that HIV-1 infection can be limited or even prevented in animals with a wide range of functional antibody titers as measured by in vitro neutralization assays when these antibodies are continuously present, as would be the case for vaccine-induced immunity (31, 37, 38).

Our chimeric gp41 NHR peptide mimetics represent one promising approach toward a prophylactic HIV-1 vaccine that targets a highly conserved functional target on the virus. However, considerable developmental hurdles remain. Despite the demonstrated potency against neutralization-sensitive tier 1 viral strains, sera showed no activity against tier 2 viruses, which represent the primary isolate class encountered in natural infection. Although D5 exhibited limited activity against these viruses, the challenge remains to boost the D5-specific antibody response to higher levels. More fundamentally, it remains to be seen whether a robust antibody response targeting the transient and sterically occluded prehairpin intermediate can be elicited. Recent studies suggest that access to this region during the viral fusion process may be limited by steric hindrance, which would be a significant barrier to the development of an effective vaccine (39, 40). Our observation that neutralization potency and the quantity of D5-like antibodies are lower in rabbits than in guinea pigs may point to increased difficulty in eliciting protective neutralizing antibody titers in higher species, including primates. Furthermore, the duration of the neutralizing antibody response has not yet been addressed, which is an important consideration for long-lasting protection. However, an important and encouraging aspect of the current study is that it shows that incremental improvements in the quality of a vaccine can be realized through iterative cycles of design.

Materials and Methods

Peptide Synthesis. All the peptides in Fig. 1 were synthesized by solid-phase synthesis. Full details on peptide synthesis and characterization are presented in *SI Materials and Methods*.

Immunization Studies. Both guinea pigs (eight animals per group) and rabbits (four animals per group) were immunized i.m. with 100 μg of peptide immunogen three times, at weeks 0, 4, and 8. For the guinea pigs, the peptide was administered together with 180 μg of aluminum hydroxyphosphate sulfate plus 40 μg of Iscomatrix (CSL) per dose, whereas for the rabbits, the peptide was administered together with 450 μg of aluminum hydroxyphosphate sulfate plus 100 μg of Iscomatrix per dose. Serum samples were collected before immunization and 3 weeks (for guinea pigs) or 4 weeks (for rabbits) after each immunization.

HIV-1 Neutralization Assays. The neutralization capabilities of immune sera were assessed in two independent single-cycle HIV-1 infectivity assays. Both are detailed in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Peptide Synthesis. All peptides were prepared as N-terminal acetylated and C-terminal amidated derivatives. IZN17, $(CCIZN17)_3$, and IZN36 were synthesized as previously described (1). All other peptides in Fig. 1 were prepared by solid-phase synthesis on Fmoc-LinkerAM-Champion resin (Biosearch Technologies) on an Apex 396 Peptide Synthesizer (Advanced Chemtek). Acylations were performed for 60–120 min with a 6-fold excess of amino acids activated with equimolar amounts of 2-(^{1}H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate or 2-(7-Aza-^{1}H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and a 2-fold molar excess of N,N-diisopropylethylamine (DIPEA). In the assembly of CCIPN17 and CCIPN36 precursors, Lys(Alloc) was incorporated at specific positions within the scaffold to allow derivatization with a LMWPEG derivative (m-dPEG acid 236; Quanta Biodesign). The allyloxycarbonyl group was deprotected by treatment of the peptide resin with 48 equivalents of phenylsilane for 2 min in anhydrous dichloromethane followed by 0.5 equivalents of Tetrakis(triphenylphosphine)palladium(0) for 30 min under argon. The procedure was repeated, and the peptide resin was then washed with 0.5% sodium diethyldithiocarbamate and 0.5% DIPEA in dimethylformamide. Acylation of the m-dPEG acid 236 was performed using 3 equivalents of the reagent activated with equimolar amounts of (benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), hydroxybenzotriazole, and 2-fold molar excess DIPEA.

At the end of the assembly, all peptides were acetylated by treatment with 10-M/L equivalents of acetic anhydride. Alternatively, to produce the biotinylated peptides used for ELISA, the sequences were elongated with a spacer, Fmoc-Ttds (13-amino-4,7,10-trioxa-tridecayl-succinamic acid) and then acylated with 3 equivalents of biotin activated with equimolar amounts of PyBOP and a 2-fold molar excess of DIPEA. At the end of synthesis, the dry peptide resins were treated with cleavage mixture (82.5% trifluoroacetic acid, 5% phenol, 5% thioanisole, 5% water, and 2.5% ethanedithiol) for 1.5 h at room temperature. The filtered peptide solution was precipitated with cold methyl-t-butyl ether. After centrifugation, the peptide pellets were washed twice with fresh cold methyl-t-butyl ether and then dried, resuspended, and lyophilized. Peptides were purified by gel permeation chromatography on a Toyopearl HW-50S column (700 × 26 mm) (Tosoh Biosciences) using 30% acetonitrile in water/0.1% trifluoroacetic acid as an eluent, followed by reverse-phase HPLC on a C4 Reprosil column (10 μm, 50 × 150 mm) (Dr. Maisch GmbH) or a C18 Xbridge column (5 μm, 50 × 150 mm) (Waters). Purified peptides were characterized by electrospray MS on a Micromass LCZ spectrometer (Waters).

Oxidation of Cysteine-Containing Chimeric N-Peptides. $(CCIZN17)_3$, $(CCIPN17)_3$, $(CCIZN36)_3$, and $(CCIPN36)_3$ were prepared by oxidation of the corresponding monomeric peptide precursors, as previously described for compound $(CCIZN17)_3$ (1). For the remaining peptides, the oxidation reaction was monitored by HPLC-MS using an ACE C4 column (3 μm, 150 × 4.6 mm) (CPS Analitica) at a flow rate of 1 mL/min at 45 °C on a Waters Alliance chromatograph equipped with an LCZ spectrometer. For $(CCIPN17)_3$, the yield of the oxidation reaction was 80%. The main product showed a mass value corresponding to three precursor peptide chains linked by three disulfide bridges (predicted M_r = 16829.6, observed average M_r = 16828.8). The trimer was purified by gel filtration chromatography on a TSKgel Toyopearl HW-50S column (26 × 700 mm) (Tosoh Biosciences).

For $(CCIZN36)_3$, the yield of the oxidation reaction was 30%. The main product showed a mass value corresponding to three precursor peptide chains linked by three disulfide bridges (predicted M_r = 22635.0, observed average M_r = 22634.5). The oxidized covalent trimer was purified by preparative HPLC on a C4 Reprosil column (10 μm, 50 × 150 mm).

For $(CCIPN36)_3$, the yield of the oxidation reaction was 50%. The main product showed a mass value corresponding to three precursor peptide chains linked by three disulfide bridges (predicted M_r = 23944.5, observed average M_r = 23943.6). The oxidized covalent trimer was purified by preparative HPLC on a C4 Reprosil column (10 μm, 50 × 150 mm).

CD Spectroscopy. All measurements were performed on a Jasco J-710 spectropolarimeter at 20 °C using a rectangular quartz cell with a path length of 0.1 cm. Spectra were acquired using an 8-s time response and a 5-nm/min scan speed and were averaged for two acquisitions. Stock solution concentration was determined by quantitative amino acid analysis. Standard measurements were performed on 10–15-μM solutions of peptide in 5 mM Hepes (pH 7), 150 mM NaCl. The percentage of α-helix was calculated according to Chen et al. (2). Thermal stability was determined at a concentration of 10 μM by monitoring the change in the CD signal at 222 nm as a function of temperature, using an hourly increase of 10 °C and an integration time of 16 s. The T_ms were determined from the midpoints of the cooperative thermal unfolding transitions. For peptides with a T_m >90 °C, thermal denaturation experiments were also performed in the presence of 2 M guanidine hydrochloride.

IgG Purification. The IgG fraction was purified from antisera and matched prebleeds of individual animals by batch protein A affinity chromatography, buffer-exchanged, and concentrated 5-fold. Protein concentration was determined by bicinchoninic acid assay.

ELISA. Serum end-point dilutions were measured against biotinylated peptides, biotin-$(CCIZN17)_3$ for testing N17-based antiserum and biotin-$(CCIZN36)_3$ for testing N36-based antiserum, bound to streptavidin (SA)-coated 96-well plates (Thermo Fisher Scientific, Inc.). The biotinylated peptides were coated at a concentration of 4 μg/mL in PBS at 50 μL per well overnight at 4 °C. Plates were washed six times with PBS containing 0.05% Tween-20 (PBST) and blocked with 3% (vol/vol) skim milk in PBST (milk-PBST). Testing samples, both before and after immune serum, were diluted, starting at 1:100, in a 4-fold series in milk-PBST and added at a volume of 100 μL per well. Plates were incubated for 2 h at room temperature, followed by six washes with PBST. Fifty microliters of predetermined dilution (1:5,000) HRP-conjugated goat anti-guinea pig (Jackson ImmunoResearch Laboratories, Inc.) or anti-rabbit (Abcam, Inc.) secondary antibodies in milk-PBST was added per well and incubated at room temperature for 1 h. Plates were washed six times, followed by addition of substrate (TMB; Virolabs, Inc.) at 100 μL per well, and stopped after 3–5 min of development. The antibody titer was defined as the reciprocal of the highest dilution that gave an OD at 450 nm value above the mean plus 2 SDs of the conjugate control wells.

DCBA. An in vitro binding assay based on fluorescence resonance energy transfer (FRET) was developed using D5 IgG conjugated to europium cryptate (Eu-D5) and a biotinylated derivative of the recombinant gp41 mimetic 5-helix (5H). As shown in Fig. S2, biotin-5H binds to a SA-conjugated allophycocyanin (APC)

substrate to form a 5H-SA-APC complex. Binding of Eu-D5 to 5H results in FRET from Eu to APC. When the reaction system is excited at a wavelength of 340 nm, the amount of bound Eu-D5 is measured at the emission wavelength of 665 nm and total Eu is measured at the emission wavelength of 620 nm. Data are reported as 10,000 × the ratio of signal at 665 nm to signal at 620 nm. Agents that bind competitively to either component cause a decrease in the ratio value.

Characterization of the Hypersensitive Virus HXB2-V570A. The viable replication-competent virus (denoted HXB2-V570A) exhibits 5- to 10-fold enhanced sensitivity against fusion inhibitors that target the prehairpin intermediate such as T20 (3), 5H (4), D5 (5) and IZN17 (6). In contrast, the neutralizing mAb b12, which binds to the CD4-binding site of gp120 (7), inhibits both viruses equally. The neutralizing antibody 2F5 (8), which binds to a region of gp41 near the CHR region, shows slightly enhanced inhibitory activity against HXB2-V570A as compared with the parental virus (Fig. S3).

HIV-1 Neutralization Assays. The neutralization capabilities of immune sera were assessed in two independent single-cycle HIV-1 infectivity assays. The p4-2R5 assay was used as previously described (9), with the following modifications: HeLa P4R5 cells were seeded at 1,000 cells per well in a 384-well plate and infected the following day with the appropriate HIV-1 or SHIV virus at a multiplicity of infection of ≈0.01 in the presence of serial dilutions of immune sera or fractionated IgG. At 48 h postinfection, cells were lysed and β-galactosidase activity was measured using a chemiluminescent substrate (GalScreen; Applied Biosystems). For analysis of purified IgG, the data are expressed as IC_{50}, defined as the IgG concentration resulting in a 50% reduction of chemiluminescence signal. For analysis of sera, the IC_{50} is defined as the reciprocal serum dilution resulting in a 50% reduced chemiluminescence signal. For the TZM-bl assay, neutralization was measured as a reduction in luciferase reporter gene expression after a single round of infection in TZM-bl cells, as previously described (10, 11). TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD), as contributed by John Kappes and Xiaoyun Wu. Briefly, 200 50% tissue culture infectious dose of virus was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 μL for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 μL of growth medium containing 75 μg/mL DEAE dextran) were added to each well. One set of control wells received cells plus virus (virus control) and another set received cells only (background control). After a 48-h incubation, 100 μL of cells was transferred to a 96-well black solid plate (Costar) for measurements of luminescence using the Britelite Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences). Neutralization titers are the dilution at which relative luminescence units (RLUs) were reduced by 50% compared with virus control wells after subtraction of background RLUs. Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by transfection in 293T cells and were titrated in TZM-bl cells as described elsewhere (11). Clade A, B, and C reference Env clones have been described previously (11–13).

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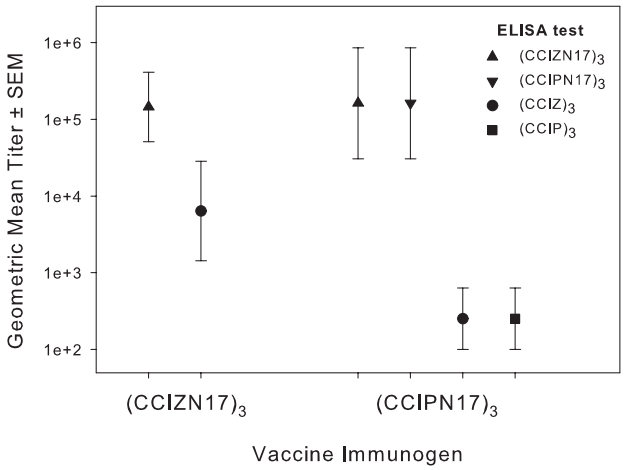

Fig. S1. Shielding the antibody response to the IZ scaffold. Guinea pigs (four animals per group) were immunized with three doses of 100 μg $(CCIZN17)_3$ or $(CCIPN17)_3$, and end-point antibody titers in PD3 sera were determined by ELISA. Geometric mean titers were calculated for the $(CCIZN17)_3$ group serum samples measured against the homologous immunizing antigen $(CCIZN17)_3$ and a construct presenting only the trimerized scaffold $(CCIZ)_3$. Likewise, geometric mean titers for the $(CCIPN17)_3$ group against homologous immunizing antigen $(CCIPN17)_3$, non-PEGylated $(CCIZN17)_3$, and trimeric scaffolds $(CCIZ)_3$ and $(CCIP)_3$ are plotted. Titers <100 were included in calculations as = 100.

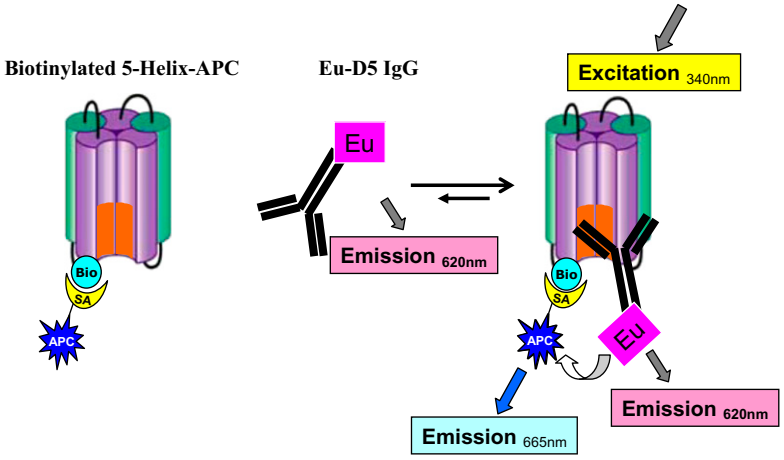

Fig. S2. Schematic outline of DCBA. The DCBA is based on a FRET readout that occurs on binding of Eu-D5 to the hydrophobic pocket presented on a biotinylated derivative of the gp41 NHR mimetic 5H (biotin-5H). Biotin-5H was labeled using an SA-APC conjugate (5H-APC). Unbound Eu-D5 exhibits a fluorescence emission (F_{em}) maximum of 620 nm on excitation at 340 nm. On binding of Eu-D5 to 5H-APC, Eu is brought into close proximity to the APC substrate, resulting in FRET and a shift of the F_{em} maximum to 665 nm. The concentration of bound antibody is calculated from the assay readout: 10,000 × (counts at 665 nm/counts at 620 nm). Inhibitors, including competitive antibodies, which bind to 5H-APC in or near the hydrophobic pocket, reduce the amount of bound Eu-D5, lowering the FRET occurrence, and thus the observed counts at 665 nm. Bio, biotin.

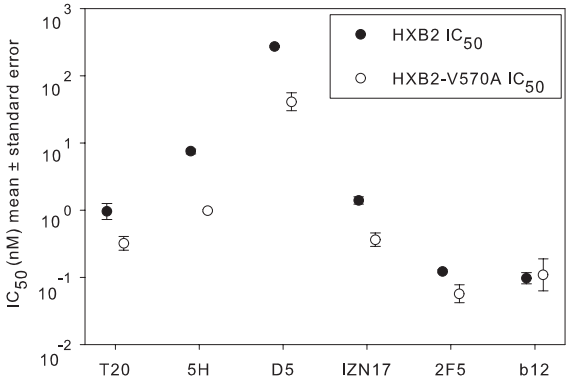

Fig. S3. Sensitivity of HXB2-V570A to neutralizing mAbs and viral entry inhibitors. The sensitivity of the mutant virus HXB2-V570A to known neutralizing mAbs (D5, b12, and 2F5) or entry inhibitors (T20, 5H, IZN17) was assessed in the p4-2/R5 neutralization assay. Parental HXB2 and HXB2-V570A were titrated against increasing concentrations of each antibody or inhibitor, and the IC_{50} for neutralization was determined. SEs of the mean are plotted but are tight to the data point in some cases. Antibodies and inhibitors that are thought to target the prehairpin intermediate are more effective at neutralizing HXB2-V570A; a slight shift is seen with antibody 2F5 (which targets a region of gp41 near the prehairpin intermediate), whereas an unrelated neutralizing antibody (b12) is equally effective at neutralizing both viruses.

Table S1. Biophysical characterization of chimeric N-peptides at neutral pH

Peptide*	% Helicity† at 10 μM	T_m, °C‡	T_m, °C in 2M GuHCl§
IZN17	99	>90	61.5
$(CCIZN17)_3$	97	>90	>90
$(CCIPN17)_3$	100	>90	>90
IZN36	100	>90	72
$(CCIZN36)_3$	95	>90	>90
$(CCIPN36)_3$	94	>90	>90

*Data for IZN17 and $(CCIZN17)_3$ are from the article by Bianchi et al. (1).
†Determined by CD spectroscopy in 5 mM Hepes (pH 7) and150 mM NaCl.
‡T_m is the midpoint of thermal denaturation transitions by CD spectroscopy.
§T_m determined in the presence of 2M guanidine hydrochloride.

Table S2. Summary of analysis on serum from guinea pigs and rabbits immunized with NHR peptide mimetics

Animal	ELISA*T = 12	DCBA IC_{50}, μg/mL† T = 12	p4-2/R5 IC_{50}, μg/mL‡ HXB2 T = 0	HXB2 T = 12	HXB2-V570A T = 0	HXB2-V570A T = 12
Guinea pig						
IZN17						
885 528	102,400	1,663	>5,063	>15,023	>5,063	>15,023
041 086	102,400	415	>7,304	>11,869	>7,304	>11,869
049 635	25,600	>3,700	>6,391	>15,355	>6,391	>15,355
589 335	25,600	230	>4,316	>9,213	>4,316	>9,213
590 263	409,600	33	>7,553	>17,347	>7,553	>17,347
886 596	102,400	410	>7,304	>17,098	>7,304	>17,098
016 578	102,400	4.1	>6,723	>11,537	>6,723	>11,537
561 883	102,400	>3,020	>9,213	>12,533	>9,213	>12,533
$(CCIZN17)_3$						
077 025	25,600	223	>6,806	>8,881	>6,806	>8,881
016 275	25,600	27	>14,525	>14,774	>14,525	2,695
043 513	102,400	1,077	>6,640	>10,375	>6,640	6,707
081 874	102,400	>1,180	>6,391	>7,129	>6,391	610
003 556	25,600	>2,180	>8,383	>9,047	>8,383	>9,047
031 378	102,400	89	>5,810	>11,333	>5,810	162
083 269	409,600	38	4,706	>8,008	5,039	>14,870
880 826	102,400	>2,420	>4,731	>10,043	>4,731	>10,043
$(CCIPN17)_3$						
071 290	102,400	>2,160	>5,727	539	>5,727	2,055
073 638	25,600	>2,560	1,807	863	>6,723	3,429
886 092	1,638,400	15.6	>5,976	>15,438	>5,976	678
045 880	1,638,400	35.5	>5,644	4,637	>5,644	105
000 576	409,600	>2,960	>6,059	>12,284	>6,059	>12,284
042 075	1,638,400	12.1	>5,976	>15,272	>5,976	824
066 883	102,400	>2,280	>4,893	1,408	>7,636	>4,808
045 584	6,400	>2,260	>5,644	>9,379	>5,644	>9,379
$(CCIZN36)_3$						
819 312	102,400	4	>5,043	>5,849	>5,043	48
823 856	<100	>3,050	>4,067	>6,330	>8,134	>12,659
830 259	102,400	4	>3,213	>4,233	>6,427	25
842 268	102,400	3	>5,125	>5,029	>5,125	27
850 000	1,638,400	2	>8,122	580	>8,122	7
855 337	102,400	35	>7,003	1,063	>7,003	9
847 525	409,600	6	>7,155	614	>7,155	5
816 317	409,600	9	>5,252	966	>5,252	6
$(CCIPN36)_3$						
863 324	102,400	14	>3,078	>6,635	>6,157	7
881 857	409,600	5	>4,199	>5,818	>8,399	19
842 114	102,400	4	>4,838	>4,346	>4,838	4
820 853	409,600	4	>5,459	>5,389	>5,459	8
839 301	102,400	0.4	>5,466	544	>5,466	5
835 124	102,400	8	>4,300	636	>4,300	8
885 056	102,400	0.7	>6,475	1,034	>6,475	12
854 560	102,400	0.9	>5,995	497	>5,995	9
IZN36						
011 262	409,600	1	>3,819	>,6,668	>7,638	16
014 582	409,600	0.3	>5,217	>5,814	>5,217	14
849 820	<100	364	>3,395	3,654	>6,789	>5,716
842 323	102,400	< 0.2	>3,446	>6,755	>6,892	41
862 804	102,400	3	>4,432	6,369	>4,432	36
866 874	102,400	6	>4,711	4,643	>4,711	39
817 809	100	>1,727	>5,308	>7,165	>5,308	>7,165
827 583	102,400	13	>7,203	3,572	>7,203	17
Rabbit						
IZN17						
40357	102,400	<0.7	>12,833	>11,111	>12,833	8,325
40358	102,400	512	>10,487	10,222	>13,333	3,825

Table S2. Cont.

Animal	ELISA*T = 12	DCBA IC_{50}, μg/mL† T = 12	p4-2/R5 IC_{50}, μg/mL‡ HXB2 T = 0	HXB2 T = 12	HXB2-V570A T = 0	HXB2-V570A T = 12
40359	25,600	1,588	12,284	>10,816	>13,750	>11,081
40360	102,400	599	>10,167	11,274	>13,083	9,021
$(CCIZN17)_3$						
40361	409,600	164	9,504	>11,493	>12,750	2,437
40362	25,600	528	9,806	5,407	>14,271	1,693
40363	409,600	557	>17,666	>15,162	>17,666	2,827
40364	102,400	801	>14,249	>17,583	>14,249	6,247
$(CCIPN17)_3$						
40365	102,400	494	>11,916	>15,249	>11,792	7,767
40366	25,600	772	>8,390	>10,406	>9,250	6,131
40367	102,400	213	>8,833	5,942	>6,862	1,187
40368	25,600	2,745	>16,916	>14,620	>16,916	>14,473
IZN36						
9	102,400	187	>5,686	>7,337	>5,686	>7,337
10	409,600	40	>4,934	>6,445	>4,934	281
11	102,400	85	>4,287	>5,204	>4,287	>5,204
12	25,600	53	>4,254	>5,822	>4,254	487
$(CCIZN36)_3$						
1	409,600	22	>6,972	>7,392	>6,972	180
2	409,600	17	>6,864	>6,781	>6,864	166
3	409,600	48	>7,283	>8,640	>7,283	352
26	1,638,400	12	>7,727	>8,238	>7,727	265
$(CCIPN36)_3$						
5	102,400	16	>5,221	>7,092	>5,221	225
6	409,600	18	>7,038	>7,358	>7,038	209
7	102,400	28	>7,837	>6,947	>7,837	184
8	409,600	17	>5,017	>6,540	>5,017	341

*End-point dilutions of fractionated IgG purified from vaccinated animal sera collected at week 12 (3 weeks PD3). ELISA was measured against biotinylated peptides bound to SA-coated plates. Coating antigens were biotin-$(CCIZN17)_3$ for N17-based immunogens and biotin-$(CCIZN36)_3$ for N36-based immunogens.

†IC_{50} is defined as the concentration of purified IgG required to reduce the amount of bound D5-Eu mAb to 5H-APC substrate by 50%. IgG was purified from week 12 sera.

‡IC_{50} is defined as the concentration of purified IgG required to reduce the chemiluminescence signal by 50%. The assay was performed on IgG fractionated from matched preimmunized (T = 0) and week 12 sera (T = 12).