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Focusing the immune response on the V3 loop, a neutralizing epitope of the HIV-1 gp120 envelope

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Abstract

Rabbits were immunized with a novel regimen designed to focus the immune response on a single neutralizing epitope of HIV-1 gp120 and thereby preferentially induce neutralizing antibodies (Abs). Animals were primed with gp120 DNA from a clade A Env bearing the GPGR V3 motif and/or a clade C Env bearing the GPGQ V3 motif, and boosted with one or more fusion proteins containing V3 sequences from clades A, B and/or C. Immune sera neutralized three of four Tier 1 primary isolates, including strains heterologous to the immunizing strains, and potent cross-clade-neutralizing activity was demonstrated against V3 chimeric pseudoviruses carrying in a Tier 1 Env, the consensus V3 sequences from clades A1, AG, B, AE, or F. The broadest and most potent neutralizing responses were elicited with the clade C gp120 DNA and a combination of V3-fusion proteins from clades A, B and C. Neutralizing activity was primarily due to V3-specific Abs. The results demonstrate that the immune response can be focused on a neutralizing epitope and show that the anti-V3 Abs induced recognize a diverse set of V3 loops.

Keywords: HIV-1; Vaccine; Neutralizing antibodies; V3 loop; gp120; Pseudovirus; Immunization

Introduction

Protective antibodies (Abs) are needed to reduce the size of a virus inoculum and block infection of target cells. The ability of Abs to afford such protection against HIV-1 (HIV) is documented by several passive immunization experiments in animals (Baba et al., 2000; Emini et al., 1990; Gauduin et al., 1997; Mascola et al., 1999; Shibata et al., 1999) and by recent data suggesting that neutralizing Abs in HIV-infected individuals protect against superinfection (Smith et al., 2006).

Since sera from some HIV-infected individuals have broad neutralizing activity (Nyambi et al., 1996; Pilgrim et al., 1997) and several human mAbs neutralize a broad spectrum of primary isolates (Binley et al., 2004), it is clear that the human B cell repertoire includes genes capable of coding for Abs that can recognize and neutralize a broad spectrum of HIV isolates. The epitopes that are known to induce broadly neutralizing Abs include the membrane proximal external region of gp41, the CD4 binding site on gp120, complex glycans on gp120, the CD4-induced epitope in and around the gp120 bridging sheet, and the V3 loop of gp120 (Zolla-Pazner, 2004).

Despite the extensive information on HIV-neutralizing Abs, it has proven difficult to induce broadly neutralizing Ab responses against HIV by immunization. This is due to several factors including the predominant induction of non-neutralizing rather than neutralizing Abs (Belshe et al., 1993; Gilbert et al., 2005; Parren et al., 1997), the masking of neutralization-sensitive epitopes (Fox et al., 1997; Krachmarov et al., 2006; Kwong et al., 2002; Pinter et al., 2004; Wei et al., 2003), the high mutation rate of HIV leading to antigenic variability, and a myriad of other factors such as the physicochemical characteristics of the virus membrane (Harada et al., 2005) and the variable affinities of the different gp120 proteins for the virus receptors (Hammond et al., 2001; Reeves et al., 2002).

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While a variety of permutations of the HIV Env have been used as immunogens (including gp120, gp140, gp145, and gp160, various oligomeric constructs, and several complete and truncated forms of Env expressed as components of recombinant viruses), the best, albeit modest, results in terms of generating cross-clade-neutralizing Abs have been achieved with strategies utilizing a DNA prime and either a recombinant adenovirus or protein boost. The promise of this approach was first demonstrated with constructs derived from T cell line-adapted HIV strains (Lu et al., 1998; Richmond et al., 1998), and has more recently been confirmed and extended using HIV *env* genes derived from various HIV strains including primary isolates (Barnett et al., 2001; Barnett et al., 1997; Beddows et al., 2005; Richmond et al., 1998; Wang et al., 2005a, 2006). Thus, in rabbits, priming with $gp120_{JR\text{-}FL}$ DNA and boosting with $Env_{JR\text{-}FL}$ induced neutralizing Abs to the relatively resistant homologous strain as well as to a limited number of other clade B primary isolates but to no viruses from heterologous clades (Wang et al., 2005a). Priming with a polyvalent cocktail of gp120 DNA plasmids and boosting with a cocktail of Env proteins from various clades induced a broader response, with cross-clade neutralizing Abs to strains from clades A, B, C, D, and E (Lian et al., 2005; Wang et al., 2006). Similarly, in guinea pigs, a polyvalent *env* DNA prime and polyvalent boost with recombinant adenoviruses, each carrying *env* genes from clades A, B and C, resulted in Abs able to neutralize strains from these clades, albeit at titers of only 1:5 (Chakrabarti et al., 2005). In each of these experiments, the boosting immunogen was a form of gp120 or gp145 which contains a multitude of B cell epitopes.

An alternative immunization approach is the construction and use of an immunogen that will focus the immune response on one or a few epitopes that are known to induce neutralizing Abs. An advantage of this approach is the potential to induce an immune response with a larger proportion, and consequently a higher titer of, neutralizing Abs. The use of selected epitopes or mimotopes for the construction of vaccines that preferentially induce protective Abs is still in its infancy, although some striking examples exist, especially with polysaccharide antigens of various pathogens (Beenhouwer et al., 2002; Buchwald et al., 2005). In the search for an HIV vaccine, several attempts have been made to graft a neutralizing epitope from the virus envelope into a foreign protein. For example, the neutralizing epitope in the membrane proximal external region of gp41 recognized by human mAb 2F5 has been grafted into influenza and the hepatitis B surface antigen (Eckhart et al., 1996; Muster et al., 1995), however these constructs failed to induce neutralizing Abs. In contrast, a peptide mimotope selected based on binding to the broadly neutralizing human anti-V3 monoclonal Ab 447, when covalently conjugated to a protein carrier, induced an Ab response which, although limited in potency and breadth, could neutralize two virus strains (Keller et al., 1993). Another strategy, using a recombinant protein prime and boosters containing V2 and V3 peptides resulted in increased titers of anti-peptide Abs and an increase in serum neutralizing Abs for the homologous and a related virus (Davis et al., 1997). To further test the concept of "immunofocusing vaccines" in order to enhance the quality and/or quantity of neutralizing Abs in immune sera, we developed an immunization regimen designed to focus the immune response on the V3 loop of gp120. To do this, we used both classical immunologic approaches to priming and selectively stimulating memory B cells (Ovary and Benacerraf, 1963) and more recent data that stress the importance of the conformation of B cell epitopes (Gorny et al., 2002).

The V3 loop was chosen because many studies have shown that anti-V3 Abs can neutralize diverse strains of HIV. Thus, it has long been known that human mAbs directed against V3 can neutralize primary isolates (Binley et al., 2004; Conley et al., 1994; Gorny et al., 2004, 1997; Hioe et al., 1997) and that polyclonal anti-V3 Abs in the sera of patients (Krachmarov et al., 2005, 2001) and immunized guinea pigs and monkeys (Chakrabarti et al., 2005; Liao et al., 2000; Yang et al., 2004) have neutralizing activity. In addition, V3 is a highly immunogenic region of the virus envelope (Carrow et al., 1991; Vogel et al., 1994). It is formed by a continuous (rather than discontinuous) stretch of amino acids, and anti-V3 Abs do not display auto-reactive activity (Haynes et al., 2005). These features make V3 a logical first target in efforts to induce a focused neutralizing Ab response. In contrast, other neutralizing epitopes of gp120 and gp41 are poorly immunogenic and/or are formed by discontinuous regions in the envelope proteins (Gorny and Zolla-Pazner, 2004; Zolla-Pazner, 2004). Though only about 20% of primary isolates are sensitive to anti-V3 Abs (Binley et al., 2004; Li et al., 2005), a V3-focused vaccine would constitute an important first step in the development of a vaccine platform that must ultimately target additional neutralizing epitopes of the HIV envelope glycoproteins.

Here, we report the results of experiments in rabbits in which new permutations to the prime/boost approach were used in order to preferentially induce anti-V3 Abs. Thus, we primed, as before, with one or more gp120 DNA constructs, all of which contained multiple B and T cell epitope. Subsequently, we used a protein boost which selectively stimulated only the HIV-specific memory B cells directed against V3 epitopes, replacing the more conventional boosting immunogens that contain all or most Env epitopes.

The results provide a proof-of-principle that it is possible, in this way, to focus the immune response on a neutralizing epitope, and they show specifically the ability to induce a vigorous anti-HIV Ab response focused on the V3 loop. This response includes the production of neutralizing Abs which can recognize and neutralize viruses and pseudoviruses carrying diverse V3 loops.

Results

Design of immunogens and immunization protocols

Two sets of rabbits were immunized. As summarized in Table 1 and detailed in Methods, the three rabbits in each group received three priming doses of codon-optimized gp120 DNA derived from *env* genes from primary isolates from clades A and/or C, and two booster doses of gp120 or one or more V3-fusion proteins (V3-FPs). The rabbits were bled before the

Table 1
Immunization groups for rabbit study

Group	Immunizing regimen	DNA prime[a] at weeks 0, 2, and 4	Protein boost[a] at weeks 10 and 14
I-1	−/B	–	$V3_B$-FP
I-2	A_R/B	gp120/Clade A (GPG**R**)	$V3_B$-FP
I-3	A_R/$gp120_R$	gp120/Clade A (GPG**R**)	$gp120_{JR\text{-}FL}$
II-1	−/ABC	–	$V3_A$-, $V3_B$- and $V3_C$-FP
II-2	A_R/ABC	gp120/Clade A (GPG**R**)	$V3_A$-, $V3_B$- and $V3_C$-FP
II-3	C_Q/ABC	gp120/Clade C (GPG**Q**)	$V3_A$-, $V3_B$- and $V3_C$-FP
II-4	A_R+C_Q/ABC	gp120/Clade A (GPG**R**) and gp120/Clade C (GPG**Q**)	$V3_A$-, $V3_B$- and $V3_C$-FP
II-5	A_R/B	gp120/Clade A (GPG**R**)	$V3_B$-FP

[a]V3 sequences contained in priming and boosting constructs. Variations in sequence from relevant consensus sequences are underlined, and the variation at the tip of the loop, position 18 (R/Q), is bolded.

CA1 clade A1 *env* gp120 DNA prime (A_R): CTRPNNNTRKGIHIGPG**R**AIYATGDIIGDIRQAHC
92BR025.9 clade C *env* gp120 DNA prime (C_Q): CTRPNNNTRKSIRIGPG**Q**AFYATGEIIGDIRQAHC
$V3_A$-FP derived from clade A strain 92UG037.08: CTRPNNNTRKSVRIGPG**Q**TFYATGDIIGDIRQAHC
$V3_B$-FP derived from clade B strain JR-CSF: CTRPSNNTRKSIHIGPG**R**AFYTTGEIIGDIRQAHC
$V3_C$-FP derived from clade C strain 93IN904: CTRPNNNTRKSIRIGPG**Q**TFYATGDIIGDIRQAHC

commencement of the immunization protocol and 2 weeks after each immunizing injection. The gp120 gene from CA1 (an R5-tropic strain of CRF011_cpx) and from 92BR025.9 (an R5-tropic strain of clade C) were chosen for preparation of the DNA prime. The CA1 strain carries a gp120 of clade A and was selected based on previous experiments showing that its envelope was immunologically representative of a cluster of unrelated primary isolates from clades A, B, D, F and G (Nyambi et al., 2000). It is noteworthy that the CA1 V3 sequence contains the GPG**R** V3 motif (Table 1) which is present in only ~4% of clade A envelopes (http://www.hiv.lanl.gov). The 92BR025.9 strain was chosen as a representative clade C virus with the GPG**Q** motif at the tip of the V3 loop (Table 1).

For the protein boosts, gp120 from the clade B JR-FL R5-tropic strain was used because it carries the V3 consensus sequence of clade B (with the GPG**R** V3 motif). The $V3_{JR\text{-}CSF}$-FP ($V3_B$-FP) was used as the boost because it had previously been shown to present the V3 epitope in its immunologically correct conformation (Kayman et al., 1994), and because the V3 of the clade B JR-CSF strain differs from the clade B consensus sequence by only one amino acid (Table 1). The $V3_A$-FP differs from the consensus sequence of clade A at two positions, and the $V3_C$-FPs carries the consensus V3 sequence of clade C (see Table 1).

Immunization with GPGR-based vaccines: antibody levels measured by ELISA

In the first experiment, both the prime and boost constructs carried the GPG**R** V3 motif. To compare the effect of priming and the boosting efficiency of gp120 vs. $V3_B$-FP, three groups of rabbits were used: *Group I-1 (−/B)*,which received no prime but was immunized with $V3_B$-FP; *Group I-2 (A_R/B)*, which received a clade A DNA gp120 prime which carries the GPG**R** V3 motif (A_R) followed by boosting with $V3_B$-FP; and *Group I-3: (A_R /$gp120_R$)*, which received a clade A DNA gp120 prime followed by boosting with gp120 protein from the JR-FL clade B strain (see Table 1). To determine the specificity of the Abs induced by the various immunization regimens, the reactivities of the sera from the various immunized animals were measured against MuLV gp70 (the protein into which the V3 sequences had been spliced to form the V3-FPs), against the YU2 gp120 core, and against the YU2 gp120 core carrying the V3 sequence (gp120 core+V3) (Wu et al., 1996b). Sera tested were derived from bloods drawn prior to immunization (pre-bleeds) and from those obtained 2 weeks after the second protein boost. The sera of animals that received V3-FP (Group I-1: −/B and Group I-2: A_R/B) made vigorous anti-gp70 responses, whereas, as anticipated, the sera of rabbits receiving no gp70 (Group I-3: A_R / $gp120_R$), and the pre-bleed sera from all three groups, contained no detectable anti-gp70 Abs (Fig. 1, left column).

To determine the levels of anti-V3 responses, serum reactivity was tested against gp120 core and gp120 core+V3 (Fig. 1, right column). As expected, sera drawn 2 weeks after the second boost from rabbits of Group I-1: −/B, which received only V3-FP, reacted only with gp120 core+V3 and displayed essentially no binding activity against gp120 core. Sera from rabbits of Group I-2: A_R/B displayed responses against gp120 core+V3 that were significantly greater than those against gp120 core (half-maximal binding titers of 1:69,398 and 1:2,909, respectively). This pattern of reactivity suggests that V3-FP was able to focus the Ab response on the V3 epitope. In contrast, the sera of rabbits primed with gp120 DNA and boosted with gp120 protein (Group I-3: A_R /$gp120_R$) displayed similar Ab reactivities against gp120 core+V3 and gp120 core (half-max. titers of 1:217,126 and 1:147,365, respectively), demonstrating that when gp120 protein is used as an immunogen, the V3 region is not an immunodominant epitope.

Immunization with GPGR-based vaccines: neutralization of primary isolates

Recently, a multi-tier approach was recommended for assessing the neutralizing Ab responses generated by candidate HIV vaccines (Mascola et al., 2005). These recommendations suggest that initially, immune sera should be tested against "Tier 1

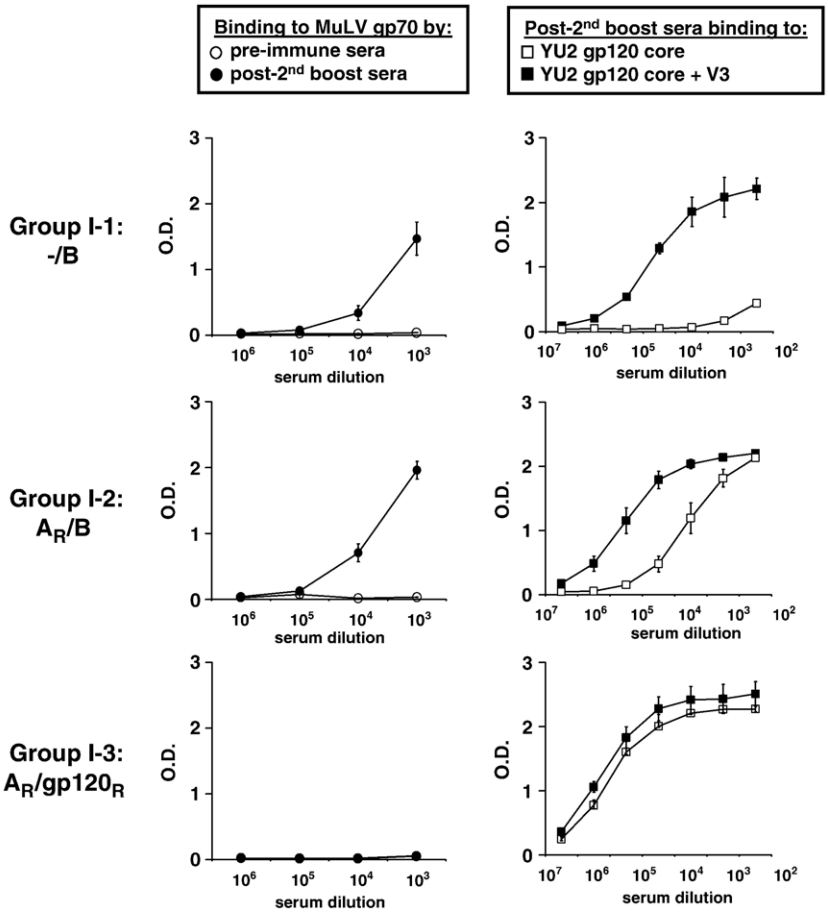

Fig. 1. Humoral immune responses of the groups of immunized rabbits described in Experiment I in Table 1 and Methods. (Left column) Activity of serum Abs binding in ELISA to MuLV gp70. ○, pre-immune sera; ●, sera drawn 2 weeks after second protein boost. (Right column) Binding activity of serum drawn 2 weeks after the second protein boost vs. YU2 gp120 core (□) or YU2 gp120 core containing V3 (■). *y*-axis represents OD; *x*-axis represents the reciprocal of the serum dilutions. Data shown are from one of two independent experiments.

viruses" which consist of "homologous virus strains represented in the vaccine and a small number of heterologous viruses that are known to be highly sensitive to Ab-mediated neutralization". Subsequently, testing should be undertaken against "Tier 2 viruses" (heterologous viruses that match the genetic subtype of the vaccine) and "Tier 3 viruses" (a multi-clade panel of Tier 2 viruses). Although no Tier 1 panels have been specified, it is generally acknowledged that Tier 1 viruses are sensitive to Abs that are specific for V3 and/or CD4i Abs; SF162 and MN are the only two primary isolates currently acknowledged and cited as Tier 1 viruses (Law et al., 2007). In the absence of a Tier 1 panel, we selected primary isolates based on previous studies showing the ability of anti-V3 mAbs at 25 μg/ml to achieve 50% neutralization of these viruses (Gorny et al., 2006). The viruses selected include CA1 (CRF011_cpx, one of the strains used in the vaccine prime), DJ263 (CRF02_AG), BX08 (clade B), and NYU129/5 (CRF02_AG). These viruses are more resistant to anti-V3 mAbs than SF162 (Gorny et al., 2006), and so should more accurately be identified as "Tier 1+" viruses; for the sake of brevity, they will be designated here as Tier 1 viruses. In addition, we tested primary isolates previously shown to be still more resistant to neutralization (Gorny et al., 2004, 2006) that were heterologous to the strains on which the immunogens were based and might therefore be categorized as Tier 2 and 3 viruses, respectively. These included JR-FL (clade B), 98CN006 (clade C), 93MW965 (clade C), and 93MW960 (clade C).

The data from two independent experiments assessing the neutralizing activity against three of four Tier 1 viruses in the sera of animals receiving the GPGR-based vaccine regimen are shown in Table 2. The data demonstrate the presence of neutralizing activity in the sera of the animals in the two groups that received both priming and boosting immunogens; the mean 50% neutralizing titers ranged from 1:13 to ≥1:320 against three of the four Tier 1 viruses tested, including viruses heterologous to those used to prepare the vaccine constructs. Significant neutralization of the CRF02_AG virus NYU129/5 was not achieved at a serum dilution of 1:20 (data not shown).

Particular attention was placed on the neutralizing activity against DJ263 in the sera of these rabbits immunized with a GPGR-based vaccine regimen since DJ263 is a virus with a clade A Env whose V3 loop carries the GPG**Q** motif. Results of one of two experiments are shown in Fig. 2 in which the neutralizing activity was titrated with sera drawn 2 weeks after the second protein boost. The sera from Group I-1: −/B displayed little or no neutralizing activity; the geometric mean titer for 50% neutralization (GMT_{50}) in this group, derived from the

Table 2
Neutralizing activity in immune rabbit sera

Group	Immunizing regimen [a]	DJ263 (CRF02_AG)		BX08 (Clade B)		CA1 (CRF011_cpx)	
		GMT_{50} [b]	% Neutralization due to V3 Abs	GMT_{50} [b]	% Neutralization due to V3 Abs	GMT_{50} [b]	% Neutralization due to V3 Abs
I-1	−/B	1:13	90% [c]	1:91	79%	<1:10	–
I-2	A_R/B	1:81	88%	1:132	69%	1:22	68%
I-3	A_R/$gp120_R$	1:38	33%	≥1:320	38%	1:47	49%

[a] Defined in Table 1.
[b] Geometric mean titers for 50% neutralization derived from all rabbit sera in each group tested in two separate experiments.
[c] Based on neutralization of DJ263 by sera from this group that contained neutralizing antibodies.

results of two experiments, was 1:13 (Table 2). In contrast, the GMT_{50} calculated from two experiments for all rabbits in Group I-2: A_R/B was 1:81. Rabbits in Group I-3: A_R/$gp120_R$ also mounted a significant neutralizing Ab response, with a GMT_{50} of 1:38 calculated from the results of two experiments. A dose–response relationship was demonstrated in the neutralization assay (Fig. 2), and, it is noteworthy that, when the immunization regimen focused the immune response on the clade B V3 loop by using the V3-FP_B boost (Group I-2: A_R/B), the neutralizing GMT_{50} reached levels comparable to those achieved by administration of the entire gp120 molecule (Group I-2: A_R/$gp120_R$) or that had otherwise required the use of a polyvalent vaccine cocktail of full-length gp120 molecules delivered as both DNA and protein (Wang et al., 2006). Moreover, the results reported here are substantially stronger than those previously achieved for neutralizing activity in the sera of immunized animals against heterologous primary isolates using other immunization approaches (Chakrabarti et al., 2005; Lian et al., 2005).

The efficacy of the boost is shown in Fig. 3: there is minimal neutralizing activity against DJ263 in sera drawn 2 weeks after the third DNA prime as opposed to the activity in the sera drawn 2 weeks after the second protein boost. These data confirm those previously published (Wang et al., 2006) showing that DNA priming induces relatively low levels of neutralizing Abs, whereas the protein boost is primarily responsible for the induction of clearly positive neutralizing Ab responses. For this reason, all subsequent data are shown for sera drawn 2 weeks after the second boost.

As noted above (Table 2; Figs. 2 and 3), there is no significant quantitative difference in the neutralizing activity against DJ263 in sera from rabbits in Groups I-2: A_R/B and I-3: A_R/$gp120_R$, however the ELISA data (Fig. 1) demonstrated a qualitative difference in the specificity of binding Abs, with the $V3_B$-FP boosted group (I-2: A_R/B) and the gp120 boosted group (I-3: A_R/$gp120_R$) having different profiles of Ab binding reactivities. To determine if there was also a qualitative difference in the specificity of the neutralizing Abs in the sera from the different groups, sera were tested for the neutralization of DJ263 at a 1:20 dilution with or without pre-incubation with a V3 peptide. Fig. 4 shows a representative experiment. The data confirm that the sera from Groups I-2: A_R/B and I-3: A_R/$gp120_R$ are quantitatively similar, with sera diluted 1:20 giving ~60–90% neutralization. However, the sera from the rabbits in these groups show a considerable qualitative difference. Thus, the majority of the neutralizing activity in sera from Group I-2: A_R/B was removed by pre-incubation with the V3 peptide. In contrast, pre-incubation with V3 peptide resulted in only a partial reduction in the neutralizing activity of sera from Group I-3: A_R/$gp120_R$. The sera from two of the animals in Group I-1: −/B showed weak neutralizing activity, and most or all of that activity was due to anti-V3 Abs, as expected. Pre-incubation of immune sera with 180 μg/ml of a scrambled peptide with the same amino acid composition as the $V3_B$ consensus 23-mer peptide did not result in any significant absorption of neutralizing activity (data not shown).

Similar peptide inhibition experiments were performed to identify the proportion of Abs that neutralize Tier 1 viruses

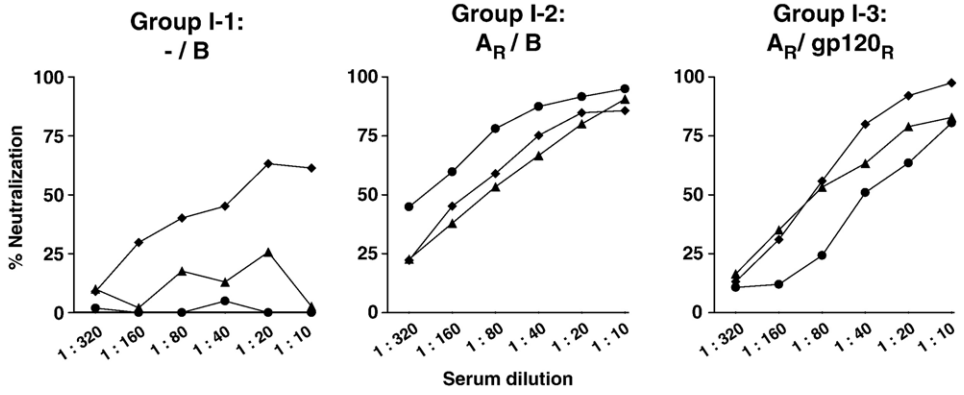

Fig. 2. Titration of neutralizing activity against CRF02_AG virus DJ263 in rabbit immune sera drawn 2 weeks after the second protein boost. Rabbit groups, summarized in Experiment I, Table 1, include Group I-1: −/B; Group I-2: A_R/B, and Group I-3: A_R/$gp120_R$. Each panel shows the results with sera from the three animals in that group, each designated with a different symbol. The percent neutralization was calculated based on the activity of each immune serum vs. the activity in the pre-bleed serum from the corresponding rabbit. Data shown are from one of two independent experiments.

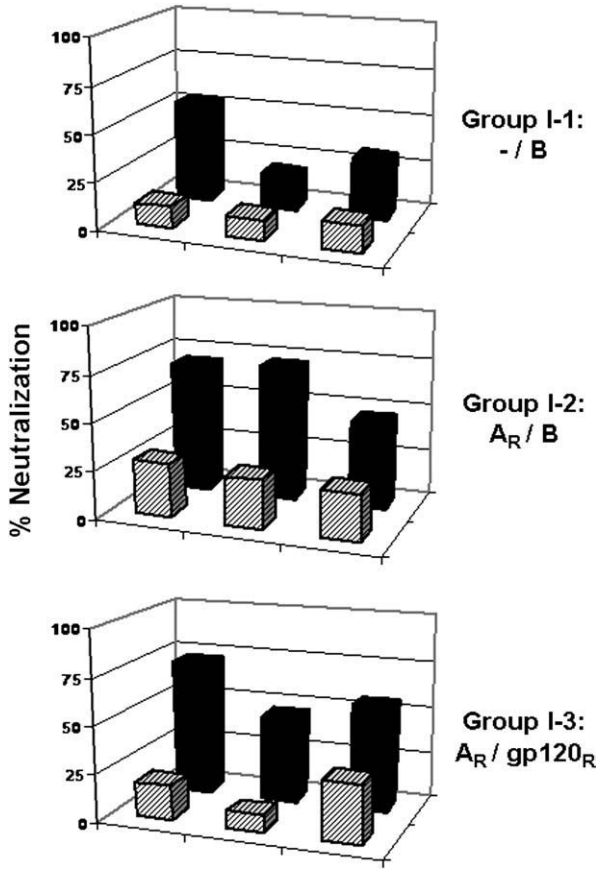

Fig. 3. Neutralizing activity in immune rabbit sera (at a final dilution of 1:20) against CRF02_AG primary isolate DJ263. Data are from the three groups of rabbits in Experiment I described in Table 1, i.e., Group I-1: −/B; Group I-2: A_R/B, and Group I-3: A_R/gp120$_R$. Data from the sera of each of the three rabbits in each group are displayed as paired bars representing sera drawn 2 weeks after the third DNA prime (hatched bars), and 2 weeks after the second protein boost (solid bars). The percent neutralization was calculated based on the activity in the immune sera vs. the corresponding animal's pre-immune sera. Data shown are from one of two experiments.

BX08 and CA1. The results again show that the use of the V3-FP results in neutralizing Abs that are preferentially V3-specific. Thus, 69% and 68% of the BX08 and CA1 neutralizing activity, respectively, in the sera of the Group I-2: A_R/B rabbits were blocked by V3 peptide, where as 38% and 49% of the comparable neutralizing activity was blocked in the sera of the Group I-3: A_R/gp120$_R$ rabbits (Table 2). Thus, while boosting with V3-FP induces a response that is quantitatively similar to that achieved with whole gp120, using a boosting immunogen with a single neutralizing epitope is able to focus the immune response such that neutralizing Abs to that epitope are preferentially produced.

Immune sera from the rabbits receiving the GPGR-based vaccine regimen were also tested against Tier 2 (JR-FL) and Tier 3 (98CN006, 93MW965, 93MW960) viruses. 50% neutralization was not detected at final serum dilutions of 1:20.

Immunization with GPGR-based vaccines: neutralization of pseudoviruses

For many primary isolates, the V3 loop is partially or fully masked by the V1/V2 loop and/or carbohydrate moieties in Env (Pinter et al., 2004; Wei et al., 2003). As noted above, viruses with an unmasked V3 loop are generally considered to be Tier 1 viruses, and about 20% of viruses are thought to be Tier 1. One such virus is the clade B strain SF162 (Krachmarov et al., 2005). Ninety percent geometric mean titers (GMT_{90}) for the neutralization of the pseudovirus (psV) carrying the SF162 Env are 1:41, 1:114, and 1:166 for animals in Group I-1: −/B, Group I-2: A_R/B, and Group I-3: A_R/gp120$_R$, respectively (Table 3).

To thoroughly assess the range of V3 diversity that is recognized by anti-V3 Abs in the rabbit immune sera, V3 chimeric psVs were constructed in which the V3 loop of SF162 was replaced with the consensus V3 sequences from clades A1, B, C, F, H, CRF01_AE (E), and CRF02_AG. The GMT_{90}s of the pre-bleed sera tested against these seven V3 chimeric psVs was <1:10 (data not shown). In the immune sera of rabbits receiving the GPGR-based immunizations, the GMT_{90}s were consistently highest against the psV carrying the clade B V3 consensus sequence (Table 3), reflecting (a) a preference for the GPG**R** motif at the tip of the loop which is homologous to the V3 motif in both the priming and boosting immunogens used, and (b) the fact that the V3 sequence of the $V3_B$-FP boosting protein differs from the consensus B V3 sequence at only a single residue at the N-terminal base of the loop (Table 1). The GMT_{90}s for Group I-1: −/B, Group I-2: A_R/B, and Group I-3: A_R/gp120$_R$ against the psV carrying the consensus V3 sequence of clade B were 1:689, 1:1717, and 1:3308, respectively. Interestingly, the titers against the chimeric psV carrying the consensus B V3 loop in the SF162 Env backbone were frequently more than one order of magnitude higher than the titers against the psV carrying the wild type Env of SF162 (which differs at three positions from the V3 consensus B sequence in the central portion of the loop [Table 3]). While the GMT_{90}s were <1:10 for neutralizing activity in the sera from animals in each group against psVs

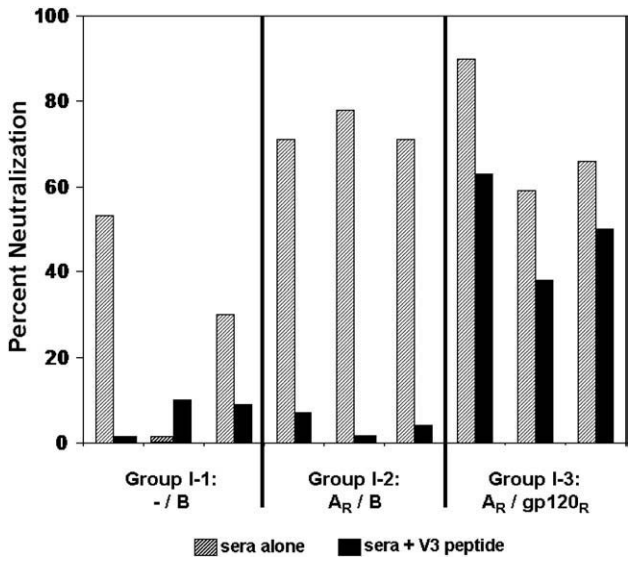

Fig. 4. Neutralizing activity against CRF02_AG primary isolate DJ263 in immune rabbit sera prior to (hatched bars) or after (solid bars) incubation of sera with 180 μg/ml of a 23-mer peptide representing the V3 consensus sequence from clade B. Data show the activity in the sera from each of the three rabbits in Groups I-1: −/B, Group I-2: A_R/B, and Group I-3: A_R/gp120$_R$, as defined in Table 1 and are derived from one of two experiments.

Table 3
90% neutralizing titers for V3 chimeric pseudoviruses

Group	Immunizing Regimen	Rabbit #	SF162wt	B	F	E	A1	AG
I-1	– / B	1	97	1320	65	24	21	<1:10
		2	33	660	<1:10	<1:10	<1:10	<1:10
		3	21	367	16	22	13	<1:10
I-2	AR / B	4	185	3358	122	46	40	11
		5	105	1498	56	274	26	25
		6	77	1720	48	48	30	15
I-3	A_R / $gp120_R$	7	187	3225	162	71	34	20
		8	170	3660	120	20	20	16
		9	145	3067	130	125	54	40
II-1	– / ABC	16	<1:10	70	<1:10	<1:10	10	<1:20
		17	<1:10	<1:10	<1:10	<1:10	<1:10	<1:20
		18	<1:10	20	<1:10	<1:10	<1:10	<1:20
II-2	A_R / ABC	19	29	710	90	78	25	30
		20	14	571	42	<1:10	19	<1:20
		21	31	1470	48	71	35	40
II-3	C_Q / ABC	22	42	594	104	91	73	103
		23	45	989	175	822	78	120
		24	162	2040	571	2165	283	403
II-4	$A_R + C_Q$ / ABC	25	18	544	33	32	20	25
		26	35	923	110	244	62	40
		27	48	345	70	14	37	60
II-5	A_R / B	28	36	488	61	165	38	30
		29	77	2959	107	<1:10	35	<1:20
		30	51	3350	388	<1:10	125	62

Titers are shown at which RLUs were reduced 90% compared to control wells containing virus alone. Data are geometric mean titers from two to three assays. Dark gray boxes show titers >1:100; light gray, 1:20–1:99, white, 90% neutralization not achieved at the lowest concentration tested. Columns show neutralizing titers against pseudoviruses carrying the wild type SF162 Env (SF162wt) and against V3 chimeric SF162 pseudoviruses carrying the consensus sequences of clades B, F, E, A1 and AG. Consensus V3 sequences inserted into the SF162 backbone Env of psVs are:

```
B       C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C
SF162wt- - - - - - - - - - - - T - - - - - - - - A - - D - - - - - - - - - -
F       - - - - - - - - - - - - H - - - - Q - - - A - - E - - - - - - K - - -
E       - - - - S - - - - T - - T - - - - Q V - - R - - D - - - - - - K - Y -
A1      - - - - - - - - - - - - R - - - - Q - - - A - - D - - - - - - - - - -
AG      - - - - - - - - - - - V R - - - - Q T - - A - - D - - - - - - - - - -
Experiments not shown (see text) also included psVs carrying the consensus
V3 sequences from the following clades:
C       - - - - - - - - - - - - R - - - - Q T - - A - - D - - - - - - - - - -
H       - - - - - - - - - - - - H L - - - Q - - - A - - D - - - - - - - - - -
```

carrying the consensus V3 sequences of clades C or H (data not shown), 90% neutralizing titers against psVs carrying the consensus V3 loops of clades A1, AG, F, and E were detected at levels of 1:20 to 1:274 in the sera of animals that had been primed and boosted with either V3-FP or gp120 (Group I-2: A_R/B and Group I-3: A_R /$gp120_R$) (Table 3). The neutralizing activity against these chimeric psVs showed a hierarchy with B ≫ F > E > A1 > AG. These data demonstrate the induction of neutralizing Abs that can recognize the V3 loops of diverse clades.

The V3 chimeric psVs with the SF162 Env backbone represent Tier 1 viruses, and, as noted, the rabbit immune sera had significant activity against many of these psVs and against three of four Tier 1 viruses. Although no activity had been detected against Tier 2 and 3 viruses, to test further for activity against Tier 2 viruses, the rabbit sera were assayed against the Tier 2 standard clade B panel of psVs (Li et al., 2005). None of the rabbit sera achieved 50% neutralization when tested at a 1:10 dilution (data not shown). Thus, neither Tier 2 viruses nor Tier 2 psVs were neutralized by the immune rabbit sera.

Immunization with multivalent vaccines: anti-V3 binding activity

A second set of rabbits was immunized using multivalent priming and/or boosting (Table 1). The multivalent approach was based on previous work showing that broader immune responses could be elicited with immunogens derived from diverse HIV clades (Chakrabarti et al., 2005; Lian et al., 2005; Wang et al., 2006). The sera of animals receiving the multivalent vaccine regimen, derived from bleeds taken 2 weeks after the second protein boost, were titrated for their binding activity against $V3_A$-, $V3_B$- and $V3_C$-FPs. The data in Table 4 demonstrate that the strongest response to all three V3-FPs was mounted by rabbits in Group II-3: C_Q/ABC.

Immunization with multivalent vaccines: neutralization of primary isolates

To determine if multivalent immunogens would help to broaden the immune response when, simultaneously, the immune

response was focused on a single neutralizing epitope, experiments were performed in which animals received either no DNA priming (Group II-1: −/ABC), a gp120 DNA prime based on the clade A_R *env* (Group II-2: A_R/ABC), a gp120 DNA prime based on the clade C_Q *env* (Group II-3: C_Q/ABC), or a combined clade A_R and C_Q gp120 DNA prime (Group II-4: A_R+C_Q/ABC). All animals in these groups received boosts of a cocktail of $V3_A$-, $V3_B$-, and $V3_C$-FPs. Group II-5: A_R/B serves as a "benchmark", recapitulating Group I-2: A_R/B in the previous set of rabbits.

The immune sera from the rabbits in this experiment were again tested first for their ability to neutralize Tier 1 primary isolates which, in this case, included CA1 (CRF02_AG) and 92BR025 (clade C), each used as the basis of the gp120 DNA prime, and BX08 (clade B), DJ263 (CRF02_AG), and NYU129/5 (CRF02_AG). While no significant neutralizing activity was detected in the sera of any of the rabbits when tested at a dilution of 1:20 against NYU129/5 or 92BR025 (data not shown), the neutralizing activity demonstrated against primary isolates DJ263 and BX08 and CA1 is shown in Fig. 5. The rabbits that received the C_Q/ABC regimen (Group II-3) displayed the strongest response against DJ263 (GMT_{50} of 1:559), the virus that carries the GPGQ V3 motif also found in the C_Q gp120 DNA prime and in the $V3_A$- and $V3_C$-FPs used to boost. In contrast, the sera from Group II-5: A_R/B showed the strongest reactivity against primary isolates BX08 and CA1 carrying the GPGR V3 motif. Thus, sera from Group II-5: A_R/B displayed a GMT_{50} vs. clade B virus BX08 of 1:246, and a GMT_{50} vs. CA1 (the CRF011_AG virus from which the A_R boost was constructed and which contains a GRGR V3 motif) of 1:111 (Fig. 5).

The sera of the animals immunized with the multivalent vaccine regimen were also tested against Tier 2 viruses including JR-FL (clade B), 98CN006 (clade C), 93MW960 (clade C), and 93MW965. Fifty percent neutralization was not detected against any of these Tier 2 primary isolates when tested at a final serum dilution of 1:20.

Immunization with multivalent vaccines: neutralization of pseudoviruses

Neutralization experiments were next performed using the panel of psVs made with the SF162 Env or chimeric forms of this Env carrying the consensus V3 sequences from clades A1, AG, B, C, F, E, C and H. The neutralization data with psVs

Table 4
Reciprocal half-maximal binding titers to $V3_A$- $V3_A$- and $V3_C$-FPs of immune sera drawn 2 weeks after the second boost

Group [a]	Immunizing regimen	$V3_A$	$V3_B$	$V3_C$
II-1	−/ABC	1765 [b]	6556	1418
II-2	A_R/ABC	1869	16,469	1869
II-3	C_Q/ABC	5580	27,332	5268
II-4	A_R+C_Q/ABC	3243	20,106	3020
II-5	A_R/B	1651	27,227	1354

[a] Groups as defined in Table 1.
[b] Reciprocals of the geometric means of the half-maximal binding titers from the sera of each of the three rabbits in each group.

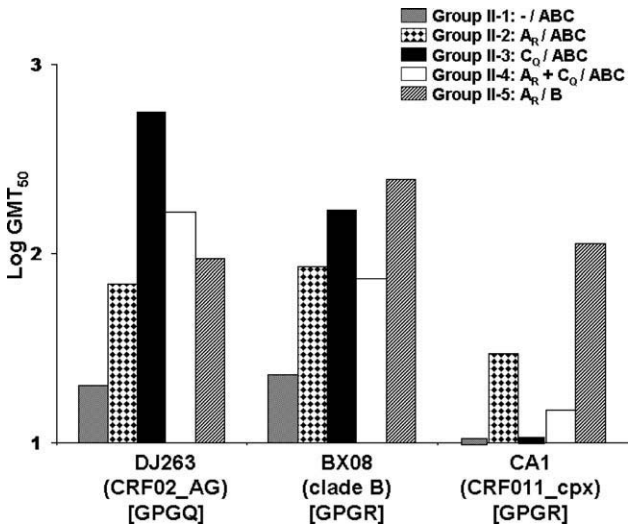

Fig. 5. Geometric mean titers for 50% neutralization (GMT_{50}) vs. three primary isolates measured in immune rabbit sera from Experiment II obtained 2 weeks after the second protein boost. Titers are shown at which RLUs were reduced 50% compared to control wells containing virus and pre-immune serum from the corresponding animal. The rabbit groups are those described in Table 1 for Experiment II. Data are compiled from two independent experiments with each virus.

(Table 3) support data from assays against the Tier 1 and 2 viruses showing that Group II-3: C_Q/ABC mounts the broadest response (Fig. 5). The response to the psV carrying the clade B V3 consensus sequence was strong (NT_{90} >1:100) in all animals that received a prime and boost, but the response to the psVs carrying the GPGQ V3 motif in consensus V3 sequences of clades F, E, A1 and AG were consistently highest when clade C gp120 DNA was used to prime and $V3_A$-, $V3_B$- and $V3_C$-FPs were used to boost (Group II-3: C_Q/ABC; Table 3) It is only in this latter group that rabbit immune sera achieve a GMT_{90} > 1:100 for the clades B, F, E, A1 and AG V3 chimeric psVs. GMT_{90} levels ≥1:10 were not achieved by sera from any of the rabbit groups against psVs carrying the clade C or H consensus V3 sequence. However, GMT_{50} levels of neutralizing Abs against these latter psVs were achieved by all groups of animals receiving both DNA prime and protein boosts, with titers ranging from 1:15 to 1:85. Group II-3: C_Q/ABC again achieved the highest levels of Abs, with GMT_{50} vs. clade C and H chimeric psVs of 1:85 and 1:53, respectively (data not shown).

The sera from these animals receiving the multiclade immunofocusing regimen were also assayed against the Tier 2 standard clade B panel of psVs (Li et al., 2005). None of the rabbit sera achieved 50% neutralization at titers of 1:10 (data not shown).

Discussion

The experiments described here demonstrate the feasibility of focusing the immune response on a single protein domain that elicits neutralizing Abs. The results serve as a proof-of-principle that the immune response can be focused on selected regions of the HIV envelope, that the majority of neutralizing Abs elicited can, indeed, be targeted to the selected epitope, and

that a broad response can be elicited with this technique. While the vaccine constructs used in this work were designed to focus the immune response on only a single HIV Env epitope, the V3 loop, the incorporation of selected additional neutralizing epitopes into recombinant vaccines may induce neutralizing Abs that produce additive, or optimally, synergistic effects.

When the immune response of rabbits was focused on the V3 epitope of the HIV gp120 envelope glycoprotein with the GPGR-based regimen, Abs were elicited that neutralized three of four Tier 1 primary viruses including a Tier 1 virus that was heterologous to the parental strains from which the immunogens were constructed and that carried the GPGR V3 loop (e.g., clade B strain BX08), and a heterologous Tier 1 virus carrying the GPGQ V3 loop (e.g., CRF02_AG strain DJ263); none of four Tier 2 primary isolates were neutralized. The multiclade immunofocusing technique also resulted in the neutralization of three of four Tier 1 viruses. Moreover, when sera from the rabbits immunized with the multiclade regimen were tested against chimeric psVs carrying unmasked consensus V3 loops from several clades, $GMT_{90} > 1{:}100$ were demonstrable against psVs carrying the consensus V3 sequences from clades A1, B, F, E, and AG.

The use of DNA priming and protein boosts has proven to be one of the best regimens for inducing anti-Env Ab responses (Barnett et al., 1997; Lu, 2006; Richmond et al., 1998), and polyvalent vaccines based on the DNA prime/protein boost approach have proven to induce broader immune responses than similar monovalent vaccines (Chakrabarti et al., 2005; Lian et al., 2005; Wang et al., 2005a). We have now modified and extended previous work using the DNA prime/protein boost approach by using polyvalent combinations in both the prime and the boost, and by focusing the Ab response on a single gp120-neutralizing epitope, the V3 loop. The results demonstrate that a cross-clade-neutralizing Ab response can be achieved by using this approach (Table 3 and Fig. 5). As previously reported (Wang et al., 2005a, 2006), low levels of neutralizing Abs were induced by the priming alone, but peak Ab responses were elicited only after the protein boosts (Fig. 3).

Since immunization regimens differ and methods for measuring neutralization vary, it is often difficult to compare results from various experiments. To facilitate comparison of our results with previous experiments, we included a group of rabbits in Experiment I that were primed with gp120 DNA and boosted with gp120 protein-a control group immunized with a regimen similar to those used previously (Richmond et al., 1998; Wang et al., 2005a). This group served as a standard for qualitative and quantitative comparisons. Based on comparison of the results from the sera of the control and experimental groups, it appears that the immunofocusing vaccine regimens employed here are at least as good as, and often better than, results obtained with vaccines targeting the many epitopes of the HIV Env. For example, in rabbits primed with $gp120_{JR\text{-}FL}$ or $gp140_{JR\text{-}FL}$ DNA and boosted with $Env_{JR\text{-}FL}$, neutralizing Abs were induced to the relatively resistant homologous JR-FL strain and to SF162, but little or no neutralizing activity was detected against other clade B primary isolates or against primary isolates from other clades (Wang et al., 2005a). The results with the multiclade immunofocusing protocol presented here also exceed, in qualitative and quantitative terms, those recently published by Law et al. (Law et al., 2007). In the latter paper, rabbits were given four priming doses of codon-optimized JR-FL gp120 DNA and three boosts with a modified form of JR-FL gp120 in which the V1V2 loop was replaced with the gp41 MPER region containing two deleted residues immediately preceding the 4E10 epitope. Sera from these immunized rabbits displayed ND_{50}s vs. a psV carrying the Env of SF162 of 1:10–1:320, however 90% neutralization was never achieved. In contrast, the sera from animals primed and boosted in the experiments described above displayed ND_{50}s against the SF162 psV that ranged from 1:190 to 1:3550 (data not shown), and ND_{90}s in all primed and boosted rabbits in the range of 1:14 to 1:190 (Table 3). The results presented above for the multiclade immunofocusing regimen also compare favorably, in terms of the titer and breadth of the response, with the results of previously published multiclade immunizations using immunogens that included all or most of the Env epitopes (Chakrabarti et al., 2005; Lian et al., 2005; Wang et al., 2006). The current study represents a step forward by showing that, in focusing the immune response on a single neutralizing epitope, a functional Ab response is achieved that is better than or comparable to that induced by Env immunogens possessing a multitude of B cell epitopes. The results suggest that focusing the immune response on a few, carefully selected neutralizing epitopes and optimizing the structure of these epitopes and the scaffolds on which they are presented could result in a stronger and broader neutralizing Ab response than that induced by Env proteins carrying the many epitopes of the Env.

Interestingly, the GPGR-based and multiclade immunization regimens used to prime and boost the rabbits in the two experiments described here resulted in approximately comparable neutralizing Ab responses against the Tier 1 clade B primary isolate BX08 and against the psV carrying the clade B V3 consensus sequence (Tables 2 and 3; Fig. 5). In contrast, immunization with clade C gp120 DNA and the polyvalent cocktail of V3-FPs (Group II-3: C_Q /ABC) elicited the broadest and/or strongest response against the Tier 1 primary isolate DJ263 (CRF02_AG) which carries the GPGQ V3 motif (Fig. 5) and against the chimeric psVs carrying V3 loops with the GPGQ motif (Table 3). These data support prior data that suggest an antigenic difference between viruses carrying the GPGR and GPGQ V3 motifs (Zolla-Pazner et al., 2004) and document that anti-V3 Ab responses induced by "GPGR viruses" favor neutralization of GPGR viruses but that anti-V3 Ab responses induced by non-B "GPGQ viruses" neutralize both GPGQ and GPGR viruses (Gorny et al., 2006; Krachmarov et al., 2005).

Group II-3: C_Q /ABC is further distinguished as the only immunized group primed with the full dose of a gp120 DNA construct carrying the GPGQ V3 motif. Interestingly, neither rabbits in Group II-2: A_R/ABC or Group II-4: $A_R + C_Q$/ABC produced Abs of comparable breadth or potency. A possible explanation for this is that these two latter groups were primed, respectively, with A_R, a construct carrying the GPGR V3 motif or with $A_R + C_Q$, a priming dose containing half the dose of each prime relative to the dose of C_Q , the clade C (GPGQ) priming

dose administered in Group II-3: C_Q/ABC. The data from each of these groups suggest that when a combination of GPGR and GPGQ immunogens are used for priming, the GPGR immunogen is dominant over the GPGQ immunogen in eliciting the Ab responses. Other explanations for these findings include the possibility that a clade C gp120 DNA prime is superior to a clade A gp120 DNA prime, and/or that the differences noted are due to other factors contributed by the individual gp120 constructs used in these experiments.

The results of the experiments reported here demonstrate that it is possible to focus the immune response on an epitope that elicits neutralizing Abs, in this case, the V3 loop. Thus, the majority of neutralizing Abs elicited with a gp120 DNA prime and V3-FP boost were directed to V3; in contrast, only the minority of neutralizing Abs elicited with a DNA prime and gp120 protein boost were directed against V3 (Fig. 4 and Table 2). It is also noteworthy that when a neutralizing Ab response was elicited with the V3-FP boost, the cross-clade-neutralizing activity could be significantly blocked by a single V3 peptide derived from the clade B consensus sequence. This stands in contrast to the work of Chakrabarti et al. (2005) in which immunization of guinea pigs was carried out with DNA encoding gp145ΔCFI of one or several clades and replication-defective recombinant adenoviruses encoding the gp140ΔCFI of the same strains. In these latter experiments, when the polyvalent regimen was used, cross-clade neutralizing Abs were elicited, but neutralizing titers did not exceed 1:5 and serum absorption with V3 peptides did not remove the neutralizing activity.

In summary, the use of vaccine constructs that are capable of immunofocusing the humoral immune response provides a platform for improving further both the strength and breadth of the Ab response. This may be accomplished by (a) defining the best combinations of parental strains from which to build the priming and boosting immunogens, (b) designing immunogens that will optimally present the neutralizing epitopes and produce Abs of higher titer and affinity, and (c) ultimately focusing the immune response on additional epitopes which have been shown to induce protective Abs.

Methods

Construction of codon-optimized HIV env DNA vaccine constructs

The codon usage of *env* genes from HIV CRF011_cpx primary isolate CA1 and clade C primary isolate 92BR025 (C1) were analyzed with the MacVector software 6.3 against codon preference in *Homo sapiens*. The codons in CA1 and C1 *env* genes that are less preferred in mammalian cells were changed to the preferred codons in mammalian systems to promote higher expression of the Env proteins. The codon optimization strategy was not limited to changes of codons for mammalian usage. Sequence optimization was also performed to make the mRNA more stable and the genes more favorable for transcriptional and translational processes. During the sequence optimization, the following *cis*-acting sequence motifs were avoided: internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or GC-rich sequence stretches, ARE, INS, CRS sequence elements, cryptic splice donor and acceptor sites, and branch points. Despite such DNA level sequence changes, the final codon-optimized CA1 and C1 *env* DNA sequences still produce the same Env amino acid sequences that occur in the parental HIV-1 primary isolates. These codon-optimized *env* genes were chemically synthesized by Geneart (Regensburg, Germany).

To make codon-optimized CA1 and C1 gp120 DNA vaccines, the codon-optimized gp120 gene inserts were first PCR-amplified from the codon-optimized CA1 or C1 *env* genes. A pair of primers, gp120.CA1-opt1 (5′ GTCGCTCGCTAGCCTGTGGGTGACCGTG 3′) and gp120.CA1-opt2 (5′ ACCTACGGATCCTTACTGCACCACTCTTCTCTTGGC 3′), were used to amplify the codon-optimized gp120 CA1 gene insert. The priming constructs, tp120.Syn-7 (5′ GTCGCTCCAGCTAGCCTGTGGGTGACCGTGTACTACGGC 3′) and gp120.Syn-10 (5′ CGACGGATCCTTACTCCACCACGCGGCGCTTGGC 3′), were used to amplify the codon-optimized gp120 C1 gene insert. Then, the optimized CA1 or C1 gp120 gene inserts were cloned into the DNA vaccine vector pJW4303 (Wang et al., 2005b) at the *Nhe*I and *Bam*HI sites downstream of a human tissue plasminogen activator (tPA) leader sequence, substituting the natural HA leader sequence. The DNA vaccine plasmids were prepared from *Escherichia coli* (HB101 strain) with a Mega purification kit (Qiagen, Valencia, CA) for *in vivo* animal immunization studies.

Protein immunogens

The V3-FPs contain a 45-amino-acid domain encompassing the V3 sequences of either JR-CSF (clade B), 92UG037.08 (clade A) or 93IN904 (clade C) (see Table 1). The V3 regions are joined to the C-terminus of a 263 amino acid fragment of the Friend murine leukemia virus (MuLV) gp70, as previously described (Kayman et al., 1994). To facilitate purification, the His-8 and Gln-9 of this gp70 protein were replaced with a sequence of six His residues. The V3 fragments in the fusion proteins contain the disulfide-bonded loop and three sites for N-linked glycosylation, one within the V3 loop and one on each flank. The clade B V3-fusion protein ($V3_B$-FP) was expressed in Chinese hamster ovary (CHO) cells from a glutamine synthetase vector, pEE14 (CellTech, Cambridge, UK) containing the human cytomegalovirus major immediate-early promoter (Kayman et al., 1994). Similarly, clade A and clade C V3-fusion proteins ($V3_A$- and $V3_C$-FPs) were cloned into pcDNA3.1zeo (−) (Invitrogen, Carlsbad, CA) and expressed in CHO cells (Krachmarov et al., 2005). All fusion proteins were purified on Ni^{2+}-nitrilotriacetic acid resin (NTA Superflow; Qiagen, Valencia, CA) as described (Krachmarov et al., 2001).

Immunization protocol

Female New Zealand White (NZW) rabbits 6–8 weeks old (with a body weight of ~2 kg) were purchased from Millbrook Farm (Amherst, MA) and housed in the animal facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School in accordance with an IACUC-

approved protocol. Groups of rabbits first received three DNA immunizations at weeks 0, 2, and 4 using a Bio-Rad Helios gene gun (Bio-Rad Laboratories, Hercules, CA). The gp120 DNA vaccine plasmids or the negative control pJW4303 vector plasmid was coated onto 1.0-μm gold beads at a ratio of 2 μg of DNA per mg of gold. Each gene gun shot delivered 1 μg of DNA to a total of 36 non-overlapping sites on the shaved abdominal skin of each rabbit at each of the three priming immunizations. The animals then received two boosts with recombinant gp120 JR-FL protein (provided by Dr. O. Sharma through the NIH AIDS Research and Reference Reagent Program, catalog no. 4598) or with one or more of the V3-FPs at weeks 10 and 14 (Table 1). A total of 100 μg per injection of recombinant gp120 protein or of V3-FP(s) was administered intramuscularly with IFA. Blood was collected prior to immunization and 2 weeks after each immunization.

Measurement of Ab levels by ELISA

To determine serum Ab reactivity, affinity-purified mammalian-expressed YU2 gp120 core and YU2 gp120 core+V3 was used, as provided by Drs. M. Tang and R. Wyatt (Wu et al., 1996a). The YU2 V3 sequence is CTRPNNNTRKSINIGPGRALYTTGEIIGDIRQAHC. The $V3_A$-, $V3_B$-, and $V3_C$-FPs described above were also used. The carrier protein gp70 was also used as a control antigen in ELISA experiments; it was expressed in CHO cells and purified from culture supernatants as previously described (Krachmarov et al., 2001). The proteins were coated onto wells of microplates (Immunolon 4, Dynatech, Chantilly, VA) overnight at 4 °C at concentrations of 1 μg/ml, with the exception of the V3-FPs which were coated at 0.4–0.5 μg/ml. Plates were washed with PBS/0.2% Tween-20 (PBST), serum samples were added with blocking buffer (5% FCS, 5% sheep serum, and 2.5% BSA in PBS) and incubated for 1 h at 37 °C. After rinsing with PBST, anti-rabbit–IgG–HRP (Bio-Rad Laboratories, Hercules, CA) was added for 30 min at 37 °C. The wells were washed with PBS, color developed with 100 μl TMB peroxidase substrate (KPL Labs, Gaithersburg, MD), the reaction was stopped with 1 M HCl, and absorbance was measured at 450 nm.

Neutralization assays

Neutralization of primary isolates

JC53-BL cells (also termed TZM-bl cells) were obtained from the NIH AIDS Research and Reference Reagent Program (catalog no. 8129). This is a genetically engineered HeLa cell clone that expresses CD4 and CCR5 and contains Tat-responsive reporter genes for firefly luciferase and *E. coli* β-galactosidase under the regulatory control of the HIV long terminal repeat. Cell lines were maintained in growth medium, consisting of Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (BioWhittaker, Walkersville, MD).

Neutralizing activity against primary isolates was measured as the reduction in *luc* reporter gene expression after a single round of virus infection in JC53-BL cells as described previously (Li et al., 2005). Briefly, 200 $TCID_{50}$ of virus was incubated with various dilutions of test serum samples for 1 h at 37 °C in a total volume of 150 μl growth medium in 96-well flat-bottom culture plates (Corning-Costar, Acton, MA). For peptide inhibition studies, a 23-mer V3 peptide representing the V3 clade B consensus sequence (TRPNNNTRKSIHIGPGRAFYTTG) was incubated for 30 min at a final concentration of 180 μg/ml with rabbit serum, and then 200 $TCID_{50}$ of virus in culture medium was added and incubated for a further 1 h at 37 °C (Bio-Synthesis, Inc., Lewisville, TX). Freshly trypsinized cells (1×10^4 cells) were added to each well and maintained in culture medium containing 1 μM indinavir sulfate (NIH AIDS RRRP, catalog no. 8145). When necessary for efficient infection, DEAE-dextran was added at a final concentration of 25 μg/ml. The background controls contained cells only, while the virus controls contained cells plus virus. After a 48 h incubation, 200 μl of growth medium was removed from each well and 50 μl of Bright Glo reagent (Promega, Madison, WI) was added. This was followed by a two min incubation at room temperature to allow cell lysis, transfer to 96-well black solid plates (Corning Costar, Acton, MA), and measurement of luminescence using a Lumimark Plus microplate reader (Bio-Rad Laboratories, Hercules, CA). The percent neutralization for immune rabbit sera was calculated relative to the effect of preimmune serum from the same rabbit. Thus, for all dilutions of sera, the percent neutralization was calculated based on the RLU in the presence of immune sera from a given animal divided by the RLU in the presence of the same dilution of preimmune serum from the same animal. The 50% neutralizing titer was determined from the linear portion of the titration curve using the method of Least Squares.

Neutralization of pseudoviruses

The *env* expression vectors for chimeric forms of SF162 *env* with various consensus V3 sequences were generated by introducing the modifications sequentially by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), as described (Krachmarov et al., 2006). Plasmids expressing the reference panel of 12 subtype B HIV-1 Env clones described by (Li et al., 2005) were obtained from the NIH AIDS RRRP (Catalog #11227). The infectious pseudotyped viruses were generated by co-transfection of 293 cells with an *env* expression vector and with the complementing vector pNL4-3.Luc.R-E− (NIH AIDS RRRP, catalog no. 3418, donated by Dr. Nathaniel Landau) (Connor et al., 1995; He et al., 1995). Transfections were performed in tissue culture dishes using *Trans*IT-LT1 Reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's protocol.

Neutralizing activity was determined as previously described (Krachmarov et al., 2001) with a single-cycle infectivity assay using virions generated from the *env*-defective luciferase-expressing pNL4-3.Luc.R-E− (Connor et al., 1995) pseudotyped with a molecularly cloned HIV *env* of interest. In brief, psVs were incubated with serial dilutions of sera from immunized rabbits for 1.5 h at 37 °C, and then added to U87-T4-CCR5

target cells plated in 96-well plates in the presence of polybrene (10 μg/ml). After 24 h, cells were re-fed with RPMI medium containing 10% FBS and 10 μg/ml polybrene, followed by an additional 24–48 h incubation. Luciferase activity was determined 48–72 h post-infection with a microtiter plate luminometer (HARTA, Inc., Gaithersburg, MD) using assay reagents from Promega, Inc. (Madison, WI). Geometric mean titers for 90% neutralization (GMT_{90}) were determined by interpolation from neutralization curves and are averages of at least two independent assays.

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