JOURNAL OF VIROLOGY, May 2011, p. 4927–4936 Vol. 85, No. 10
0022-538X/11/$12.00 doi:10.1128/JVI.00081-11

Two Closely Related Env Antigens from the Same Patient Elicited Different Spectra of Neutralizing Antibodies against Heterologous HIV-1 Isolates▽

Michael Vaine,[1] Maria Duenas-Decamp,[2] Paul Peters,[2] Qin Liu,[1] James Arthos,[3] Shixia Wang,[1] Paul Clapham,[2] and Shan Lu[1]*

Department of Medicine[1] and Program in Molecular Medicine,[2] University of Massachusetts Medical School, Worcester, Massachusetts 01605, and Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892[3]

Received 12 January 2011/Accepted 2 March 2011

Identification of immunogens capable of eliciting broadly neutralizing antibody (NAb) responses against HIV-1 is a major goal toward the development of an AIDS vaccine. Despite significant progress in understanding the structural features of the HIV-1 envelope glycoprotein (Env) and the discovery of multiple broadly neutralizing monoclonal antibodies with defined antigenic structures, the design of optimal Env immunogens to elicit broad NAbs remains a major challenge. As the structural determinants of Env immunogenicity remain unclear, we assessed two closely related Env antigens isolated from the same HIV-1-infected patient with different phenotypic features to identify what may result in a favorable immunogenic profile. One Env, B33, isolated from brain, was highly macrophage tropic with a high CD4 affinity, while the other, LN40, isolated from the lymph nodes, was poorly macrophage tropic with a low CD4 affinity. Using a DNA prime-protein boost approach, rabbits primed with LN40 Env antigen had a NAb response against heterologous primary isolates, while B33 Env antigens were capable of eliciting NAbs against only homologous and sensitive viral isolates. Further analysis revealed that the specificity of NAbs elicited by the LN40 antigen mapped to limited residues within or flanking the CD4 binding site. Certain key structural determinants were identified that could differentiate primary Env immunogens based on their potential to elicit broader NAbs. This progress will facilitate the rational design of effective HIV-1 vaccine formulations with optimal Env antigens.

Protective antibodies are expected to play key roles in the development of an effective prophylactic AIDS vaccine, but overcoming the high degree of antigen diversity is a major challenge in eliciting broadly neutralizing antibody (NAb) responses against HIV-1. A less-studied but theoretically attractive approach for AIDS vaccine development is the use of polyvalent Env formulations (17). Given the high degree of sequence variation among primary HIV-1 isolates, the prevailing view for many years has been that it is difficult to produce a polyvalent HIV vaccine that can elicit broad antibody responses to encompass circulating viral isolates with diverse genetic backgrounds. It has been technically difficult to consider producing a polyvalent HIV vaccine formulation using randomly selected Env proteins, because a subunit-based vaccine, using recombinant HIV-1 Env proteins alone, was unable to elicit meaningful NAbs against primary viral isolates (10, 22). In fact, two failed phase III efficacy trials using a recombinant subunit protein-based HIV vaccine approach included only two Env antigens for each formulation, one of which was from a T cell line-adapted (TCLA) virus.

However, our recent progress in both animal and human immunogenicity studies has demonstrated that the DNA prime-protein boost immunization approach is more effective than DNA or protein alone in eliciting improved antibody responses with clearly positive NAb activities against primary Env antigens, including resistant ones (26, 27, 30, 31, 33). More significantly, polyvalent formulations with multiple primary Env antigens were more effective in eliciting broader NAbs than were monovalent primary Env antigens (27, 33). In a phase I clinical study, the polyvalent DNA prime-protein boost HIV vaccine, using five primary Env antigens, was able to elicit broad NAbs against a wide range of primary Env antigens of viruses isolated from subtypes A to E (32), indicating the promising potential of polyvalent Env HIV vaccines.

In the above studies, primary Env antigens included in the polyvalent formulations were randomly selected based on what was available at the start of the study, while the investigators tried to maintain a balanced representation of major HIV-1 subtypes. Since then, additional primary Env antigens have become available with well-characterized phenotypic features associated with the original viral isolates. It will be interesting to determine whether any certain phenotypes of the original viral isolates can preselect Env antigens with a greater ability to elicit NAb responses, and also whether a polyvalent Env formulation, with such rationally selected Env antigens, will achieve even broader and more potent NAbs than the early generation of polyvalent formulations with randomly selected Env antigens.

In the current report, studies were conducted to determine whether differences in immunogenic potential exist between two previously reported primary Env antigens with closely re-

* Corresponding author. Mailing address: Room 304, LRB, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Lazare Research Building, Worcester, MA 01605. Phone: (508) 856-6791. Fax: (508) 856-6751. E-mail: shan.lu@umassmed.edu.

▽ Published ahead of print on 16 March 2011.

lated gene sequences and completely different phenotypic features. Clade B primary Env antigens LN40 and B33 (19) were isolated from a single HIV-1-infected individual. These antigens share over 90% amino acid identity; however, despite the high degree of sequence homology, they display different phenotypic properties. The B33 Env is highly macrophage tropic, maintains a high affinity for CD4, and is sensitive to neutralization by monoclonal antibody (MAb) IgG1 b12 but resistant to MAb 2G12 (7, 19–21), while the LN40 Env has the opposite phenotype of B33, as it is poorly macrophage tropic, has only a low affinity for CD4, and is resistant to the MAb IgG1 b12 but sensitive to MAb 2G12 (7, 19–21).

In this study, rabbits were immunized with one of these two primary Env antigens via the DNA prime-protein boost approach. Rabbit immune sera were analyzed for the level and specificity of binding antibodies and NAb activity. A difference was observed in the heterologous NAb responses elicited by these two primary Env antigens. Further characterization of NAbs with mutated amino acids on the targeted viral Env sequences led to the identification of key Env sites that are responsible for sensitivity to NAbs elicited in different immune sera.

MATERIALS AND METHODS

HIV-1 gp120 DNA vaccines. Gene segments coding for the gp120 region were produced by PCR from the parental NA420 LN40 (LN40) or NA420 B33 (B33) full-length *env* genes (19). LN40 and B33 gp120 genes were then subcloned separately into the pJW4303 DNA vaccine vector for the DNA priming phase of the immunization, as previously reported (30). DNA plasmid was produced in the HB101 strain of *Escherichia coli*, then isolated and purified using the Qiagen Plasmid megakit (catalog number 12183).

HIV-1 gp120 protein vaccines. Recombinant HIV-1 gp120 protein vaccines were produced from Chinese hamster ovary (CHO) cells. The JR-FL gp120 protein produced by Progenics was provided by John Warren at the Division of AIDS, NIAID, NIH. The other gp120 Env from subtypes A (UG21-9), B (92US715), C (MW959), and E (TH14.12) were all produced in our laboratories at the University of Massachusetts Medical School (UMMS) and NIH. Secreted proteins from stably transfected CHO cell lines were harvested and purified over a lectin affinity column.

Rabbit immunizations. New Zealand White rabbits (6 to 8 weeks of age) were purchased from Millbrook Farm (Amherst, MA) and housed in the animal facility managed by the Department of Animal Medicine at UMMS. Animal studies were conducted according to a UMMS IACUC-approved study protocol. Rabbits were immunized three times at weeks 0, 2, and 4 with either LN40 gp120 or B33 gp120 DNA vaccine via a Bio-Rad Helios gene gun to shaved abdominal skin (36 μg each time). Rabbits were then boosted twice with a 5-valent gp120 recombinant protein formulation from subtypes A (UG21-9), B (JR-FL and 92US715), C (MW959), and E (TH14.12). Half of the animals were boosted at weeks 8 and 12, and the remaining received a boost at weeks 38 and 42. Each animal received 50 μg of total protein in incomplete Freund's adjuvant (IFA) intramuscularly for each boost. Sera were collected prior to the start of the whole study and 2 weeks after each immunization.

Enzyme-linked immunosorbent assay (ELISA). JR-FL gp120 protein produced by Progenics Pharmaceuticals and provided by NIAID was applied onto 96-well microtiter plates (Costar 3369) at 1 μg/ml in 100 μl of phosphate-buffered saline (PBS) for 1 h at room temperature. LN40 and B33 gp120 proteins were produced by transient transfection in 293T cells. On day 3 posttransfection, the supernatants were harvested and cleared of cell debris by low-speed centrifugation. Supernatants containing LN40 or B33 gp120 (100 μl) were then directly applied onto microtiter plates. Plates were washed five times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4°C in PBS containing 4% (weight/weight) whey (whey dilution buffer) and 5% powdered milk. The following morning plates were washed five times in EWB, and serially diluted rabbit serum that was collected 2 weeks after each immunization was added to the wells in a volume of 100 μl. Plates were washed five times in EWB, and 100 μl of biotinylated anti-rabbit secondary antibody (Vector Labs BA-1000) at 1.5 μg/ml was incubated on the plate for 1 h at room temperature. Plates were washed five times with EWB and incubated with 100 μl of a streptavidin-horseradish peroxidase construct (Vector Labs SA-5004) at 500 ng/ml. Plates were washed five times with EWB and developed for 3 min in 100 μl of a 3,3′5,5′-tetramethylbenzidine substrate solution (Sigma T3405). The reaction was stopped with 25 μl of 2 N H_2SO_4.

Neutralization assays. HIV pseudovirus was first produced and titrated as previously described (18). Briefly, equal molar quantities of a plasmid expressing the HIV-1 gp160 of interest and the pSG3ΔEnv HIV backbone were cotransfected into HEK 293T cells. At 48 h after transfection, supernatants were harvested and cleared of cell debris by low-speed centrifugation. Produced pseudovirus was titrated on TZM-bl cells by using a 2-fold increase above background levels of luciferase activity as a positive cutoff.

Neutralization assays were performed as previously described (18). Briefly, pseudovirus was generated through cotransfection of vector coding for a gp160 *env* gene and the $pSG3^{\Delta env}$ backbone (NIH AIDS Research and Reference Reagent Program) in 293T cells. A total of 200 50% tissue culture infective doses of pseudovirus were incubated with the rabbit sera for 1 h at 37°C. The virus-sera mix was then added to 10^5 TZM-bl cells at a final concentration of 20 μg/ml to DEAE-dextran. Plates were incubated at 37°C for 48 h and developed with luciferase assay reagent according to the manufacturer's instructions (Promega). Neutralization was calculated as the percent change in luciferase activity (relative luciferase units [RLUs]) in the presence of preimmune sera versus that of luciferase activity in the presence of immune sera [(preimmune RLUs − immune RLUs)/(preimmune RLUs)] × 100.

For peptide adsorption experiments, the same protocol was applied as described above with one additional step. Prior to the exposure of sera to the pseudovirus, the sera were incubated with 25 μg/ml of subtype B consensus V3 peptide (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) for 1 h at 37°C.

Mutagenesis. All site-directed mutagenesis was performed using the Stratagene QuikChange II kit according to the manufacturer's instructions. Mutagenesis was verified by sequencing the gp120 gene region.

Competitive binding assays. Competitive binding assays were performed as previously described (4, 5) with minor modifications. Pseudovirions bearing the JR-FL Env and vesicular stomatitis virus (VSV) glycoprotein were produced with the $pSG3^{\Delta Env}$ backbone in 293T cells. Microtiter plates were coated with 50 μl of MAb at 5 μg/ml for 1 h at room temperature. Plates were then blocked in PBS with 3% bovine serum albumin overnight at 4°C. Rabbit serum was heat inactivated at 56°C for 30 min, serially diluted, and incubated with pseudovirus correlating to 2.5 ng of p24/well for 1 h prior to the addition to the virus-sera mixture to the ELISA wells. The pseudovirus-sera mixture was then incubated in the ELISA wells for 3 h at room temperature. Plates were washed 5 times with sterile PBS and overlaid with 10,000 TZM-bl cells per well. Plates were then incubated for 48 h at 37°C. Luciferase activity was determined per the manufacturer's instructions (Promega). The competition titer is reported as the serum dilution at which the luciferase signal was reduced by 50% compared to a serum negative control. When competition with the coreceptor binding site antibody 17b was tested, the pseudovirus was incubated with soluble CD4 (sCD4) at 5 μg/ml for 30 min at 37°C prior to the exposure of sera.

Statistical analyses. Neutralization data were subjected to one of several statistical analyses in order to identify significant differences. Using the neutralization data reported in Table 2, below, the number of animals from the B33- and LN40-primed groups capable of neutralizing 1 of the 14 viruses tested at greater than 50% neutralization was subjected to a Wilcoxon rank-sum test. The neutralization capacity of LN40-primed rabbit sera (see Table 3, below) was analyzed to determine statistical differences between the neutralization of the LN40 parental virus and the mutant isolates. This was accomplished by subjecting the data to an analysis of variance (ANOVA) followed by a Bonferroni-adjusted multiple comparison using the parental LN40 isolate neutralization as the control group. Statistical analysis to identify significant differences in neutralization of various chimeric LN40/B33 isolates (see Fig. 5B, below) was done by comparing responses to the neutralization of the parental B33 control virus to the neutralization capacity of LN40-primed sera using Student's *t* test.

RESULTS

Envelope phenotypes and immunization regimen. The LN40 and B33 Env antigens were selected for this study due to their high degree of sequence homology (Fig. 1) but opposing phenotypic properties (Table 1). They represent the extremes in macrophage tropism for HIV-1 R5 envelopes. Briefly, the B33

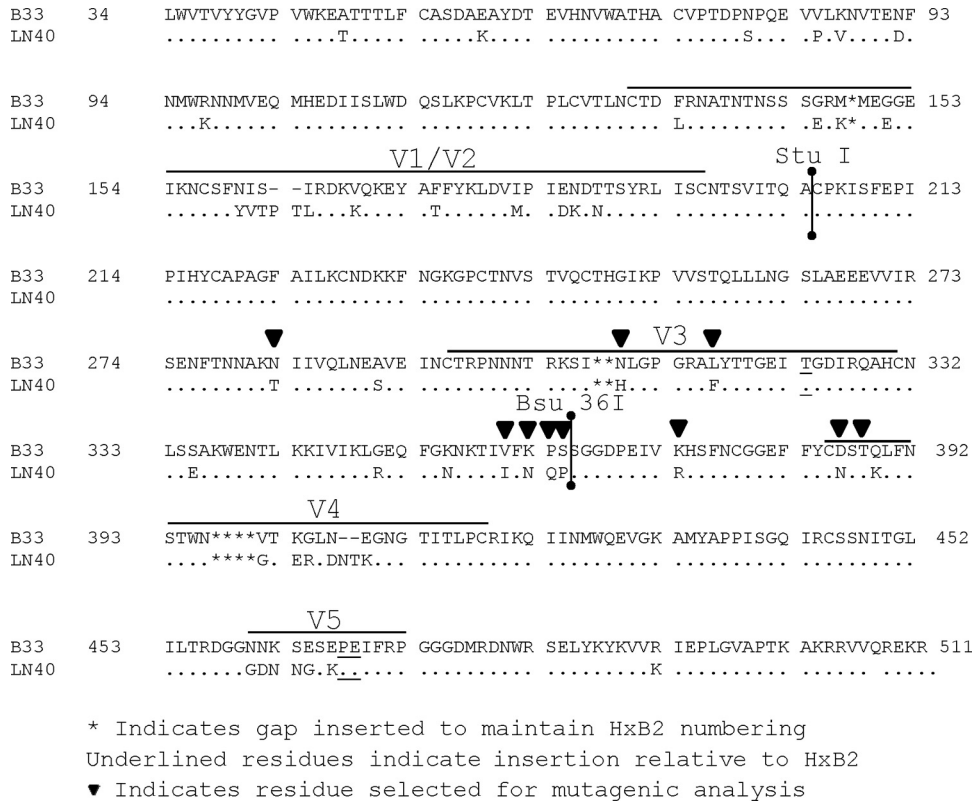


FIG. 1. Amino acid sequences of gp120 Env proteins for HIV-1 B33 and LN40 isolates. Variable loops and key cloning sites for creating the chimeric Envs are marked.

isolate has been shown to be highly macrophage tropic, sensitive to neutralization by MAb b12, and resistant to MAb 2G12. The LN40 isolate, however, is not macrophage tropic, is resistant to b12, and is sensitive to 2G12. Their phenotypes are influenced by changes within and proximal to the CD4 binding site (CD4bs) that result in a higher affinity for B33. The different phenotypes for these two Envs are determined solely by residues in gp120. Finally, a large range of mutants of these Envs are available and have been extensively studied (6, 7, 19–21).

The immunogenicities of these two Env antigens were evaluated in rabbits by using a standard DNA prime (3 times)-protein boost (2 times) approach, as previously reported (26, 27, 31, 33). Because the key phenotypic differences of these two viral isolates have been mapped to the gp120 region in previous studies (6–8), the gp120 form of Env was used for both DNA and protein vaccine components in the current study. More significantly, our previous reports demonstrated that gp120 DNA priming was able to greatly enhance the NAb activities in immunized rabbit sera, while an immunization approach with gp120 protein alone was much less effective (26, 27). The expression of both LN40 and B33 gp120 DNA vaccines was confirmed *in vitro* by Western blot analysis of gp120 produced in 293T cells transiently transfected with either of the two gp120 DNA vaccine plasmids (Fig. 2). A 5-valent protein boost consisting of gp120s from clades A, B, C, and E was given twice, based on our recent finding that a polyvalent Env protein boost using this same 5-valent gp120 protein was more effective than the single-valent matched gp120 boost in eliciting broader NAb activities in rabbits (27). Half of the animals from each group were boosted on weeks 8 and 12 while the other half of the animals were boosted on weeks 38 and 42 (Fig. 3), but a delay in protein boost did not cause any significant difference in either binding or functional antibody

TABLE 1. Envelope characteristics

Characteristic	LN40	B33
Macrophage tropic	No	Yes
CD4 affinity	Low	High
Neutralization by MAb b12	Resistant	Sensitive
Neutralization by MAb 2G12	Sensitive	Resistant

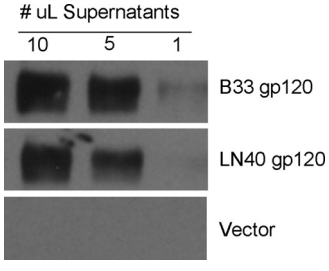


FIG. 2. Western blots of gp120 proteins transiently expressed in 293T cells that were transfected by one of the following: B33 gp120 DNA, LN40 gp120 DNA, or empty DNA vector.

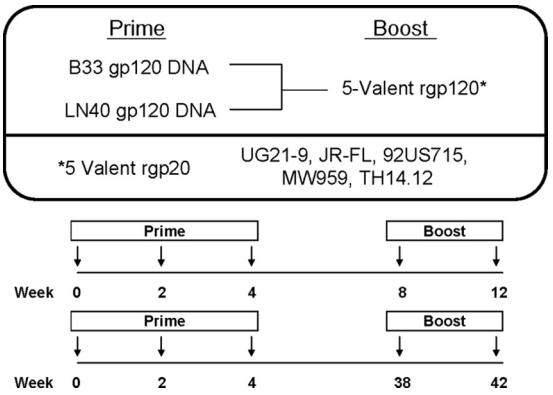


FIG. 3. Study design and immunization regimen. Rabbits received three priming immunizations of either LN40 gp120 or B33 gp120 DNA vaccines at weeks 0, 2, and 4. Protein boosts consisting of a 5-valent mixture of gp120s were given as an early boost at weeks 8 and 12 or as a late boost at weeks 38 and 42.

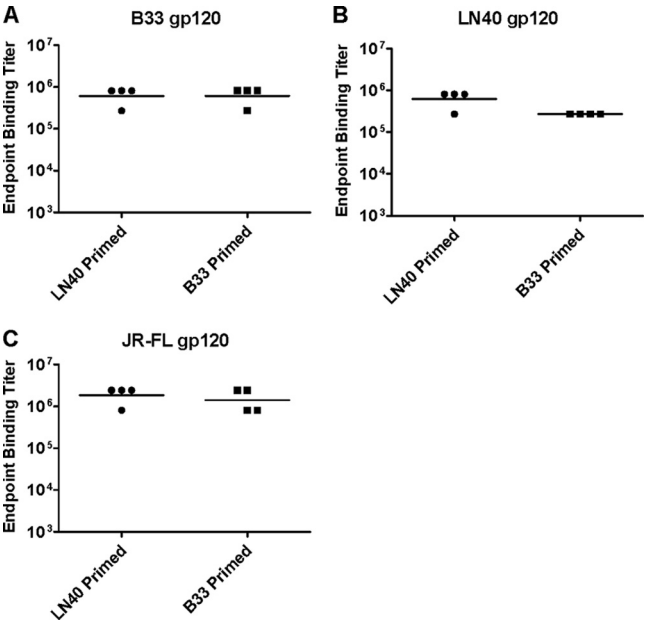


FIG. 4. Endpoint binding titers of serum antibody responses against HIV-1 gp120 antigens. Serially diluted rabbit sera collected 2 weeks after the last protein boost were assayed for binding against homologous and heterologous clade B gp120s. (A) Endpoint titer against B33 gp120. (B) Endpoint titer against LN40 gp120. (C) Endpoint titer against JR-FL gp120. The endpoint titer was defined as the last serum dilution giving at least double the optical density of a preimmune control.

responses (see below). Because all animals received identical protein boosts, any differences seen in the resulting antibody responses should be due to effects of the initial priming immunizations with either LN40 gp120 or B33 gp120 DNA vaccines.

Env-specific binding antibody titers. The peak level binding antibody response was evaluated against that to the homologous LN40 and B33 gp120s as well as a heterologous clade B JR-FL gp120, using rabbit serum samples collected at 2 weeks after the final protein boost. High-titer binding antibodies were observed against all three clade B gp120s (Fig. 4). No differences in the binding titers were observed against B33 gp120 regardless of the priming immunization given (Fig. 4A). Sera from animals primed with the LN40 gp120 recognized the homologous LN40 gp120 protein with an approximately 3-fold-higher titer than animals primed with the heterologous B33 gp120 (Fig. 4B). However, against the completely heterologous JR-FL gp120, no differences in the endpoint binding titers were observed between immunization groups (Fig. 4C). All prebleed sera against the above three gp120 antigens were below the limit of detection in this same assay (data not shown).

Neutralizing activity in sera from B33- and LN40-immunized rabbits. Despite the observation that immunization with B33 and LN40 gp120 antigens generated similar levels of high-titer binding antibody responses, the neutralizing activity in sera from rabbits primed with LN40 gp120 was much broader (Table 2). All animals, regardless of whether they were primed with B33 or LN40 gp120 DNA vaccines, were capable of neutralizing the sensitive or TCLA isolates, SF162 and NL4-3, indicating that a functional neutralizing antibody response can be elicited through priming with both B33 and LN40 gp120s. However, when the ability to neutralize other more resistant clade B primary isolates, including those from the standard tier 2 panel, is taken into account, only animals primed with the LN40 gp120 DNA vaccine were capable of neutralizing some of these viruses. Differences in the neutralizing activity of sera elicited by each gp120 DNA vaccine became apparent against the moderately sensitive clade B isolate SS1196.1. Three of the four animals primed with LN40 gp120 were capable of neutralizing this virus at a 1:10 dilution. However, none of the animals primed with B33 gp120 were capable of neutralizing this isolate. When evaluating the neutralizing activity of the elicited sera against more resistant primary isolates, specifically those tier 2 isolates (14), differences in the neutralizing activities between the two immunization groups were quite clear. While the overall breadth and potency remained limited within the LN40 gp120 DNA vaccine-primed animals, it is notable that only rabbits in the LN40-primed group were capable of neutralizing any of the primary isolates tested. All four of the LN40-primed animals were capable of achieving 50% neutralization of the 6535.3 isolate at a 1:10 dilution. Three of the four animals elicited an antibody response capable of neutralizing CAAN5342.A2 and QH0692.42 at the same dilution. Additionally, half of the LN40-primed animals were capable of neutralizing REJO4541.67. In contrast, the only positive neutralizing activity we observed in animals primed with the B33 gp120 DNA vaccine was against the 6535.3 isolate, where rabbit 657 achieved greater-than-50% neutralization at a 1:10 dilution. When these data were subjected to a Wilcoxon rank-sum test, we determined that the sera from LN40-primed animals neutralized significantly more viruses than sera from animals that were primed with B33 ($P = 0.025$).

To verify these results, we purified the rabbit IgG over a protein A column and reevaluated the neutralizing activity against the tier 2 clade B panel. Results from this study mirrored those seen when only serum was used; animals primed with LN40 gp120 elicited a more potent NAb response than animals primed with B33. Based on the 50% inhibitory concentration achieved with purified IgG at the same concentration, LN40 priming resulted in NAb activities that were gen-

TABLE 2. Neutralization of heterologous clade B isolates by sera from LN40- and B33-primed animals

Virus	% neutralization[a] at a 1:10 serum dilution in:							
	LN40-primed animal no.:				B33-primed animal no.:			
	491	652	653	654	492	655	656	657
Sensitive/TCLA								
SF162	96	94	90	89	93	94	89	89
NL4-3	97	97	98	87	80	72	69	73
Resistant/primary								
SS1196.1	66	56	86	—[b]	—	—	—	—
6535.3	85	68	88	51	—	—	—	73
AC10.0.29	—	—	—	—	—	—	—	—
CAAN5342.A2	54	60	63	—	—	—	—	—
PVO.4	—	—	—	—	—	—	—	—
QH0692.42	52	51	57	—	—	—	—	—
REJO4541.67	—	53	52	—	—	—	—	—
RHPA4259.7	—	—	—	—	—	—	—	—
SC422661.8	—	—	—	—	—	—	—	—
THRO4156.18	—	—	—	—	—	—	—	—
TRJO4551.58	—	—	—	—	—	—	—	—
TRO.11	—	—	—	—	—	—	—	—
WITO4160.33	—	—	—	—	—	—	—	—
Negative control, MLV	—	—	—	—	—	—	—	—

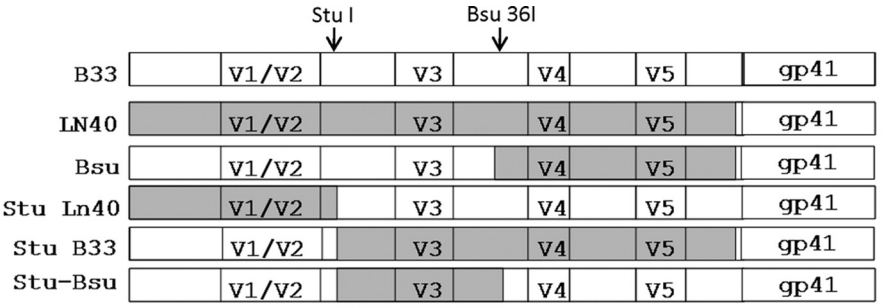
[a] The Wilcoxon rank-sum test was used to compare the number of positive neutralization results (of 13 total) for resistant/primary viruses between sera from groups of LN40 gp120- and B33 gp120-primed animals. They were significantly different ($P = 0.025$).
[b] —, less than 50% neutralization at a 1:10 serum dilution.

erally 2 to 10 times more potent (in one case 50 times more potent) than the neutralization seen in animals primed with B33 (data not shown).

Evaluating the specificity of neutralizing activity against pseudotyped viruses expressing chimeric Env antigens. In order to understand why the LN40-primed animals appeared to elicit a unique NAb response capable of neutralizing a greater breadth of viruses with increased potency, studies were organized to map the specificity of the observed neutralizing activity. Neutralization of the homologous B33 and LN40 viruses with rabbit sera immunized with the matching gp120 DNA vaccines indicated that neutralizing activity elicited by each gp120 was strain specific with regard to their respective homologous isolates. Sera elicited by an LN40 gp120 DNA prime were capable of neutralizing the homologous LN40 virus but not the B33 virus ($P < 0.0001$). A similar pattern was seen with sera from animals primed with the B33 gp120 DNA vaccine, as they were able to neutralize the homologous B33 isolate but not the LN40 isolate ($P < 0.0001$) (Fig. 5B).

This allowed us to further dissect the specificity of the neutralizing activity by using a series of previously described chimeric Env antigens with segments of Env sequences from both parental LN40 and B33 Env antigens (7). A total of four pseudotyped viruses expressing different LN40/B33 chimeric Env antigens were tested (Fig. 5A). Similar to the above neutralization results with heterologous clade B isolates, none of the B33-primed animals achieved over 50% neutralization for any of the four chimeric viruses tested (Fig. 5B). The LN40-immunized animals, however, were able to neutralize two of the four chimeric viruses tested (Fig. 5B). One of these, Stu-B33, contains the C1, V1/V2, and N terminus of C2 from the B33 Env and the remaining C-terminal portion from the LN40 Env. At a 1:10 serum dilution, 50% neutralization of this virus was achieved by sera from three of the four LN40-primed animals. The fourth animal exhibited a 32% increase in the neutralization potency of Stu-B33 over the parental B33 virus. The neutralization of this chimera relative to that of the B33 parental isolate was significantly higher ($P = 0.0001$). It was also found to be significantly higher relative to neutralization of this chimera by animals primed with B33 ($P = 0.0065$), suggesting that the chimerization does not create a universally more sensitive isolate. The second chimeric virus, Stu-Bsu, which was sensitive to neutralization by sera from the LN40-primed animals, contained the further-reduced segment from LN40 Env with only the C-terminal portion of C2, the V3 loop, and the N-terminal portion of C3 in the B33 Env backbone. Again, sera from three of the four animals neutralized this chimeric virus, and similar to what was seen with the Stu-B33 chimera, the fourth LN40-primed animal was more capable of neutralizing this Stu-Bsu chimeric isolate than the B33 parental isolate (Fig. 5B). Similar to the neutralization of the Stu-B33 chimera, LN40-primed animals neutralized Stu-Bsu significantly more potently ($P = 0.0002$) than the B33 parental isolate. Significance was also achieved relative to neutralization of this chimera by the B33-primed sera ($P = 0.0025$), again confirming that the mutant did not become universally neutralization sensitive. The other two chimeric viruses, Bsu and Stu LN40, which only had either the N-terminal segment or the C-terminal segment of LN40, respectively, were resistant to neutralization by sera from LN40 gp120 DNA vaccine-primed animals. Together, these results suggested that the critical region for NAb activity in LN40 gp120-primed rabbit sera was within the LN40 segment spanning the middle section of gp120 from the C-terminal portion of C2 to the N-terminal portion of C3.

A



B

Virus	LN40 Primed				B33 Primed				LN40 primed sera: B33 virus vs other viruses p-value	LN40 primed Sera vs B33 primed Sera p-value
	R491	R652	R653	R654	R492	R655	R656	R657		
B33	5	16	17	14	52	65	56	73	-	0.0001
LN40	63	69	67	60	11	10	21	20	<0.0001	<0.0001
Bsu	0	20	15	14	44	8	9	41	0.89	0.2631
Stu LN40	16	0	0	0	13	0	20	19	0.11	0.1903
Stu B33	68	61	64	46	8	21	40	32	0.0001	0.0065
Stu Bsu	68	55	54	46	4	20	25	30	0.0002	0.0025
Numbers indicate percent neutralization at a 1:10 serum dilution										

FIG. 5. Neutralization of chimeric viruses by rabbit sera primed with B33 or LN40 gp120 DNA vaccines. (A) Schematic designs of chimeric gp120 regions. White regions indicate Env portions derived from B33. Gray regions indicate Env portions derived from LN40. (B) Neutralizing activities against wild-type or chimeric Env pseudotyped viruses by immune sera from either LN40 gp120- or B33 gp120-primed rabbits. Numbers indicate the percent neutralization of the target viruses at a 1:10 serum dilution. Student's *t* test was used for statistical analysis to compare (i) the differences between neutralization by LN40 gp120-primed sera against B33 virus and neutralization by the same sera against other pseudotyped viruses and (ii) the differences between neutralization by LN40 gp120-primed sera and B33 gp120-primed sera against the same viruses.

V3 peptide adsorption of sera from LN40-immunized animals. After having narrowed down the region of neutralizing specificity from the parental LN40 Env, an absorption study was conducted with a clade B consensus V3 region peptide (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) to ensure that the NAb activity observed was not simply due to recognition of the V3 loop. The immune sera were treated with the consensus B peptide at 25 μg/ml prior to exposure to the viruses. When this V3 adsorption was done against the highly V3-sensitive SF162 isolate, a greater-than-97% reduction in the NAb titer was observed with sera from all of the LN40-primed animals (Fig. 6A). However, V3 adsorption had very little effect on the neutralization of the homologous LN40 virus, with less than a 10% observed reduction in neutralization for all four rabbit sera in the LN40 gp120-primed group (Fig. 6B). When the Stu-Bsu chimera was tested in the same analysis, a slightly larger proportion of the neutralizing activity (4% to 26% specific inhibition) was adsorbed with the V3 peptide (Fig. 6C), but the majority of the neutralizing activity directed against this chimera remained intact, indicating that the main neutralizing specificity was not targeted to the V3 loop. The same analysis was conducted against QH0692, one of the primary isolates that were capable of being neutralized by sera from the LN40-primed animals (Table 2). Similar to what was seen with V3 adsorption against the Stu-Bsu chimeric virus, there was only a slight drop in the ability to neutralize this primary virus, and the majority of the neutralizing activity remained intact (Fig. 6D). The above results demonstrated that neutralizing specificities outside of the V3 loop are responsible for the broader neutralization against heterologous primary viruses observed with sera of LN40 gp120 DNA-primed animals.

To further exclude V3 as the main reason for the difference in NAb responses observed in sera from LN40 gp120-primed animals, mutations for the only 2 amino acid residues that are different in two V3 regions between LN40 and B33 were made in the crown of the V3 loop of LN40 Env (H310N and F317L) to make it identical to that of B33 Env. Only a low to moderate decrease in NAb sensitivity was observed for the LN40 virus compared to its autologous sera (Table 3).

Identification of residues responsible for the sensitivity of the neutralizing activity in sera from LN40 gp120-primed animals. After eliminating the V3 loop as the main target of the broader neutralization observed with the sera from LN40 gp120-primed animals, fine mapping was performed to determine exactly where on the Stu-Bsu region of the LN40 Env the NAb activities were targeted. Mutagenesis was performed to determine the effects of point mutations in and around this region. Closer inspection of this region revealed that it contained contact residues for CD4 as well as residues that flank the CD4 binding loop at the C-terminal end. This area had previously been found to confer sensitivity to MAb b12 (7). Because of this, pseudotyped viruses were produced with mutations at key residues in this region which were previously reported to be involved in CD4 binding (8, 9), and the effects of these mutations on neutralization of the parental LN40 virus were analyzed (Table 3).

The first mutation (T283N) was created at position 283, which is known to be part of the CD4 binding site (13). The threonine (T) at this position in LN40 Env was mutated to the asparagine (N) found in B33 Env. When pseudotyped viruses LN40N with LN40 T283N Env were tested against serum from LN40-primed rabbits, almost all neutralizing activity was eliminated, relative to the parental LN40 virus ($P < 0.001$). Interestingly, when the reverse mutation was made in the parental B33 virus B33T (B33 N283T Env), the sera from two of the LN40-primed animals gained some of

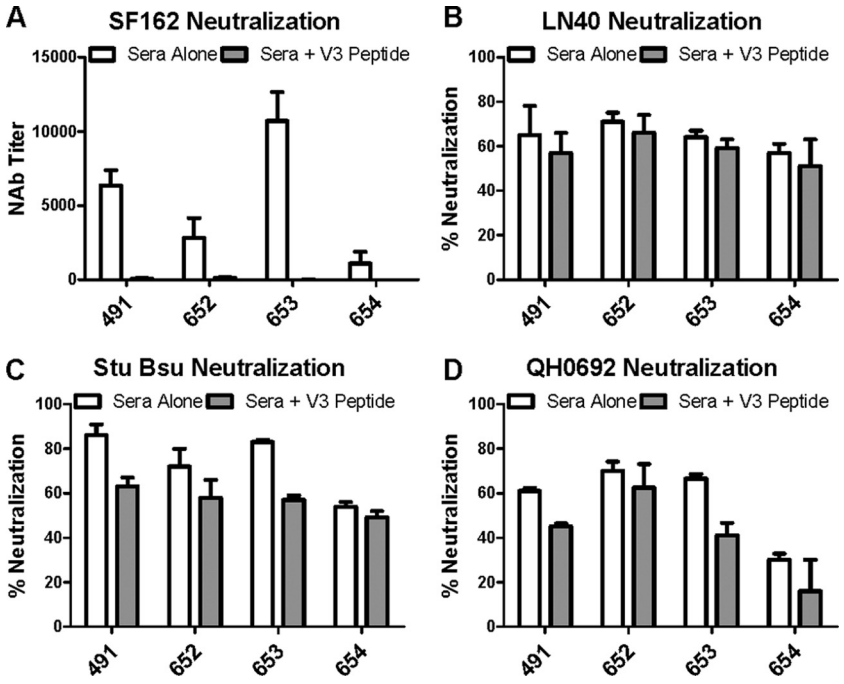


FIG. 6. Effects of V3 adsorption on the neutralization of homologous and heterologous viruses. Prior to incubation with virus, sera samples were added with a clade B consensus V3 peptide at 25 μg/ml to determine the role of V3-directed antibodies in the neutralization activity of sera from LN40-primed animals. (A) Effect of V3 adsorption on the neutralization of SF162. The *y* axis indicates the neutralizing antibody titer. (B) Effect of V3 adsorption on homologous LN40. The *y* axis indicates the percent neutralization at a 1:10 serum dilution. (C) Effect of V3 adsorption on chimeric Stu-Bsu virus. The *y* axis indicates the percent neutralization at a 1:10 dilution. (D) Effect of V3 adsorption on the neutralization of QH0692 at a 1:10 serum dilution.

the neutralizing activity against this B33 mutant relative to parental B33 virus (Table 3).

Additional mutations were created within known CD4 and b12 binding pockets to evaluate their effects on the neutralizing activities of our vaccine sera. Previous work implicated arginine (R) residue 373 and a glycan at asparagine (N)386 in mediating resistance to b12 in the parental LN40 virus (7). In the current study, R373 of parental LN40 Env was mutated to

TABLE 3. Neutralization of mutant isolates with sera from LN40-primed rabbits

Virus	Mutation(s)	% neutralization[a] at 1:10 dilution of sera in animal no.:				*P* value[b]
		491	652	653	654	
Parental WT						
LN40		**71**	**79**	**66**	**66**	(Control group)
B33		14	20	14	11	<0.001*
CD4bs (position 283)						
LN40 N	T283N	8	17	20	19	<0.001*
B33 T	N283T	**50**	**51**	21	23	<0.001*
b12 binding region						
LN40 K	R373K	**71**	**61**	**52**	42	0.09
LN40 D	N386D	**55**	**71**	**57**	46	0.13
LN40 V	T388V	**65**	**76**	**59**	**54**	1
LN40 KD	R373K, N386D	21	21	14	0	<0.001*
LN40 KV	R373K, T388V	22	27	20	8	<0.001*
Flanking CD4bs						
LN40 VKPS	I360V, N362K, Q363P, P364S	44	**64**	43	41	0.001*
V3 loop						
LN40 NL	H310N, F317L	**58**	**51**	41	22	<0.001*

[a] Values shown in bold indicate >50% neutralization.
[b] *P* values were determined by ANOVA followed by Bonferroni-adjusted multiple comparisons against the LN40 control group. *, significant difference.

the lysine (K) that is found at this position in B33 Env, to produce the mutant LN40K (R373K). In LN40, N386, as part of the NXT motif, is a potential N-linked glycosylation site, and two types of mutations were made to prevent the glycosylation. The first mutant (LN40D) was made to replace asparagine with aspartic acid to create the N386D mutant. The second mutant (LN40V) replaced the threonine at position 388 with a valine (T388V), which still eliminated the glycosylation motif while leaving the asparagine residue at position 386 intact. When these mutated Env antigens LN40 R373K, N386D, and T388V were evaluated for their effects on neutralization, only marginal reductions in neutralization were observed compared to the wild-type LN40 Env. In most instances, we observed less than a 10% reduction in neutralization against each of these mutants, with *P* values ranging from 0.09 to 1.00 (Table 3).

However, when these mutations were combined into one Env antigen, a dramatic effect on neutralization was recorded. With the combination mutants LN40KD (R373K plus N386D) and LN40KV (R373K plus T388V), neutralizing sensitivity to the sera of LN40 gp120 DNA vaccine-primed animals was almost completely lost ($P < 0.001$) (Table 3). Therefore, residues that were found to be critical in modulating resistance to b12 also appear to modulate resistance to the antibodies generated through immunization with LN40 gp120 antigen.

As further evidence pointed to antibodies targeted to the CD4 binding site as being the cause of the neutralizing activity in sera from LN40 gp120-primed animals, additional mutations that flank the CD4 binding loop were evaluated. Because this region is highly polymorphic, a combined mutation at four sites was produced, including I360V, N362K, Q363P, and P364S, to form the LN40-VKPS Env mutant. When this mutant was tested for its effect on neutralization, a less dramatic but still modulating role in mediating the neutralization sensitivity of the virus was observed, resulting in an average drop of 22% neutralization relative to the parental LN40 Env virus. The data presented above thus implicate sites within and proximal to the CD4bs as responsible for LN40 neutralization.

In order to further confirm that CD4 binding site antibodies were responsible for mediating NAb activities in sera from LN40 gp120 DNA vaccine-primed rabbits, a competition assay was done, as previously reported, against human MAb b12, which is specific for the CD4 binding site (4, 26). By using purified IgG from rabbit immune sera, b12-competing antibodies were detected in the sera of three of four LN40 gp120-primed rabbits 2.2214 to 2.492 μg/μl IgG to prevent 50% of virus binding to b12), but not in sera from B33 gp120-primed animals (less than 50% competition was observed in all four animals in the B33 group). This provided further evidence that unique specificities of antibodies were elicited in LN40 gp120-primed animals. This finding is consistent with the above results showing that mutations in the CD4 binding site resulted in a significant drop in functional NAb activities.

In order to provide a direct visual understanding on the relative locations of mutations produced in the current study, Fig. 7 highlights such mutations in an HIV-1 Env crystal structure model. The T283N mutation is right at the center of the CD4bs, which may explain the more dramatic effect of this mutation as tested in the current study. CD4bs-flanking and b12 contact residues are more peripheral, and thus only the combination of several mutations could lead to major changes in the neutralizing sensitivities. The V3 loop is more distant and only resulted in moderate reductions in neutralization when mutated, but it may function as another neutralizing target in susceptible viruses.

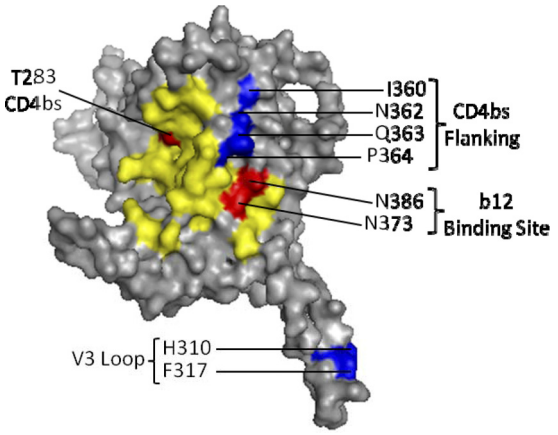


FIG. 7. Crystal structure of gp120 liganded with sCD4 and X5. Yellow residues indicate the binding footprint of MAb b12. Red positions indicate residues critical for neutralization of LN40 by autologous sera. Positions highlighted in blue indicate residues which either modulate, but do not ablate, neutralization of LN40 by autologous sera (CD4bs flanking) or are part of the V3 loop. Letters indicate the residue found at that position in LN40. Numbers indicate the position of that residue according to the HxBc2 numbering system.

DISCUSSION

Functional antibody is considered one of the best-characterized correlates of immune protection in licensed human vaccines that are used successfully in clinical practice (1). With regard to the development of an effective vaccine against HIV-1, eliciting protective antibodies, especially in the form of broadly neutralizing antibodies against a wide range of primary viral isolates, has been proven an exceptionally difficult task (3, 12, 25, 29). Previous attempts to utilize a traditional recombinant Env protein vaccine approach to elicit protection have failed in efficacy trials (10, 22), and the antibody responses in human volunteers recruited into these studies were unable to neutralize primary viral isolates (10, 11). Initially, it was thought that the monomeric gp120 was the culprit for this failure. Recent studies have indicated that the delivery approach may also play a critical role in determining the quality of antibody responses. In side-by-side comparison studies conducted in animals, DNA immunization to deliver an HIV-1 gp120 antigen is more effective in eliciting conformationally sensitive antibodies against Env and, thus, better neutralizing antibodies against primary viral isolates than recombinant gp120 protein (27). Furthermore, the sequential DNA prime-protein boost immunization approach was even more effective in expanding the breadth of neutralizing antibody responses (26, 33). Such findings were confirmed in a pilot human clinical study in which the DNA prime-protein boost vaccination approach was effective in eliciting broad NAb responses against a wide range of primary viral isolates (32), and further analysis showed that this approach was more effective than the protein-

alone approach in eliciting human antibody responses against conformational epitopes important for neutralization (28). The results from the recently completed RV144 clinical trial, which used a different, canarypox prime-gp120 protein boost approach, demonstrated partial protection against HIV acquisition in humans (23).

With such progress confirming the utility of gp120 antigens and the DNA prime-protein boost antigen delivery approach, it was critical to understand whether there is any difference regarding the immunogenicity of various primary HIV-1 gp120 antigens collected from HIV-1-infected patients and, if there is, to determine the structural basis for such a difference. The current study took advantage of two unique Env antigens derived from the same individual at the same time point of HIV-1 infection, thereby making them much more similar to each other than to any gp120 in a panel of heterologous isolates. Nevertheless, despite a close sequence relationship, these two envelopes differed profoundly in their biological properties (Table 1). It was not clear, though, whether such sequence homologies or their different properties would affect the capacity of these two Env proteins to behave similarly or differently as immunogens.

Dissection of the binding antibody response revealed very little difference between the LN40 and B33 gp120s when used in the priming phase of a DNA prime-protein boost regimen. High titers of cross-reactive binding antibodies were seen regardless of what was used as the priming immunization. However, in contrast to this similarity, the quality of the neutralizing antibody response differed quite significantly, suggesting functional antibodies do not always match binding antibody responses.

The current study is the first report in which one of a pair of closely related HIV-1 Env antigens has been demonstrated to be more effective than the other in priming broadly neutralizing antibody responses. This unique system allowed more specific epitope mapping to identify the critical sites that were responsible for the enhanced neutralization observed in the sera from LN40-primed animals. While the V3 loop-specific antibodies may be also involved in the neutralizing activities observed in the heterologous neutralizing activities in LN40 gp120-primed animals, the CD4 binding site of gp120, either at certain key CD4 contact residues or around the CD4 binding site, appeared to be at least a main target of the heterologous neutralizing activities elicited in sera from LN40 gp120-primed rabbits. These data are in agreement with other studies that have indicated that the CD4 binding site is a critical target recognized by sera from HIV-infected individuals with broadly neutralizing activities (2, 16, 24) and certain broadly neutralizing monoclonal antibodies (34).

Despite the importance of CD4bs antibodies, it has been difficult to elicit antibodies against this region because it is highly conformational, consisting of residues far apart in different segments of the HIV-1 gp120 sequence. In our recent studies, we found that DNA immunization, as a way to achieve better preservation of protein conformation due to its *in vivo* expression of antigens, was able to elicit unique serum antibodies that could compete against the CD4bs-targeting monoclonal antibody b12 in both rabbits and humans (26–28). The current results further indicated that selection of the gp120 antigen is also important, because LN40 gp120-primed animals had b12 competing antibodies that were not detected in B33 gp120-primed animals. At the same time, sera from LN40-primed animals were able to neutralize heterologous viral isolates, while B33 could not. Mutations at key residues involved in or around the CD4 binding site proved that the sequence difference between LN40 gp120 and B33 gp120 may be responsible for the different NAb profiles between these two Env antigens. Findings from the current study provide useful information for future studies for confirming whether the similar sequence determinants may also be observed in more primary Env sequences, which will determine their neutralizing sensitivities or immunogenicities to elicit better NAb responses.

One unexpected finding was that the LN40 Env, with a low affinity to CD4, was able to elicit heterologous NAbs, while the high-CD4-affinity B33 Env was not a very good immunogen. This result implies that either high CD4 affinity may not be equal to more opened access to CD4bs or that easy access to this region may not necessarily lead to higher conformation-based antibody responses directed toward this region. In fact, if the LN40 Env has a less-open CD4 binding site structurally and this more-limited access promotes the generation of neutralizing antibodies to this domain, our data suggest that having more limited access could "direct" the antibody immune response to this domain, which is more relevant for antibodies with neutralizing activity. If this is the case, instead of previous efforts to make the CD4 binding site more available through elimination of glycosylation sites and variable loops, we may instead want to look at modifications that reduce, but not preclude entirely, access of CD4 to this domain. This may limit the number of nonneutralizing antibodies being elicited to the CD4bs while selectively promoting the expansion of B cells that have specificities to neutralizing sites within the CD4 binding domain.

The finding that a low-CD4-affinity Env (LN40) is a better immunogen also reminded us of previous reports that CD4-independent Env antigens from at least two studies were found to be good immunogens for the induction of neutralizing antibodies (15, 35). Further studies should examine if there is a general pattern that low-CD4-affinity Env and CD4-independent Env may entail a better chance to act as good immunogens to elicit broad heterologous NAb responses. More importantly, a more complete understanding of the structural basis for such better immunogenicity is needed in order to guide the creation of optimal Env antigens that can be used for vaccine development.

ACKNOWLEDGMENTS

This study was funded in part by NIH grants R01AI065250, P01AI082274, U19AI082676, and R01MH064408.

We thank Jill M. Grimes Serrano for critical reading and editing of the manuscript.

REFERENCES

1. **Amanna, I. J., N. E. Carlson, and M. K. Slifka.** 2007. Duration of humoral immunity to common viral and vaccine antigens. N. Engl. J. Med. **357:**1903–1915.
2. **Binley, J. M., et al.** 2008. Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. J. Virol. **82:**11651–11668.
3. **Burton, D. R., and R. A. Weiss.** 2010. A boost for HIV vaccine design. Science **329:**770–773.
4. **Crooks, E. T., et al.** 2007. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. Virology **366:**245–262.

5. **Derby, N. R., et al.** 2006. Antibody responses elicited in macaques immunized with human immunodeficiency virus type 1 (HIV-1) SF162-derived gp140 envelope immunogens: comparison with those elicited during homologous simian/human immunodeficiency virus SHIVSF162P4 and heterologous HIV-1 infection. J. Virol. **80:**8745–8762.
6. **Duenas-Decamp, M. J., and P. R. Clapham.** 2010. HIV-1 gp120 determinants proximal to the CD4 binding site shift protective glycans that are targeted by monoclonal antibody 2G12. J. Virol. **84:**9608–9612.
7. **Duenas-Decamp, M. J., P. Peters, D. Burton, and P. R. Clapham.** 2008. Natural resistance of human immunodeficiency virus type 1 to the CD4bs antibody b12 conferred by a glycan and an arginine residue close to the CD4 binding loop. J. Virol. **82:**5807–5814.
8. **Duenas-Decamp, M. J., P. J. Peters, D. Burton, and P. R. Clapham.** 2009. Determinants flanking the CD4 binding loop modulate macrophage tropism of human immunodeficiency virus type 1 R5 envelopes. J. Virol. **83:**2575–2583.
9. **Dunfee, R. L., et al.** 2006. The HIV Env variant N283 enhances macrophage tropism and is associated with brain infection and dementia. Proc. Natl. Acad. Sci. U. S. A. **103:**15160–15165.
10. **Flynn, N. M., et al.** 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J. Infect. Dis. **191:**654–665.
11. **Gilbert, P. B., et al.** 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J. Infect. Dis. **191:**666–677.
12. **Johnston, M. I., and A. S. Fauci.** 2008. An HIV vaccine: challenges and prospects. N. Engl. J. Med. **359:**888–890.
13. **Kwong, P. D., et al.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659.
14. **Li, M., et al.** 2005. Human immunodeficiency virus type 1 Env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125.
15. **Li, Y., et al.** 2008. Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses. J. Virol. **82:**638–651.
16. **Li, Y., et al.** 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat. Med. **13:**1032–1034.
17. **Lu, S., J. M. Grimes Serrano, and S. Wang.** 2010. Polyvalent HIV vaccines. Current HIV Res. **8:**622–629.
18. **Montefiori, D. C.** 2004. Evaluating neutralizing antibodies against HIV, SIV and SHIV in luciferase reporter gene assays, p. 1–15. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margullies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology, vol. 12. John Wiley, Hoboken, NJ.
19. **Peters, P. J., et al.** 2004. Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. J. Virol. **78:**6915–6926.
20. **Peters, P. J., et al.** 2008. Variation in HIV-1 R5 macrophage-tropism correlates with sensitivity to reagents that block envelope: CD4 interactions but not with sensitivity to other entry inhibitors. Retrovirology **5:**5.
21. **Peters, P. J., et al.** 2006. Non-macrophage-tropic human immunodeficiency virus type 1 R5 envelopes predominate in blood, lymph nodes, and semen: implications for transmission and pathogenesis. J. Virol. **80:**6324–6332.
22. **Pitisuttithum, P., et al.** 2006. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J. Infect. Dis. **194:**1661–1671.
23. **Rerks-Ngarm, S., et al.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. **361:**2209–2220.
24. **Scheid, J. F., et al.** 2009. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature **458:**636–640.
25. **Stamatatos, L., L. Morris, D. R. Burton, and J. R. Mascola.** 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? Nat. Med. **15:**866–870.
26. **Vaine, M., et al.** 2008. Improved induction of antibodies against key neutralizing epitopes by HIV-1 gp120 DNA prime-protein boost vaccination compared to gp120 protein only vaccination. J. Virol. **82:**7369–7378.
27. **Vaine, M., S. Wang, A. Hackett, J. Arthos, and S. Lu.** 2010. Antibody responses elicited through homologous or heterologous prime-boost DNA and protein vaccinations differ in functional activity and avidity. Vaccine **28:**2999–3007.
28. **Vaine, M., et al.** 2010. Profiles of human serum antibody responses elicited by three leading HIV vaccines focusing on the induction of Env-specific antibodies. PLoS One **5:**e13916. doi:10.1371/journal.pone.0013916.
29. **Walker, B. D., and D. R. Burton.** 2008. Toward an AIDS vaccine. Science **320:**760–764.
30. **Wang, S., et al.** 2005. Enhanced immunogenicity of gp120 protein when combined with recombinant DNA priming to generate antibodies that neutralize the JR-FL primary isolate of human immunodeficiency virus type 1. J. Virol. **79:**7933–7937.
31. Reference deleted.
32. **Wang, S., et al.** 2008. Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine **26:**1098–1110.
33. **Wang, S., et al.** 2006. Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. Virology **350:**34–47.
34. **Wu, X., et al.** 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science **329:**856–861.
35. **Zhang, P. F., et al.** 2007. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. Proc. Natl. Acad. Sci. U. S. A. **104:**10193–10198.