

**Levels of N-linked Glycosylation on the V1 Loop of HIV-1 Env Proteins  
and Their Relationship to the Antigenicity of Env from Primary Viral Isolates**

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## ABSTRACT

A good understanding about the structure and function of the envelope glycoprotein (Env) from primary human immunodeficiency virus-1 (HIV-1) isolates is important in facilitating the development of effective neutralizing antibody responses as a component of an effective HIV-1 vaccine. In the current study, the antigenicity of a panel of diverse HIV-1 primary Env from different clades of HIV-1 Group M was analyzed using rabbit sera produced by either 3- or 9-valent gp120 DNA vaccine formulations. Both the 3- and 9-valent gp120 DNA vaccine formulations elicited HIV-1 gp120-specific antibodies in immunized rabbits. However, we observed two levels of primary envelope antigenicity to the same set of rabbit immune sera and that the level of glycosylation, particularly in the V1 loop, may contribute to such diversity. Bioinformatics analysis on the distribution and average number of the N-linked glycosylation sites in all variable regions (V1–V5) was conducted. A linear plot demonstrated that the average number of potential N-glycosylation sites in the V1 and V4 loops correlates to the size of the loop. These data provide further evidence on the complexity of primary HIV-1 Env antigens and offers new insight into the mechanisms that HIV-1 uses to escape protective immune responses.

## **LIST OF ABBRIVIATIONS**

CD4i	CD4 induced epitopes
CMI	cell-mediated immune
CTL	cytotoxic T lymphocytes
Env	envelope glycoprotein
ELISA	Enzyme-linked immunosorbent assay
HIV	human immunodeficiency virus
mAb	monoclonal antibody
NAb	neutralizing antibody
NZW	New Zealand White
SIV	simian immunodeficiency virus
TCLA	T-cell line adapted
tPA	tissue plasminogen activator

## **INTRODUCTION**

Since scientists first reported on clinical evidence of acquired immunodeficiency syndrome or AIDS in 1981, this epidemic has spread rapidly throughout the world. An estimated 33 million people are infected with human immunodeficiency virus-1 (HIV-1), the virus that causes AIDS. In 2006, approximately 4.3 million people became newly infected with HIV and approximately 2.9 million people died of AIDS-related illnesses worldwide [1]. Despite years of research, the development of a safe and effective vaccine to prevent the transmission of HIV-1 remains a major challenge [2, 3].

Typically, in order for a vaccine to be successful, it should elicit both humoral and cellular immune responses. While the humoral response yields virus-neutralizing antibodies to prevent viral particles from infecting new cells, the cell-mediated immune (CMI) response mobilizes specific cytotoxic T lymphocytes (CTL) to target and kill cells that express viral antigens. By using non-human primate models, it has been suggested that anti-HIV CTL could play an important role in suppressing viral load after viral challenge [4-6]. It remains unknown whether this CTL-induced suppression is sufficient to prevent transmission of HIV in high risk human populations, especially in light of the recent setback with the STEP study [7]. Historically, antibody responses have proven critical in inducing protective immunity against a wide range of infectious diseases. Despite efforts in the last two decades, however, there has been little progress in generating broadly cross-reactive neutralizing antibodies through immunization against HIV-1. This is likely due to the diversity of the HIV-1 subtypes and high frequency of mutations in HIV-1 [8]. Extensive studies on the diversity of HIV-1 subtypes isolated

from different regions of the world have classified HIV-1 into three major groups, designated as M (Main) [9-15], O (outlier) [16], and N (Non-M, Non-O or New) [17]. Group M, which comprises the majority of HIV-1 isolates, can be further divided into genetic subtypes or clades, designated as A, B, C, D, F, G, H, J and K [10, 18-21]. However, there are other mechanisms that may also contribute to the failure of inducing potent protective antibodies against HIV-1, including the low immunogenicity of HIV-1 Env protein as shown in clinical trials [22].

In addition, HIV-1 Env is a critical viral protein in allowing HIV-1 to evade humoral immune responses, thereby permitting the virus to establish a persistent infection within the host. The gp120 core of HIV-1 Env from both primary isolates and laboratory-adapted HIV-1 (TCLA) strains is very similar as their sequence variability is concentrated in the variable regions (V1–V5) of Env. These variable regions appear to be major targets for neutralizing antibody (NAb) responses [23], and are considered essential in the neutralization resistance of HIV-1 primary isolates. Many HIV-1 vaccines have been designed to target the V3 region, one of the principal neutralizing domains. However, the V3 region is frequently masked by other Env structures including high levels of glycosylation.

Studies examining simian immunodeficiency virus (SIV) have provided critical evidence showing that glycosylation in the V1/V2 region of the SIV<sub>mac</sub> Env protein is critical for immune evasion by SIV infection in non-human primates [24, 25]. It has been proposed that the removal of major variable loops, such as V1/V2 and V3, on HIV Env

would expose the core structure of HIV-1 Env [26-30]. Previously, we tested the immunogenicity of DNA vaccines that express mutant Envs (dV123) that have the V1/V2 and V3 regions deleted [26]. As expected, dV123 DNA vaccines elicited higher antibody binding responses when compared to their wild type counterparts. Unfortunately, no improvement in neutralization activities was observed in immune sera [26] as it is possible that the deletion of these variable regions of HIV-1 Env is too drastic to keep the sensitive neutralizing epitopes intact. Recently, a deletion of the V2 loop on HIV-1 isolate, HIV<sub>SF162</sub>, made it more susceptible to neutralization [31, 32]. Both rabbits and macaques inoculated with the HIV<sub>SF162</sub> Env gp140 antigens with the deleted V2 domain, through either DNA and protein immunization, produced higher NAb responses than those who received the wild type HIV<sub>SF162</sub> gp140 [33]. It remains to be seen whether a V2-deletion can be equally effective when applied to additional HIV primary Env antigens. Information is still limited regarding the structure-function relationship for primary HIV-1 envelope antigens, especially for those non-clade B patient isolates.

The current report is a pilot study on the antigenicity of a panel of diverse HIV-1 primary gp120 glycoproteins from different clades of Group M HIV-1 isolates. Data from this study indicates that different levels of antigenicity among primary HIV-1 Env exist and that levels of glycosylation, particularly in the V1 loop region, may contribute to such diversity. Computer analysis of all the available gp120 protein sequences of HIV-1 from the HIV Los Alamos database (<http://www.hiv.lanl.gov/>) further demonstrated a strong correlation between the size of the V1 loop and the number of potential glycosylation sites. These data improve our understanding of the structure-function relationship of HIV-1 Env

antigens from primary viral isolates and may facilitate the development of HIV vaccines that induce broad NAb responses.

## MATERIALS AND METHODS

**Construction of DNA plasmids expressing HIV-1 gp120 Env from clade A-G primary isolates.** The DNA vaccine inserts coding for the extracellular portion of seven representative clades from the nine primary HIV-1 gp120 Env clades A through G were first PCR amplified from the full-length *env* genes with the high fidelity PFU polymerase, as previously reported [26] (Fig. (1)). A pair of consensus PCR primers was designed to amplify all nine of the different gp120 genes: the plus strand primer GP120-p-f1 (5' p-cttggtgggtcacagtctattatgggggtacc 3') and the minus strand primer GP120-p-b1 (5' ggtcggatccttactccaccactcttctctttgcc 3'). The PCR products with 5' blunt-end were then digested with BamHI to create a cohesive 3' end before ligation into the DNA vaccine vector pJW4303 [34, 35]. The pJW4303 vector was prepared by NheI digestion, followed by treatment with Klenow fragment to blunt the end and then cut with BamHI. The NheI site was regenerated after ligation with the Env inserts and in-frame with the tissue plasminogen activator (tPA) leader sequence in pJW4303. The DNA vaccine plasmids were grown in *E. coli* HB101 and their expression was confirmed by Western blot analysis using transiently expressed gp120 from 293T cells (see below). The DNA vaccine constructs are referred to as pJW4303/A1-, A2-, B-, C1-, C2-, D-, E-, F- and G-gp120 (Fig. (1) and Table 1).

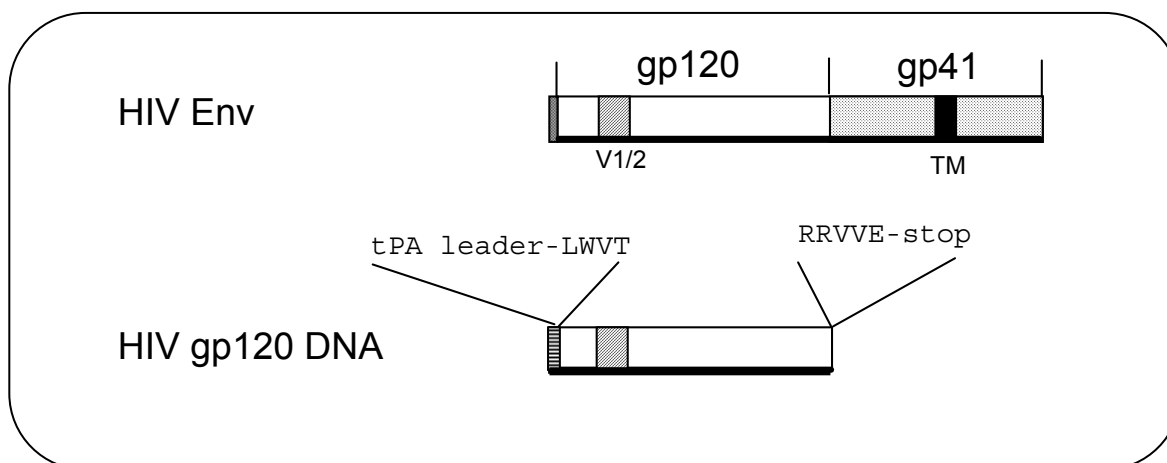


Fig. (1) A schematic diagram of the gp120 DNA vaccine insert cloned into the vector, pJW4303, after the tPA leader sequence. The amino acid sequences of Env at both N- and C-terminal junctions are shown in single letter code. V1/2: the combined V1/V2 loop; TM: transmembrane domain of HIV Env.

Table 1. List of primary HIV-1 isolates

Env DNA vaccines	Primary HIV-1 envelope clone	Sequence subtype <sup>a</sup>	GeneBank accession no	Sex of patient	Country	Risk factor <sup>b</sup>	Disease status <sup>c</sup>	Antiviral therapy	Biological phenotype <sup>d</sup>	Coreceptor usage
A1	92RW020.5	A	U08794	F	Rwanda	Het	AS	No	NSI	CCR5
A2	92UG037.8	A	U09127	F	Uganda	Het	AS	No	NSI	CCR5
B	92US715.6	B	U08451	M	U.S.	IVDU	AS	AZT	NSI	CCR5
C1	92BR025.9	C	U09126	M	Brazil	Hemo	AS	No	NSI	CCR5
C2	93MW965.26	C	U08455	F	Malawi	Het	AS	No	NSI	CCR5
D	92UG021.16	D	U27399	F	Uganda	Het	AS	No	SI	CXCR4
E	93TH976.17	"E"	U08458	M	Thailand	Het	AS	No	NA	CCR5
F	93BR020.17	F	U27401	M	Brazil	Bi	AS	No	SI	NA
G	92UG975.10	"G"	U27426	F	Uganda	Het	AIDS	AZT	NSI	CCR5

**NOTE**

a Quotation marks indicate the mosaic nature of subtypes E and G envelope genes.

b Het, heterosexual contact; IVDU, intravenous drug use; Hemo, hemophilic patient; Bi, bisexual.

c Disease status at the time of viral isolation. AS, asymptomatic.

d SI, syncytium inducing; NSI, non-syncytium inducing.

***In vitro* expression of primary gp120 HIV Env antigens by DNA vaccines.** The expression of the primary Env antigens from DNA vaccine plasmids was examined in transiently transfected 293T cells [36]. 293T cells were transfected at approximately 50% confluence on 60 mm plates with calcium phosphate co-precipitated with 10 µg of plasmid

DNA. The transfected cells were harvested 72 hours post-transfection. The expressed Env proteins were then used for ELISA or Western blot analysis.

**DNA Immunization.** Female New Zealand White (NZW) rabbits of 6-8 weeks old (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 USDA regulations and IACUC approved protocol. The animals received three monthly DNA immunizations by a Bio-Rad Helios gene gun (Bio-Rad, Hercules, CA). The Env-expressing DNA plasmids or the pJW4303 vector plasmid were coated on to the 1.0 micron gold beads at a ratio of 2 µg of DNA/mg of gold, as previously reported [37]. Rabbits were anesthetized, according to an IACUC approved protocol, and their abdominal skin was shaved prior to gene gun inoculation. Each shot delivered 1 µg of DNA, and a total of 36 non-overlapping shots were delivered to each rabbit. Three rabbits in the Group 1 (R31-R33) were immunized with three different primary Env-expressing DNA vaccines with the same amount of DNA plasmid for each DNA vaccine construct. In Group 2, two rabbits (R34 and R35) were inoculated with all nine gp120-expressing DNA vaccines (clades A to G). Two rabbits in Group 3 served as the negative control and received only the pJW4303 vector plasmid (Table 2). Serum samples were collected immediately before and 4 weeks after each immunization.

Table 2. Rabbit groups immunized with various polyvalent DNA vaccines expressing primary gp120s

Rabbits	gp120 DNA vaccines	No. of vaccines	Amount per DNA vaccine	Total amount
<i>Group One</i>				
R31	A1, B, C1	3	12 µg	36 µg
R32	A2, C2, D	3	12 µg	36 µg
R33	E, F, G	3	12 µg	36 µg
<i>Group Two</i>				
R34	A1to G	9	4 µg	36 µg
R35	A1to G,	9	4 µg	36 µg
<i>Group Three</i>				
R29	Vector only	1	36 µg	36 µg
R30	Vector only	1	36 µg	36 µg

**ELISA (Enzyme-linked immunosorbent assay).** Rabbit sera were tested for Env-specific IgG antibody responses by ELISA. Microtiter plates were coated with ConA (5 µg per well) for 1 hour and then washed 5 times with washing buffer (1XPBS at pH 7.2 with 0.1% Triton X-100). Transiently expressed Env antigens at 1 µg/ml were added (100 µl for each well) and incubated for 1 hour at room temperature. Blocking was done with 200 µl/well of 4% milk-whey blocking buffer for 1 hour at room temperature. After five washes, 100 µl of serially diluted rabbit sera were added and incubated for 1 hour. The plates were then washed five times and incubated with 100 µl of biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted at 1:1000 for 1 hour. Then, horseradish peroxidase conjugated streptavidin (Vector Laboratories) diluted at 1:2000 was added (100 µl /well) and incubated for 1 hour. After the final washes, 100 µl of fresh TMB substrate (Sigma, St. Louis, MO) was added per well and incubated for 3.5 min. The reaction was stopped by 25 µl of 2 M H<sub>2</sub>SO<sub>4</sub>, and the plates were read at the optical density (OD) of 450 nm.

**Western blot.** The Env antigens transiently expressed from 293T cell supernatants and cell lysate were subjected to SDS-PAGE and blotted onto PVDF membrane. Blocking was done with 0.1% I-Block (Tropix, Bedford, MA). The immunized rabbit sera were used as detecting antibodies at 1:500 dilution and incubated for 45 minutes. Subsequently, the membranes were washed with blocking buffer and then reacted with AP-conjugated goat anti-rabbit (Tropix) at 1:5000 dilution. After the final washes, Western-light substrate was applied to the membranes for 5 minutes (Tropix). Once the membranes were dried, Kodak films were exposed to the membrane and developed by an X-OMAT processor.

**Env protein sequence and glycosylation analysis.** The sequence variability of the N-linked glycosylation sites was analyzed by searching potential glycosylation sites on all five major variable loops of HIV-1 Env. We first downloaded complete HIV-1 gp120 sequences from the Los Alamos HIV Database (<http://www.hiv.lanl.gov/>) and obtained 4084 sequences in total (as of December 16, 2006) and then performed multiple sequence alignment using CLUSTALW (version 1.8). We then analyzed the resulting alignments and obtained the boundaries of the variable regions (V1 to V5) from their neighboring constant regions. Programs were written to perform text extraction of each of the five variable regions, and to compute the number of glycosylation sites for each existing length and their average. The programs in Java are available upon request to the corresponding author. The distribution and average number of glycosylation sites from each variable region have been studied by linear correlation plots.

## RESULTS

**Construction of DNA vaccines expressing primary envelope glycoproteins and DNA immunization of NZW rabbits.** In the current study, nine primary HIV-1 envelope glycoproteins were randomly selected from 7 different subtypes (clades A to G) of HIV-1 Group M (Table 1) isolated from one of the early sets of primary HIV-1 Env clones reported in literature [18]. Due to the large and growing population of patients infected with clades A and C, two isolates were included from each of these two clades.

The gp120 gene inserts were PCR amplified from the full-length *env* genes with the high fidelity PFU polymerase and subcloned into the DNA vaccine vector pSW4303 vector, as previously reported [38] (Fig. (1)). The ability of the DNA vaccines to express gp120 antigens was confirmed in transiently transfected 293T cells before being used to immunize the animals (data not shown).

The immunogenicity of primary HIV gp120 3- or 9-valent DNA vaccine formulations were studied in NZW rabbits (Table 2). Each animal received 3 monthly DNA immunizations by a gene gun at Weeks 0, 4, and 8. The total amount of gp120 DNA at each immunization was fixed at 36  $\mu\text{g}$ , therefore, the dose for each individual gp120 DNA plasmid in the 3- and 9-valent formulations was 12 $\mu\text{g}$  and 4 $\mu\text{g}$ , respectively, for each plasmid. The control group received an empty DNA vaccine vector, following the same immunization schedule.

**Antigenicity analysis of primary HIV-1 gp120 proteins by ELISA.** Peak level antibody responses in immunized rabbit sera collected four weeks after the last (third) DNA immunization were analyzed by ELISA. In order to make sure that the amount of coating gp120 antigen used throughout the assay was comparable for each gp120 antigen, the quantity of each primary Env antigen coated onto each well was first determined by a titration assay using pooled sera against a standard curve of a known amount of recombinant Env antigens (data not shown) and a same amount of Env antigen was used for each ELISA experiment.

Positive gp120-specific IgG was identified by ELISA in both the 3- and 9-valent gp120 DNA vaccine groups against the primary HIV-1 Env antigens (see below). Serially diluted sera from each rabbit were tested against various *in vitro* expressed HIV-1 gp120 antigens from 293T cells, as previously reported [39]. Interestingly, we found that the antigenicity of primary gp120 Env antigens used in this study can be divided into two groups based on their levels of reactivity with the same set of rabbit sera. Five primary gp120 antigens (A1, A2, C1, D and G), as coated on the ELISA plates, showed high reactivity with five of the DNA immunized rabbit sera (R31 to R35 which includes both 3- and 9-valent sera), irrespective of the gp-120 that was included in the DNA formulation (Fig. **(2A)**). However, the other four primary gp120 antigens (B, C2, E and F) reacted at much lower levels with the same five rabbit sera (Fig. **(2B)**) even though the same serum dilution (1:1000) was used in all of these assays. End-point titration ELISAs showed titers ranging from 1:4000 ~ 1:16,000 for Env antigens B, C2, E and F, and 1:32,000 ~ 1:64,000 for Env antigens A1, A2, C1, D and G.

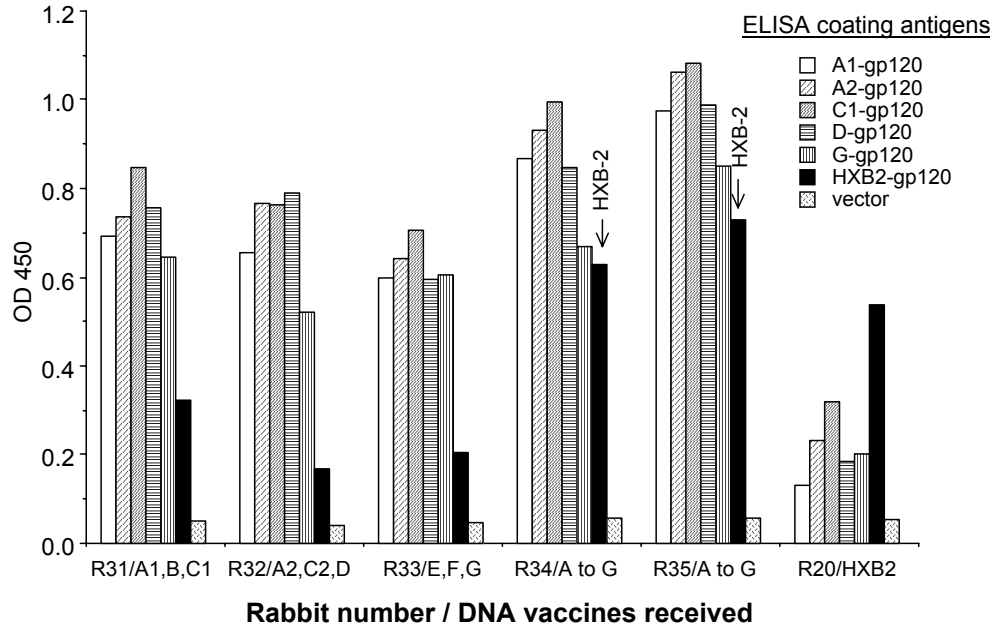
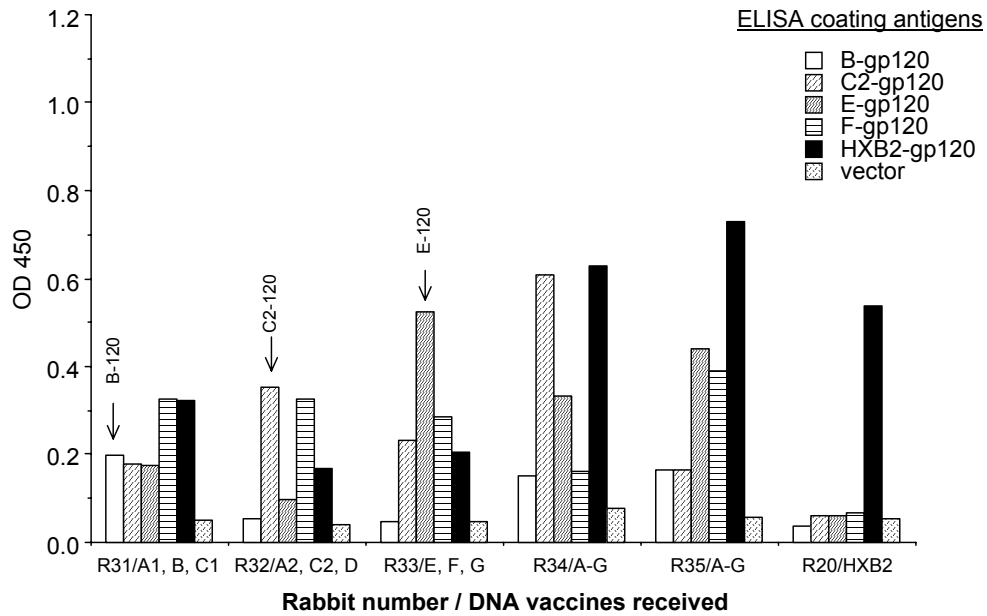
**A****B**

Fig. (2) ELISA reactivity of individual primary gp120 antigens to rabbit sera inoculated with various polyvalent gp120 DNA vaccines. Individual rabbit sera are referred to as R31-R35 and R20 and the DNA vaccine each rabbit was immunized with is denoted after the animal number and referred to as A1, C, C1, C2, D, E, F, G and the TCLA HIV-1<sub>HXB2</sub> strain. Therefore, Rabbit #31 was immunized with A1-gp120, B- and C1- (R31/A1, B, C1). (A) Highly reactive primary gp120 antigens: A1, A2, C1, D, G along with HIV<sub>HXB2</sub> and vector control. (B) Poorly reactive primary gp120 antigens: B, C2, E, F with HIV<sub>HXB2</sub> and vector control. The arrows mark the homologous capture antigens that were included in the immunization formulations. The OD values were the average of multiple wells in an ELISA measurement.

These data suggest that there is difference in antigenicity among primary HIV-1 envelope glycoproteins as measured in a solid phase antibody assay. Nine primary gp120 antigens were then ranked based on their average antigenicity against the five rabbit sera (R31-R35) included in the study showing a significant difference in antigenicity for Env proteins A1, A2, C1, D and G compared to B, C2, E and F (Fig. (3)).

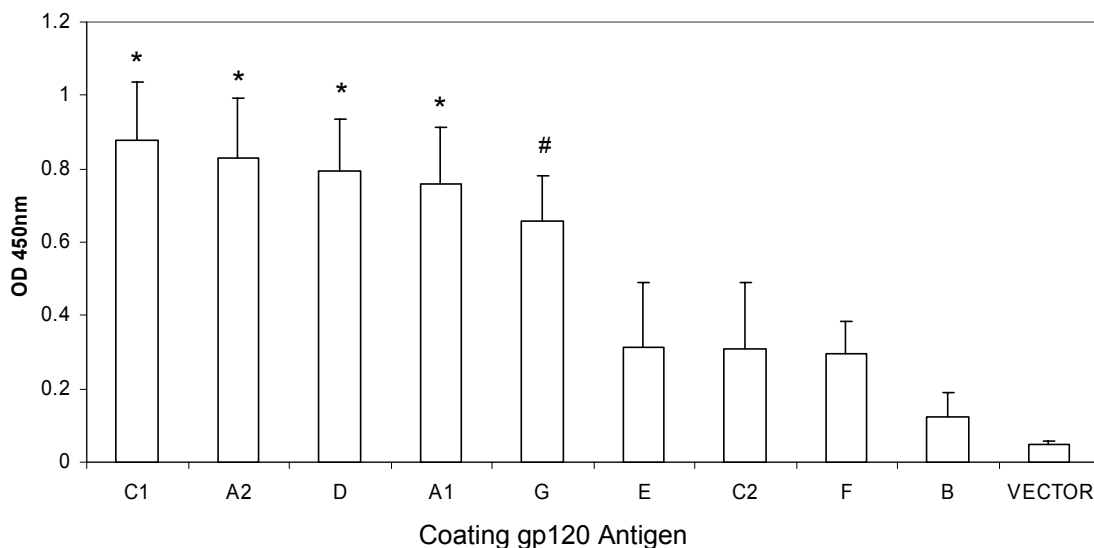


Fig. (3) Summary of gp120 antigen-specific responses to sera from rabbits immunized with different gp-120 DNA vaccines. Individual gp120 (A1, A2, B, C1, C2, D, E, F and G) antigens were evaluated for their reactivity to sera from every rabbit (R31-R35) as determined by ELISA. These gp120 antigens are ranked by the average activity of the pooled rabbit sera ( $\pm$  standard deviation) and presented in this figure from high (left) to

low (right). \* or # indicate statistical difference ( $p < 0.05$ ) compared with gp120 antigens B, C2, E and F or with gp120 antigens B and F, respectively.

It is clear that although the overall levels of antigenicity were reduced, the low reactive gp120 antigens reacted best with rabbit serum raised with the polyvalent DNA vaccines containing the homologous antigen (Fig. **(2B)**, marked by arrows). For example, B-gp120 antigen reacted best with serum from R31, which received the 3-valent DNA vaccine containing the B-gp120 antigen, when compared to sera from rabbits not vaccinated with a B-gp120 antigen vaccine (i.e., R32 and R33) (Fig. **(2B)**). Similarly, C2-gp120 reacted well with serum from R32, and E-gp120 reacted well with serum from R33. However, sera from rabbits that received the 9-valent DNA vaccine formulation (R34 and R35) displayed various reactivity toward each individual gp120 antigen (Fig. **(2B)**), probably reflecting cross reactivity against multiple antigens in the 9-valent sera.

The specificity of sera from rabbits immunized with the polyvalent gp120 DNA vaccine formulations was further demonstrated by their poor reactivity against the Env antigen from the TCLA HIV-1 isolate, HIV<sub>HXB2</sub>. While the HIV<sub>HXB2</sub>-gp120 antigen reacted strongly with serum from autologous HIV<sub>HXB2</sub>-gp120 immunized rabbit, R20, which was prepared in a separated study using the same DNA immunization schedule including dosing, it reacted only moderately with sera from rabbits R31-R33, which received the 3-valent vaccine (Fig. **(2A)**). Further confirming the poor reactivity between Env antigens from TCLA and primary isolates, serum from R20 (HIV<sub>HXB2</sub>-gp120 immunized) recognized its autologous HIV<sub>HXB2</sub>-gp120 antigen, but only moderately

recognized the gp120s from the primary viral isolates (Figs. **(2A)** & **(2B)**), confirming previous findings that anti-gp120 antibody responses are strain-specific [40, 41].

It is worth noting that the sera from 9-valent immunized rabbits (R34 and R35) showed improved reactivity against the HIV<sub>HXB2</sub>-gp120 Env antigen than observed with the sera from 3-valent immunized rabbits and even to a greater degree than was observed for the sera from the HIV<sub>HXB2</sub>-gp120 immunized rabbit (compare R34 and R35 vs. R20) (Figs. **(2A)** & **(2B)**). Our ELISA data showed that overall, the 9-valent rabbit sera (R34 and R35) had a higher reactivity when compared to the 3-valent sera (R31-R33) against the same sets of coating Env antigens A1, A2, C1, D, G, HXB2 (Fig. **(2A)**) or B, C2, E, F, HXB2 (Fig. **(2B)**).

**Levels of glycosylation may affect the antigenicity of primary gp120 proteins.**

The reactivity of primary gp120 antigens was further examined by Western blot analysis. Each individual antigen, harvested from 293T cells transiently transfected with primary gp120 DNA vaccines, were probed by immunized rabbit sera. We found that the Western blot analysis was in agreement with the ELISA data. One typical sample is shown in Fig. **(4)**. Highly reactive antigens (e.g. A2-gp120 & C1-gp120) showed a strong band pattern and poorly reactive antigens (e.g. B-gp120) showed a weak band pattern (Fig. **(4A)**). In order to determine if the low reactivity of B-gp120 was the result of heavy glycosylation of the gp120 antigen, B-gp120 protein was treated with Endo F (N-Glycosidase F deglycosylation kit, Boehringer Mannheim). After Endo F treatment, all of the gp120 proteins migrated to a lower position during electrophoresis than their untreated gp120

counterparts, as shown in Fig. (4B). The downshift in molecular weight verifies that the glycans had been removed from the gp120 protein, as a result of treatment with Endo F. A stronger band for the deglycosylated form of B-gp120 was discovered when compared to the fully glycosylated B-gp120, suggesting that glycosylation may have masked certain antigenic determinants on this HIV-1 Env protein. A similar pattern was observed for another low reacting antigen from our ELISA assay, C2-gp120 (Fig. (4B)). In contrast, removal of glycans from A2-gp120, a highly reactive antigen, as indicated by the ELISA (Fig. (2A)), did not result in a significant increase in its band intensity with rabbit immune serum, despite confirmation of deglycosylation, as shown by a decrease of in its molecular weight (Fig. (4B)). The relative density of gp120 bands on Western-blot as measured by the Kodak EDAS 290 system were calculated using A2 as 100% (Fig (4)).

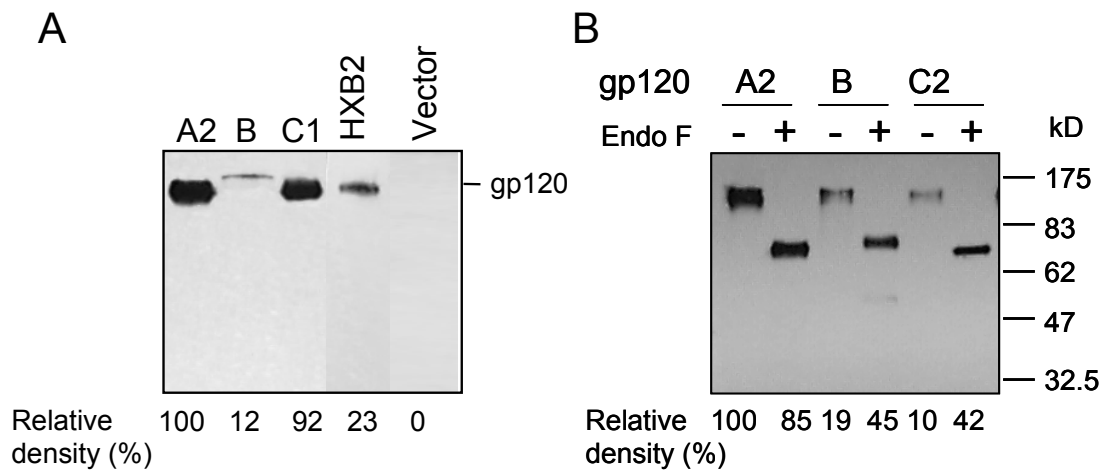


Fig. (4) (A) Western blot analysis of equal amounts of primary gp-120 Env antigens (A2, B and C1) from transiently transfected 293T cells, the TCLA HIV-1<sub>HXB2</sub>-gp120 and vector control were probed with anti-HIV sera from Rabbit serum #34 elicited with the 9-valent gp120 A through G DNA vaccine. (B) Western blot analysis of equal amounts of primary gp120 Env antigens (A2, B, C2) before (-) and after (+) the removal of N-linked glycosylations by Endo F. The relative density of gp120 bands as measured by the Kodak EDAS 290 system were calculated by using A2 as 100%.

**The number of potential N-linked glycosylation sites is correlated to the size of the V1 loop on primary HIV-1 envelope glycoproteins.** When the distribution of total potential N-linked glycosylation sites on nine primary HIV-1 gp120 glycoproteins included in our study were analyzed, we found that the V1 loop was the most variable region with the number of variations ranging from 3 to 7 sites. The second most variable region was the V4 loop with 3 to 6 sites while the remaining regions varied by only 1 or 2 glycosylation sites among the primary Env antigens included in this study (Table 3). Interestingly, 3 of the 4 low antigenic gp120 antigens (B, C2 and E) had higher numbers of N-glycosylation sites within the V1 region (6, 5 and 7 sites, respectfully) than observed in the highly reactive antigens (Table 3).

Table 3 Distribution of N-linked glycosylation sites on HIV-1 gp120 glycoproteins

HIV-1 isolates		Distribution of potential N-glycosylated sites in HIV-1 gp120										
<u>Clade</u>	<u>Env clone</u>	<u>C1</u>	<u>V1</u>	<u>V2</u>	<u>C2</u>	<u>V3</u>	<u>C3</u>	<u>V4</u>	<u>C4</u>	<u>V5</u>	<u>C5</u>	<u>total</u>
A1	92RW020.5	1	3	2	6	1	2	6	1	2	0	24
A2	92UG037.8	1	4	3	7	1	3	4	1	1	0	25
B	92US715.6	2	<b>6</b>	2	7	1	4	4	1	2	0	29
C1	92BR025.9	1	3	3	8	1	2	4	1	0	0	23
C2	93MW965.26	1	<b>5</b>	3	6	1	3	4	2	1	0	26
D	92UG021.16	1	4	2	7	0	3	4	1	2	0	24
E	93TH976.17	1	<b>7</b>	1	6	0	2	3	1	1	0	22
F	93BR020.17	1	4	2	6	1	3	3	2	2	0	24
G	92UG975.10	1	4	1	6	1	4	3	2	2	0	24
B	HXB2	1	3	2	8	1	3	4	1	1	0	24

A closer look at the distribution of these N-glycosylation sites within the V1 region, using the same sequence alignment method by Gao et al. [18], showed that the V1 loop can be further divided into 3 segments (Fig. (5)). The segment at the C-terminal, including a potential N-glycosylation site, N-C-S, is well conserved among many different viral isolates. The N-terminus has some variation but is still fairly conserved with 1 to 2 N-glycosylation sites. It is likely that glycosylation at both ends of the V1 loop may not be dispensable and may be needed to “anchor” the V1 loop. The most striking variations are found in the middle or the “tip” region of V1 loop. The length of V1 tip is highly variable, as is the number of N-glycosylation sites. All four low reacting gp120 antigens have more than one N-glycosylation site at the V1 tip: gp-120-B has 3 sites, C2 has 2 sites, E has 4 sites and F has 2 sites (Fig. (6)). The other 5 highly reactive gp120 antigens (A1, A2, C1, D and G) have only one N-glycosylation site at the V1 tip region.

	<u>N-side anchor</u>	<u>Tip of V1 loop (highly variable)</u>	<u>C-side anchor</u>	<u>N-Glycan</u>	<u>Total amino acid</u>
<b>Consensus</b>	<u>N C T</u> ? ? ? ? N ? T . . . . .	. . . . .	G E I K <u>N C S</u>		
A1	D C . . . . <u>N A T</u> . . . . .	A S <u>N V T</u> N E M . . . . .	. . . R <u>N C S</u>	1/3	17
A2	D N S Y <u>N I T N N I T</u> . . . . .	N S I T <u>N S S</u> V N M R . . . . .	E E I K <u>N C S</u>	1/4	29
B	<u>N C T</u> N L R . <u>N D T</u> . . . . .	<u>N T T R N A T N T T S</u> S E T M M E E . . . . .	G E I K <u>N C S</u>	3/6	34
C1	H C . . . . <u>S N R T</u> . . . . .	I D Y N <u>N R T</u> D N M G . . . . .	G E I K <u>N C S</u>	1/3	24
C2	<u>N C T</u> . . N A <u>N G T</u> . . . . .	N N <u>N G T</u> V N V <u>N D T</u> M Y . . . . .	G E I K <u>N C S</u>	2/5	28
D	<u>N C T</u> E W K . <u>N A T</u> T . . . . .	. . . . . <u>N A T</u> N E G I . . . . .	G M I . <u>N C S</u>	1/4	23
E	T C T <u>N A T</u> L <u>N C T</u> . . . . .	<u>N L T N G N K T T N V S</u> N I I G <u>N L T</u> D . . . . .	G V R . <u>N C S</u>	4/7	36
F	D C T N I A T <u>N G T</u> . . . . .	<u>N D T</u> I A T <u>N D S</u> L K E D P W . . . . .	A E Q . <u>N C S</u>	2/4	31
G	H C A <u>N V T N N Y T</u> . . . . .	. . . . . E L A <u>N T S</u> I G N R . . . . .	G E I K <u>N C S</u>	1/4	27
HXB2	K C T D L K . <u>N D T</u> . . . . .	N T N S S S G R M I M E K . . . . .	G E I K <u>N C S</u>	1/3	29

**HIV-1 V1 loop**

Fig. (5) Alignment of V1 loop segments from ten different HIV-1 Env glycoproteins against the consensus sequence. The number of N-glycan sites that are at the tip of the V1

loop divided by the total numbers of N-glycan sites within the entire V1 segment. The sequence gaps are marked ( . ).

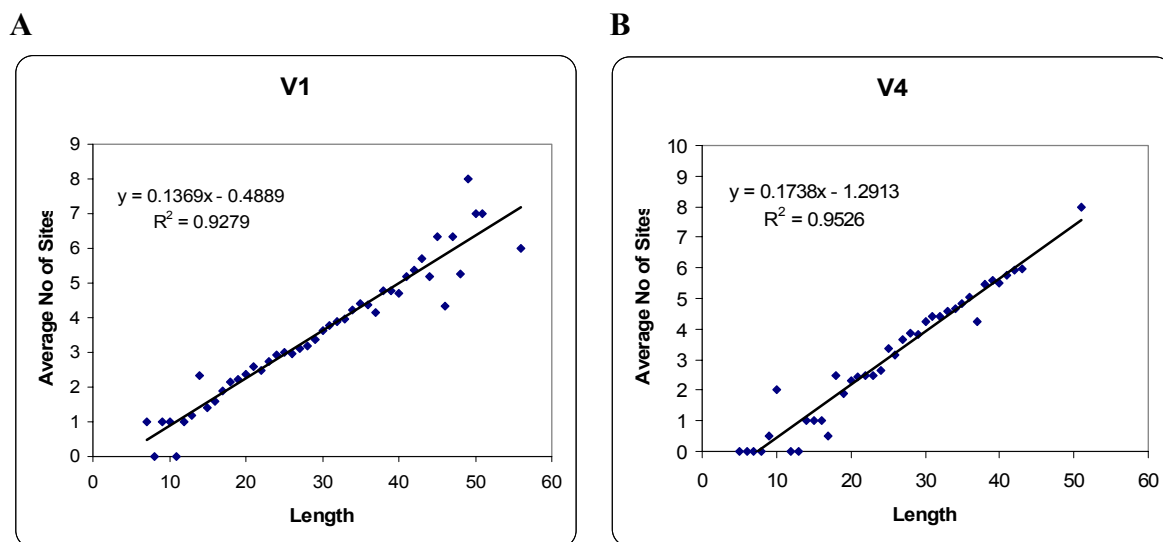


Fig. (6) The relationship between the length of a defined HIV-1 Env segment and the number of potential N-linked glycosylation sites within that segment. (A) A total of 3022 primary gp120 Env glycoproteins were analyzed. A strong positive relationship exists between the length of the entire gp120 and the number of N-glycosylation sites within that segment. (B) Different V1 and V4 sequences show the strong linear relationship ( $R^2 > 0.9$ ) between average number of N-glycosylation sites and length of segment among all variable (V1-V5) segments tested.

We then studied whether it is a common feature for the HIV-1 V1 loop to have increased N-glycosylation relative to the length of V1 loop, and if so, whether this relationship is also present for other variable loops of HIV-1 Env protein. The protein sequence variability and the number of the N-linked glycosylation sites were analyzed by using protein sequence database downloaded from the Los Alamos HIV Sequence Database. Protein sequences from variable regions (V1-V5) were extracted from each of the 4084 complete HIV-1 gp120 sequences after performing alignment using CLUSTALW (version 1.8). Programs were written to search for potential N-glycosylation sites and then to compute the number of glycosylation sites for each variable loop length and the average

number of sites for each variable. The ranges of both the variable loop size and the number of glycosylations are shown in Table 4.

Table 4. Summary of sequence analysis of gp120 and variable loops regions in known HIV-1 Envelope sequences

	Sequence Range	Glycosylation Range	R <sup>2</sup> value
V1	7-56	0-8	<b>0.93</b>
V2	7-69	0-6	0.69
V3	10-40	0-3	0.59
V4	5-51	0-8	<b>0.95</b>
V5	3-27	0-4	0.89

Analysis of the number of N-glycosylations for each of the variable loops showed that, in general, the longer the variable loop, the more N-glycan sites within the variable regions of HIV-1 Env proteins. This can be best described by the linear plots showing the average numbers of potential N-glycan sites within each segment of HIV-1 Env sequences against the length of that segment (Fig. (6)). Although the average number of N-glycosylation sites generally increases with increasing loop size for all variable regions, only V1 and V4 show a strong linear correlation between these two variables ( $R^2 = 0.93$  and  $0.95$ , respectively) but not for other variable regions such as the V3 loop (Table 4 & Fig. (6)). The linear plots showing the average number of glycosylation sites from the different lengths of the V1 and V4 loops are shown in Fig. (6A & 6B), respectively.

## **DISCUSSION**

The data in this report address an important question in the biology of HIV-1 envelope glycoproteins (Env). It is known that heavy glycosylation of Env plays a role in the masking of key neutralizing domains [42]. However, such studies are still limited to a few selected primary Env antigens, mainly from clade B isolates. The effect of glycosylation on the antigenicity of Env, i.e., the reactivity with Env-specific antibodies, has not been well studied, especially with non-clade B Env proteins.

In the current pilot study, we first subcloned nine gp120 genes from the primary HIV-1 Env clones into a DNA vaccine vector which can be used for both gp120 antigen expression and induction of antibody responses against the autologous gp120 antigens. We used 3- and 9-valent gp120 formulations to study different immune responses among antigens in each immunized animal, in order to minimize animal variations when each animal receives only one gp120 antigen. We then studied the reactivity between individual gp120 antigens and the rabbit sera elicited with matched or unmatched gp120 antigens.

Our results indicate that there is a difference in the antigenicity among different primary gp120 antigens. By using the same 3-valent and 9-valent rabbit sera, about half of the tested gp120 proteins had high reactivity and another half showed poor reactivity, suggesting that different primary gp120 proteins have different levels of antigenicity. We further demonstrated that N-linked glycosylation may be at least partially responsible for the poor reactivity of some gp120 proteins because deglycosylation can clearly improve the reactivity of these “low antigenic” gp120 proteins. This finding also supports the

concept that the core structures of HIV-1 gp120 antigens are similar but the reactivity against the core structure can only be demonstrated when the glycosylation is removed.

Further bioinformatics analysis on a large panel of primary HIV-1 Env confirmed that there is a trend of increased N-linked glycosylation with increased sizes of variable loops for HIV-1 envelope glycoproteins. This relationship may be completely random as it may be the result of random chance. However, the V1 and V4 loops demonstrated a striking correlation between the length and level of glycosylation. It is well known that V1/V2 loop plays key role in the making of important neutralizing domains and the length of this loop can vary significantly. It is the most variable one among all five variable loops by two mechanisms: mutations at a given amino acid position and the addition/deletion of a cluster of amino acids to make the size of V1/V2 loop highly variable. As shown in our study, the V1 portion is more variable than the V2 portion of this loop but remarkably, the levels of glycosylation, as measured by the number of potential N-glycan sites, are proportional to the size of V1 loop.

This result is consistent with reports showing that monkeys infected with SIV lab mutants lacking certain N-linked glycosylation sites in the V1 region had a large number of new viral *env* sequences either with the original N-glycan site restored or as newly created N-glycan sites that are adjacent to the mutated sites [24, 25]. The importance of the V1 region has been previously reported [43-45]. While there are approximately 200 monoclonal antibody epitopes (some overlapping) covering many areas of the HIV gp120

protein, there are only a limited number of monoclonal antibody (mAb) sites available against the V1 region [46].

The V1/V2 dual loop structure on the HIV Env protein has been described as masking important elements on the V3 loop and CD4 induced epitopes (CD4i) [47]. It is possible that in order for a persistent viral infection to evade NAb, a large, flexible and variable structure (i.e., the V1/V2 loop) superimposed on an existing, functional envelope core may be required and that heavy glycosylation of the V1/V2 becomes necessary for HIV to escape neutralization. It has been reported that variants of SIV<sub>mne</sub> with an additional glycosylation site in the V1 loop were not neutralized as well by some infected monkey sera when compared to the parental virus [48]. This SIV<sub>mne</sub> vaccine study also reported that the majority of amino acid changes in virus variants, recovered from the E11S gp160 vaccine immunized but not protected monkeys, occurred in the V1 region. The most predominant sequence change across these variant clones included an extra N-linked glycosylation site, when compared to the sequence of the original vaccine strain, E11S [49, 50].

It is very interesting to observe that gp120 antigens from primary viral isolates are dramatically different from the TCLA HIV<sub>HXB2</sub> strain, as shown in the current study. While it is well known that the TCLA virus is more sensitive to neutralization than the primary isolates, our data suggest that large differences occur between these types of viruses in respect to their antigenicity. This finding provides good evidence as to why gp120 from TCLA strains can not be used effectively as diagnostic reagents to detect a

positive HIV serology in patients with suspected HIV infections. At the same time, it also supports the use of primary gp120 antigens to detect positive HIV antibody responses.

In the current study, the 9-valent rabbit sera (R34 and R35) showed a higher overall reactivity when compared to the 3-valent sera (R31-R33) against the same set of coating primary antigens, as shown by ELISA. These results indicate that the polyvalent formulation was able to expand the breadth of reactivity which supports the use of polyvalent formulation for vaccine development.

It remains a challenge to develop promising HIV-1 vaccine candidates that can show protection in efficacy trials [2, 3]. There has been notable progress in inducing protective immune responses based on CMI responses [4, 6, 22, 51], however, none has been able to induce a sterilizing immunity which requires an early and complete block of viral infection to the target cells – a task usually depending on the strong anti-viral NAbs [52, 53]. An effective vaccine against infection with HIV-1 is likely to require both humoral and CMI immune responses. Vaccine formulations that lack the ability to produce both types of immune responses may fail to protect against HIV-1 infection and this may have been the reason for the recent failure of the phase 2 Merck STEP investigational vaccine, V520, a genetically modified type 5 adenovirus containing three HIV genes, *gag*, *nef* and *pol*, designed to elicit only CMI responses [7].

There has been little progress in generating broadly cross-reactive NAbs against HIV-1 through immunization. This has been attributed to the diversity of HIV-1 subtypes

and any vaccine based solely on one HIV-1 Env antigen would not be effective in preventing infection by other strains of HIV-1 [8]. Recently, by using the DNA prime and protein boost approach, progress has been made in the induction of NAb responses against HIV<sub>JR-FL</sub> [54, 55], a relatively difficult to neutralize virus, and other primary HIV-1 isolates [39, 56]. In order to produce broadly reactive neutralizing antibodies, we have shown that polyvalent Env formulations are, in fact, effective [39, 57]. However, with randomly selected Env antigens in these polyvalent formulations, there is still a good percentage of primary HIV-1 isolates that can not be neutralized by the sera elicited with our polyvalent Env formulations. A better understanding of the fine structure determinants that may affect the susceptibility of HIV-1 viruses to neutralizing antibodies is important for the development of more effective HIV vaccines. We need to understand not only the immunogenicity of different primary Env antigens, but also their antigenicity which may be more important in determining their resistance to neutralizing antibodies. The approach presented in this pilot study demonstrates the feasibility of using DNA vaccines as a tool to study both the immunogenicity and antigenicity with the same primary HIV Env antigens.

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