Antigenicity and Immunogenicity of a Trimeric Envelope protein from an Indian clade C HIV-1 isolate

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Keywords: Immunogen design, Indian clade C envelope, broadly neutralizing antibodies, Multi-clade HIV-1 vaccine, RV144 trial.

Background: RV144 trial has established importance of studying diverse HIV-1 envelopes for immunogen design.

Results: HIV-1 93IN101 gp145 exposes conserved regions of envelope protein and induces broadly neutralizing antibodies.

Conclusion: Characterization of trimeric gp145 from an Indian isolate demonstrates its potential use as a multi-clade candidate HIV-1 vaccine to combat AIDS.

Significance: Encourages studies on various Indian clade C envelope proteins.

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) isolates from India mainly belong to clade C and are quite distinct from clade C isolates from Africa in terms of their phylogenetic makeup, serotype and sensitivity to known human broadly neutralizing monoclonal antibodies. Since many of these properties are associated with the envelope proteins of HIV-1, it is of interest to study the envelope proteins of Indian clade C isolates as part of the ongoing efforts to develop vaccine against HIV-1. To this end, we purified trimeric uncleaved gp145 of a CCR5 tropic Indian clade C HIV-1 (93IN101) from the conditioned medium of 293 cells. The purified protein was shown to be properly folded with stable structure by circular dichroism. Conformational integrity was further demonstrated by its high affinity binding to soluble CD4, CD4 binding site antibodies such as b12 and VRC01, quaternary epitope specific antibody PG9 and to CD4 induced epitope specific antibody 17b. Sera from rabbits immunized with the gp145 elicited high titer antibodies to various domains of gp120 and neutralized a broad spectrum of clade B and clade C HIV-1 isolates. Similar to other clade B and clade C envelope immunogens, most of the Tier 1 neutralizing activity could be absorbed with the V3 specific peptide. Subsequent boosting of these rabbits with a clade B HIV-1Bal gp145 resulted in an expanded breadth of neutralization of HIV-1 isolates. The present study strongly supports the inclusion of envelopes from Indian isolates, in future cocktail of HIV-1 vaccines.

INTRODUCTION

The development of an effective vaccine against HIV-1 has been a major challenge in the containment and eradication of acquired immunodeficiency syndrome (AIDS) pandemic. While significant broadly effective cellular immune response has been achieved using HIV-1
gag gene based viral vectors (1–3), it has been difficult to elicit significant humoral immune response resulting in induction of broadly neutralizing antibodies (bNAb) capable of conferring sterilizing immunity against HIV-1. Efforts towards the latter have been mainly directed against the HIV-1 envelope protein (Env) which consists of glycoproteins: gp120 and gp41 existing as non-covalently bound trimers on the surface of the virus. The vaccine strategies have been complicated by the high genetic variability of env among the global isolates of HIV-1 as well as the evolution of neutralization resistant viruses within an individual during the course of infection. Most of the Env based vaccines, which have been tested in pre-clinical studies with non-human primates and in human clinical trials, have failed to generate bNAb (4–6). However, approximately 20% of individuals chronically infected with HIV-1 develop bNAb over a period of three years. Several monoclonal antibodies which neutralize a broad spectrum of isolates from different clades of HIV-1 have been isolated from such individuals (7, 8). Interestingly, it has been shown in macaque animal models that a transfusion of cocktail of such bNAb can protect against viral transmission if they are present at the time of challenge (9–15). Thus it should be possible to achieve protective immunity against HIV-1 with an appropriate vaccine regimen involving induction of both strong humoral and cellular immune response against HIV-1. These bNAb mostly bind to the conserved sites on the Env gp120 or gp41, essential for viral fitness, such as the CD4 binding site, co-receptor binding site or fusion intermediate state (7). While a lot of emphasis is directed towards the CD4 binding site antibodies based gp120 immunogens (16–18), the conserved CD4-induced transition form of gp120-gp41 trimer has not received enough attention. The gp120-gp41 complex becomes a six helical bundle at the time of virus attachment to the cells through interaction with the primary receptor CD4 and the co-receptor CCR5 or CXCR4. This transitional state, which occurs during process of infection, lends itself to attack by neutralizing antibodies and thereby prevent infection. However, many of these conserved sites are not easily accessible as these are protected by extensive glycosylation and are presented as conformation specific quaternary epitopes on the native trimer. In order to generate recombinant stable trimeric immunogen, various strategies have been used so far. Most studies have relied on abolishing the gp120-gp41 cleavage of precursor gp160 to express their soluble form gp140, with or without additional trimerization domains (16). These immunogens which form stable gp140 trimers have been used as immunogens in animal model systems (19). Recent reports on the antigenicity of di-sulfide linked cleaved trimers called SOSIP trimers of an African clade A Env have been shown to bind well with a number of potent neutralizing human monoclonal antibodies (20).

The partial success of RV144 HIV-1 vaccine trial has demonstrated a vital role of purified envelope proteins in future AIDS vaccine design (21). The vaccine regimen used in RV144 trial consisted of a recombinant canary pox vector expressing the gag and env genes of HIV-1 and a mixture of clade B and E HIV-1 envelope proteins. The trial was conducted in 14000 volunteers from high risk population in Thailand and had an efficacy of 31.2% (22). Such a protective response was not seen when the recombinant canary pox vector or the HIV-1 gp120 proteins alone were used in the human clinical trials (5). While neutralizing antibodies to HIV-1 were not detected in the RV144 vaccinees, a strong positive correlation was seen between levels of binding antibodies to the V2 domain of gp120 with protection in the trial participants (23). This showed that in addition to the neutralizing antibodies, non-neutralizing binding antibodies to gp120 play poorly understood but important role in the efficacy of the AIDS vaccine. Thus the search is still on to identify a potential envelope immunogen which either alone or as a cocktail will enhance efficacy of such vaccines.

In view of the extensive variations in the sequences of HIV-1 isolates of different clades, it is important to understand the antigenic and immunogenic properties of the envelope proteins of HIV-1 from different geographical areas and serotypes. In this regard, the most widely studied envelope proteins are from HIV-1 clade B followed by the African clades A or C (17, 20, 24–30). Indian clade C envelope proteins have not been well characterized for their structural and immunological properties. Genetic analysis of the
gp160 sequences shows that the Indian clade C HIV-1 isolates have not diverged much and form a closely associated branch in the phylogenetic tree (31–33). They are also unique in that, they are difficult to neutralize by many bNAbs and are categorized mostly as Tier 1B or Tier 2 (34). Recent studies indicate that the Indian clade C viruses are mostly resistant to the CD4 binding site antibody b12 (32, 35–37), suggesting possible differences in the Env conformation. The membrane proximal external region (MPER) targeting antibody 10E8 has a unique feature of no autoreactivity and neutralizes 98% of tested viruses (38). Mutation of highly conserved Asp at 671 position renders the virus resistant to 10E8. Sequence analysis of Indian clade C HIV-1 show the presence of Ser at 671 position instead of Asp, indicating that these variant viruses may belong to 1.17% 10E8 resistant group (32, 36). Hence it is of interest to study the gp120 and gp145 trimers from Indian isolates of HIV-1 to understand their role in global AIDS vaccine strategies.

In the present study, we have produced conformationally stable trimers of uncleaved gp145 (gp120 with the ectodomain of gp41) of HIV-1 93IN101, isolated from a chronically infected Indian patient (39). The stability of trimeric envelope and its binding to known bNAbs, which are essential criteria for mimicking the native viral surface trimers, has been examined. Immunization of rabbits with 93IN101 gp145 trimer elicited high titered antibody response capable of neutralizing a number of clade B and clade C isolates. Moreover, sequential boosting of 93IN101 gp145 immunized rabbits with clade B HIV-1 Bal gp145 protein broadened the neutralizing antibody response to few Tier 2 isolates. These results suggest that trimeric Env protein of Indian HIV-1 isolates may represent unique candidate to include in future vaccine designs to combat HIV-1 infection.

EXPERIMENTAL PROCEDURES

Antigens, antibodies, plasmids and cell lines- Broadly neutralizing human monoclonal antibodies (MAb) PG9, PG16 (40), 4E10 (41) and 2F5 (42) were purchased from POLYMUN Scientific GMBH. MAb 17b (43) was a gift from Dr. James E. Robinson. HIV-1 gp120 MAb b6 was a gift from Dr. Dennis Burton. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 MAb (2G12) from Dr. Hermann Katinger (44), HIV-1 gp120 MAb (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas (45), HIV-1 gp120 MAb (VRC01) from Dr. John Mascola (46), HIV-1 MAb NIH 45-46 G54W IgG (47), pCAGGS SF162 gp160 from Drs. L. Stamatatos and C. Cheng-Mayer (48) and TZM-bl cells from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (49). GS015 (50) envelope plasmid was provided by Dr. Vicky Polonis. Four domain human sCD4 purified from stable CHO cells was obtained from Advanced BioScience Laboratories Inc (ABL Inc., MD) (51). HIV-1 Bal gp145 (Genbank Accession: M63929) was obtained from ABL Inc., MD.

Envelope plasmid construction- HIV-1 gp120 and gp145 sequences used in the study were derived from the infectious molecular clone: 93IN101, a CCR5 tropic clade C virus isolated from a patient chronically infected with HIV-1 in India (Accession number AY669738.1). The schematic representations of DNAs used in the study are shown in Figure 1. The codon optimized gp120 and gp145 sequences were cloned in to expression vector under the control of CMV promoter in-frame with a N-terminal TPA (Tissue Plasminogen Activator) signal peptide sequence. The vector also encodes a Puromycin resistance gene as a selectable marker. The gp120-gp41 primary and secondary cleavage sites were mutated by replacing arginine with serine residues at positions 503 and 511 to abolish the cleavage of gp145 protein. The modifications were introduced by means of site directed mutagenesis using Pf Turbo polymerase (Stratagene).

Development of HEK-293 cells expressing 93IN101 gp120 and gp145- HEK-293 cells were transfected with the expression plasmid containing gp120 or gp145 using Lipofectamine 2000 (Life Technologies Inc) following the manufacturer’s protocol. After 24 h of transfection, the cells were trypsinized and seeded in to 96 well flat bottom plates with 1000 cells per well. Twenty four hours later puromycin was added to the medium to a final concentration of 5 µg/ml. After four days, cells were fed by replacing 100 µl of medium with 100 µl of fresh medium containing puromycin. The wells with stable clones were screened for
gp120 or gp145 expression using a HIV-1 gp120 antigen capture assay (ABL Inc, MD). High envelope producing single cell clones were selected by limiting dilution and adapted to grow in serum-free medium (293-SFMII, Life Technologies Inc) with 10 µg/ml of Puromycin. For gp145 production, the cells were grown in hollow fiber units (Fibercell Systems, MD) with 30,000Da molecular weight cut off filters. The supernatants were harvested from the extra cellular space and used for the purification of gp145 trimer.

Purification of 93IN101 gp120 and gp145-
The gp120 and gp145 proteins were purified by affinity chromatography from the clarified culture using Galavantus Nivalis Lectin (GNL) agarose beads (Vector laboratories) in buffer with 0.1 % Triton X-100, 500 mM NaCl and 10 mM Tris-HCl pH 8. Polyprep column (BioRad) was packed with 5 ml GNL agarose beads and the concentrated, buffered culture supernatant was passed through the column under gravity flow. After washing with PBS, the gp120 and gp145 were eluted using 400 mM α-methyl-mannopyranoside (Sigma). Non-specific proteins were further removed by anion-exchange chromatography using mono-Q sepharose beads (GE Healthcare). At 150 mM NaCl, only the non-specific proteins bound to the mono-Q beads and gp145 was recovered in the flow through fraction. A gel filtration chromatography step was introduced in order to selectively purify the trimeric gp145 fraction (52). Two columns were connected in tandem: Superdex 200 followed by Superose 6 (GE Healthcare). Columns were equilibrated using 1 X PBS with 0.5 M NaCl followed by injection of sample containing 12 to 13 mg gp145 in 1 ml volume. Fractions were run on BlueNative PAGE as described below. The fractions with only trimers were pooled together and concentrated using Amicon 50 Kda centrifugal filters (Millipore). The concentrated protein fraction was passed through PD-10 gel filtration column (GE Healthcare) in order to buffer exchange into PBS. The purified 93IN101 gp120 and gp145 were stored in aliquots at -80 ºC. All protein concentrations were determined by Bradford protein assay Kit (Pierce). Endotoxin levels were checked in the purified trimeric 93IN101gp145 using LAL assay (Lonza).

SDS-PAGE, Blue-Native PAGE and EGS cross-linking analysis- Purified proteins were resolved in 4-16% Tris Glycine SDS Criterion TM gels (BioRad). For Native PAGE, Novex 4-15% Bis Tris gels (Life Technologies Inc) were used as per manufacturer's instruction. EGS cross-linking was performed as described by Chen et al (53). The cross-linked proteins were analyzed on 3-8% Tris acetate SDS gels under reducing conditions.

Circular Dichroism- Far UV spectral analysis was performed using a J815 CD spectrometer (Jasco). Each protein was loaded at a concentration of 0.35 mg/ml in water and the spectrum was recorded from 250 nm to 190 nm at 20 ºC. An average value of three scans was plotted after subtracting the background value taken in water.

ELISA- For testing antigenicity, Nunc 96 well Maxisorb Immuno Module (Thermo Scientific) plates were coated overnight at 4°C with 125 ng/well of gp145 plus 125 ng/well BSA (Bovine serum albumin) in final volume of 100 µl/well using 50 mm bicarbonate buffer pH 9.6. The coating solution was aspirated and the wells were blocked for 1 h at room temperature with 200 µl of SuperBlock solution (GE Healthcare). Serial dilutions of human monoclonal antibodies in the diluent (ABL Inc., MD) were added to the wells and incubated for 1 h at 37 ºC. The plates were then washed 4 times in PBST (1XPBS, 0.05% Tween 20) followed by 1 h incubation at 37 ºC with HRP conjugated antibody to human IgG (KPL Inc., MD). The wells were washed four times with PBST and 100 µl/well of TMB substrate (KPL Inc., MD) was added to the wells. The reaction was stopped after 30 minutes with 100µl/well of 2N Sulfuric acid. Absorbance at 450 nm was then measured using Molecular Devices spectrophotometer. For 17b ELISA, 17b antibody was coated along with BSA as described above. After blocking, various dilutions of gp145 or gp120 with or without pre-incubation with CD4 were added to the wells. The bound gp120 or gp145 was detected with human anti-gp120 HRP conjugate (ABLinc., MD). For peptide ELISA, 96 well Nunc plates were coated with 200 ng/well of the peptide and the ELISA was performed as described above. The V1, V2 and V3 peptides of 93IN101 gp120 were used in ELISA and neutralization assays (V1peptide:
RNVSRRNVSSYNTYNGSVEEIKNC, V2peptide: SFNATPEVDRKQRMYALFYGLDIVPLNKK
NSSENSSEYRLINC, V3peptide: TRPNNNTRKSIRGPQTFYATGDIGDIRQA HC).

For full length gp160 linear peptide ELISA, 15 amino acid long linear peptides with 11 amino acid overlap were synthesized for the complete 93IN101 gp160 protein sequence (Infinity Biotech and Resource Inc., PA). Each peptide was coated in individual wells of 96 well ELISA plates overnight at a concentration of 500ng/well. Plates were blocked for 1 h at room temperature. Rabbit serum diluted at 1:50 in diluent was added to all wells of the peptide coated 96 well plates. After incubation at 37 °C for 1 h, the plates were washed four times with PBST buffer. Secondary incubation was performed in presence of HRP conjugated antibody to rabbit IgG. The wells were then washed with PBST four times followed by addition of 100 µl/well aqueous TMB substrate. Color development was stopped using 2 N sulfuric acid after 30 min. The absorbance was measured at 450 nm.

Kinetics of gp145 binding to antibodies and CD4- The interaction of 93IN101 glycoproteins with CD4 and various monoclonal antibodies was measured using Octet RED system (Pall ForteBio Corp, CA), Bio-Layer Interferometry. The proteins were biotinylated (1:1 ratio) with EZ-Link NHS-PEG-4-Biotin according to the manufacturer’s protocol (Thermo Scientific). Streptavidin (SA) sensors were loaded at 1000 RPM shake using 10 µg/ml of biotinylated gp120 or gp145. Baseline was established using 1 X kinetics buffer (PBS with 0.02% Tween and 0.1 mg/ml BSA). Association was carried out by dipping the loaded sensors in various concentrations of IgG in 1 X kinetics buffer for 300 s, 600 s or 900 s depending on the on rate of each IgG. Dissociation was observed by dipping in buffer alone. Alternatively biotinylated sCD4 was attached to the streptavidin sensor and the kinetics of binding was analyzed with varying concentrations of gp120 or gp145 in solution.

Rabbit immunizations- Rabbits were housed in the animal facility of Advanced BioScience Laboratories Inc (ABL Inc, MD) under protocols approved by the Institutional Animal Care and Use Committee. Four weight and age matched female New Zealand white rabbits were prescreened for background reactivity before being immunized thrice with 50 µg of 93IN101 gp145 in 1 ml Adjuplex (Sigma) adjuvant once every four weeks. Inoculation was carried out as follows: 300 µl intradermally (50 µl into each of 6 sites), 600 µl intramuscularly (300 µl into each hind leg) and 100 µl subcutaneously (neck region). Serial bleeds were collected 2 weeks post each immunization. After the last 93IN101 gp145 immunization, gp145 specific IgG levels were monitored for 10 weeks followed by four HIV-1 Bal gp145 protein immunizations in a similar fashion. Another group of four rabbits were immunized with HIV-1 Bal gp145 four times with interval of four weeks each as additional control.

gp145 specific IgG quantification- 93IN101 gp145 was coupled to CNBr activated sepharose beads (GE Healthcare) according to manufacturer’s guidelines. Polyprep columns (BioRad) were packed with the gp145 coupled sepharose beads. Pooled terminal bleeds from all four rabbits (1ml each), was loaded on to the column and eluted in MAPSII elution buffer. The purified IgG was quantified by bradford’s assay and used as a standard curve for gp145 specific serum IgG quantification in ELISA. gp145 (125ng/well) and BSA were coated overnight in 96 well plates overnight. Following 1 h blocking at 37°C, various dilutions of serial bleeds of rabbits post-immunization was incubated in 37°C. After four PBST washes, anti-rabbit IgG HRP conjugate (KPL Inc. MD) was added at 1:20,000 dilution, absorbance was measured as described above.

Pseudovirus neutralization- In order to make autologous pseudovirus, rev-vpu-env cassette was amplified from the full length molecular clone of 93IN101 and cloned using pcDNA3.1 Directional TOPO cloning kit (Invitrogen) as described previously (54). HEK-293T cells were co-transfected with Rev/Env containing vector and pSG3Del Env. Supernatant was harvested 48 h after transfection and stored in aliquots at -80 °C. Serial dilutions of purified rabbit IgG were mixed with 200 TCID50/ml pseudovirus and incubated for 1 h, followed by addition of 10,000 TZM-bl cells/well in 96 well plates along with DEAE-dextran at a final concentration of 10 µg/ml. Luciferase activity was

Indian clade C HIV-1 Env gp145 based Immunogen
measured 48 h after infection using bright glow luciferase assay system (Promega).

Peptide absorption of neutralizing antibodies- IgG from various rabbit sera were purified using Protein-A agarose column (BioRad). In 96 well plates, 50 µg/ml of IgG was serially diluted in DMEM (1:2) followed by incubation at 37 °C for 1 h with and without 15µg/ml of V1, V2 or V3 peptides. GS015 pseudovirus was diluted in DMEM to 200 TCID50/ml and added to the wells followed by 1 h incubation at 37 °C in CO2 incubator. TZM-bl cells (10,000 cells/well) were then added with DEAE-dextran at a final concentration of 10 µg/ml. Luciferase activity was measured after 48 h of infection as described above.

RESULTS
Expression and purification of 93IN101 gp120 and gp145- Indian clade C HIV-1 gp120 and gp145 proteins (Schematic representation in Fig. 1A) were expressed in HEK-293 cells and purified as described in the experimental procedures section. The initial step of purification by lectin affinity chromatography resulted in a mixture of dimers and trimers, as shown in native PAGE analysis (Fig. 1B). The trimeric fraction was then purified by gel filtration on Superdex 200 and Superose 6) The SDS-PAGE analysis indicates that the purified gp120 and gp145 proteins are more than 90% pure. The structure of the pooled trimeric gp145 was further established by intra-molecular cross-linking of gp145 with the bifunctional cross-linker EGS followed by SDS-PAGE analysis under reducing conditions. Under saturating amounts of the cross-linker, only the trimer band was noted (Fig. 1B), thereby confirming that the purified gp145 was predominantly trimeric. The purified trimeric gp145 was used for all further studies described below and referred to as gp145. The purified trimeric 93IN101 gp145 contained less than 20 Units of endotoxin per milligram, measured using LAL assay, which did not cause any adverse effects during animal immunizations.

Analysis of gp120 and gp145 by Circular Dichroism- The secondary structure analysis and the proper folding of purified gp120 and gp145 were examined by circular dichroism at room temperature. Both the gp120 and gp145 had similar folded structures, typically consisting of alpha helices and beta sheets (Fig. 2), indicating that during purification the structure of these proteins were preserved. This will be critical for the protein-protein interaction experiments.

Characterization of CD4 and co-receptor binding sites on purified Env- Interaction of CD4 with the purified gp120 and gp145 was examined by bio layer interferometry (Octet Red), with biotinylated CD4 bound to the sensors and 93IN101 gp120 and gp145 in solution. Serial dilutions of either 93IN101 gp120 or gp145 were incubated with the CD4 bound sensors and the KD was determined. The results in figure 3 indicate that 93IN101 gp145 binds soluble CD4 (sCD4) with 200 fold higher affinity than the gp120 (0.15nM vs. 30.73 nM). This higher affinity is due to ten fold faster association (1.8E+05 1/Ms vs. 0.2E+05 1/Ms) and four fold slower dissociation rate (0.24E-04 1/s vs. 6.62E-04 1/s) of gp145 from the CD4 bound sensor. This may be due to the cooperative binding of CD4 to the trimeric gp145 compared to gp120 as reported previously (55).

The human monoclonal antibody 17b binds to the CD4 induced co-receptor overlapping site on gp120. The conformational change in gp120-gp41 trimers on the virion surface induced by CD4 binding leads to the exposure of the CCR5 or CXCR4 co-receptor binding site. To explore the co-receptor binding site in the purified Envs, the binding of 17b to 93IN101 gp120 and gp145 was examined. For this purpose, the 17b antibody was immobilized on Octet Protein G sensors and the interactions were measured with both gp120 and gp145 in the presence of two fold molar excess of sCD4. As seen from the sensograms in figure 3 B, the rate of dissociation of bound gp120 and gp145 from the 17b immobilized sensors is very slow both in the presence and the absence of CD4. Nevertheless it is evident that in presence of sCD4, there is an increase in the rate of association of 17b with gp120 as well as gp145, showing the intact co-receptor binding site and conformational flexibility of the purified proteins.

The CD4 binding site is the target for number of broadly neutralizing human monoclonal antibodies (56). This site is highly conserved among HIV-1 isolates across different clades from various geographical regions. It is of interest to compare the affinity of known CD4 binding site
antibodies to the gp120 and gp145 of 93IN101 virus. The binding of neutralizing (b12, VRC01, NIH 45-46 G54W) and non-neutralizing (b6) CD4 binding site antibodies to 93IN101 gp120 and gp145 was analyzed by ELISA using gp120 or gp145 coated plates (Fig. 4A). Comparable binding of b6 and NIH 45-46 G54W to both gp120 and gp145 was observed, the neutralizing monoclonal antibody b12 and VRC01 bound only to gp145, suggesting a conformational exposure of the site in gp145 but not in gp120. When the binding kinetics was assayed using Octet, all four CD4 binding site antibodies had a two fold faster Kon to gp145 as compared to gp120 (Fig. 4B). Thus both gp120 and gp145 seem to have intact CD4 binding site with better exposure in gp145 as it binds consistently to all antibodies tested.

Recognition of 93IN101 gp145 by glycosylation dependent human monoclonal antibodies- It is well known that lentivirus envelope proteins are glycosylated and in particular this post-translational modification helps HIV-1 to escape from immune pressure. It is worthwhile to test binding affinity of purified Env with broadly neutralizing human monoclonal antibodies 2G12, PG9 and PG16, which target specific glycosylation sites on gp120. The purified 93IN101 gp145 did not bind with the 2G12 antibody in ELISA. This is not surprising in view of the lack of glycosylation at position 295 of 93IN101 gp160 which is required for the binding of the neutralizing human monoclonal antibody 2G12 (57). The quarternary V2/V3 epitope and glycosylation (N160) dependent antibodies PG9 and PG16 did not bind with 93IN101 gp120. While PG9 bound with gp145 very well, PG16 binding was very poor in ELISA (Fig. 5A) similar to the observations of Barouch et. al. (19) and Stamatatos et. al. (58). The interaction was further examined by Octet with gp145 immobilized to the sensors (Fig. 5B), which showed that both PG9 and PG16 IgGs interacted with gp145 but the dissociation rate is ten times faster with PG16 compared to PG9. This may account for the lack of binding in ELISA with PG16.

Binding to Anti-gp41 MPER antibodies- Sequence comparison between the clade B HXB2 gp41 and 93IN101 (Fig. 1A) shows that the 2F5 epitope is not conserved in 93IN101 gp41. As expected, 2F5 did not bind 93IN101 gp145 both in ELISA and Octet. The other MPER directed bNab, 4E10 bound poorly in ELISA (Fig. 5A) with 93IN101 gp145 and had a high Kp (57.8 nM) as determined by Octet (Fig. 5B). This shows that although the site is present in purified gp145 it is not optimal for high affinity binding to 4E10. The neutralizing human monoclonal antibody 10E8 against HIV-1 gp41 bound poorly in ELISA and did not show any binding to 93IN101 gp145 trimer in Octet binding studies (data not shown).

Neutralization of 93IN101 pseudovirus- In view of the differences in the binding of purified trimeric 93IN101 gp145 to well characterized neutralizing human monoclonal antibodies, the ability of these antibodies to neutralize 93IN101 pseudovirus was tested in TZM-bl cells (Table 1). Those antibodies that showed strong binding to gp145, namely the CD4 binding site antibodies VRC101 and NIH 45-46 G54W also neutralized the pseudovirus with lower IC90s than others. In support with results obtained from ELISA and Octet binding studies, PG9 monoclonal antibody neutralized 93IN101 virus strongly (1.6 µg/ml) while only weak neutralization was noticed with PG16 (20.9 µg/ml). The IC90 value for sCD4 is very high (57.2 µg/ml), similar to that reported by Binley et al (35) indicating that perhaps 93IN101 is partly CD4 resistant.

Immunogenicity in rabbits- While a number of clade B and African clade C gp120 and gp140 proteins have been tested in animal models and human clinical trials, the envelope proteins of HIV-1 isolates from India, which form a unique serotype, have not been studied for the immunogenicity in detail. A study using gp120 from two Indian isolates reported limited immune response in guinea pigs (59) while there are no reports on the immunogenicity of Indian gp140 proteins. To this end, four New Zealand white rabbits were immunized with 50 µg of purified 93IN101 gp145 trimers in Adjuplex adjuvant at four week intervals as depicted in the schematic representation of immunization scheme (Fig. 6A). Adjuplex has been shown to elicit high titer antibodies in rabbits and rhesus macaques (16, 60). Significant antibody titers were seen in all the rabbits even after the second immunization and remained high during the course of the study (Fig. 6B). In order to delineate the immune response to linear epitopes of gp145, the sera were analyzed in
ELISA with overlapping peptides (15 mers with 11 amino acid overlap) of 93IN101 gp160 (Fig. 7A). Similar profiles are seen in all the rabbits. As can be seen from the figure 7A, there was a robust immune response to the C1, V1, V2, V3 and immunodominant region of gp41 domains of gp145. A similar peptide scan with clade B HIV-1 Bal peptides was also carried out (data not shown) which showed high titer immune responses to the V1 and V2 domains of HIV-1 Bal gp120. We synthesized the complete linear 93IN101 V1, V2 and V3 peptides and tested serial dilutions of the rabbit sera by ELISA for reactivity with these peptides. A strong reactivity was observed with V3 peptide, moderate reactivity with V2 peptide and less reactivity with the V1 peptide (Fig. 7B).

To determine the neutralization efficiency of these antibodies, we purified total IgG from the rabbits and tested in the TZM-bl based pseudovirus neutralization assay. As shown in Table 2, neutralization of the homologous 93IN101 pseudovirus was observed in one immunized rabbit. In addition, purified IgG from all four animals neutralized clade B viruses SF162, Bal and pNL4-3 as well as African clade C virus GS015. The specificity of the neutralization of HIV-1 by the rabbit sera was also analyzed by absorption with V1, V2 and V3 peptides of 93IN101 gp120. The neutralization of the GS015 pseudovirus by IgG from all four of the immunized rabbits was completely absorbed by the V3 peptide (Data not shown). It is well known than Tier1 neutralization is due to V3 specific antibodies (35, 61). Collectively these data demonstrate that the 93IN101 gp145 is a potent immunogen and elicits high titer humoral immune response to all the regions of gp120 and gp41 of HIV-1.

To test whether the neutralizing activity can be broadened by a combination of heterogenous envelope proteins, the 93IN101 gp145 immunized rabbits were subsequently immunized four times with HIV-1 Bal gp145 in Adjuplex at four week intervals (Fig. 6A). A strong neutralization of HIV-1 Bal was seen in 93IN101 immunized rabbits and this was enhanced after boosting with HIV-1 Bal gp145 (Table 2). This enhanced HIV-1 Bal neutralization was markedly higher than the neutralization observed using IgG from rabbit sera immunized with Bal gp145 alone (Table 2). Weak neutralization of the difficult to neutralize autologous 93IN101 virus was also observed by IgG purified from all rabbits. Immune sera collected two weeks after the last Bal gp145 immunization were also tested for their ability to neutralize a panel of Tier 1 and Tier 2 pseudoviruses (62, 63). As shown in Table 3, a high neutralizing antibody titer was observed in these sera to Tier 1 HIV-1 isolates. In addition, Tier 2 isolates were also neutralized by sera from two of the immunized rabbits after Bal gp145, with low to moderate titers. In this assay, significant neutralizing antibody titers were noticed against clade B and African clade C HIV-1 isolates following just 93IN101 gp145 immunization (week 13), suggesting that the Indian clade C envelope proteins may be strong candidates for future vaccine design to fight the global AIDS epidemic.

**DISCUSSION**

One of the major problems to be addressed in AIDS vaccine development is the variability of the envelope protein among the global isolates of HIV-1. The envelope proteins are the targets of well characterized human antibodies that broadly neutralize HIV-1 from different clades. The vaccine development is further made difficult by the evolution of virus in response to the early development of neutralizing antibodies to HIV-1 in infected individuals. In the recently concluded RV144 human clinical trial, a mixture of clade B and clade E gp120 proteins were used in a prime boost regimen, presumably to generate broadly reactive protective immune response in the vaccinees. The study was conducted in Thailand where HIV-1 clade E is widely prevalent. In other geographical regions of the AIDS pandemic like Africa and Asia, where there is a great need for an AIDS vaccine, the most dominant forms of the virus belong to clade C genotype. While the immunogenicity of purified envelope proteins from African clade C have been well studied, little is known about the purified envelope proteins of clade C HIV-1 isolates from India. A limited immunogenicity study of Indian clade C gp120 along with other clade C gp120 isolates has been performed and no characterization of gp145 which is the target of gp120 and gp41 neutralizing
Indian clade C HIV-1 Env gp145 based Immunogen

antibodies. In order to achieve these stated goals, we expressed and purified stable trimeric gp145 envelope protein from a patient chronically infected with HIV-1 from India. The infectious molecular clone of the CCR5 tropic 93IN101 HIV-1 has been well characterized (35, 39). In this report we showed for the first time that the 93IN101 gp145 protein purified from the conditioned medium of HEK 293 cells forms highly stable trimer. For structural comparison, 93IN101 gp120 was also purified and studied simultaneously. The gp145 had better exposure of the CD4 binding site epitopes than gp120. Previous reports show a complex interaction between CD4 and the gp160 trimers present on the virion surface of HIV-1 isolates from India in pseudovirus neutralization assays by monoclonal antibodies. In addition, Berman et al (59) observed that one of the purified Indian clade C gp120 did not bind CD4 similar to the 93IN101 gp120 reported in the present study. The purified 93IN101 gp120 had 200 fold lower $K_d$ than gp145. Studies with other clade B and African clade C isolates indicate that CD4 binding with gp120 is of higher or equivalent affinity as compared to gp140 (19, 64), suggesting the Indian clade C is distinct. Further, high affinity binding of 93IN101 gp145 to CD4 binding site monoclonal antibodies b12, VRC01 and NIH 45-46 G54W suggests that the gp145 had the conformational integrity to expose the CD4 binding site to the immune system. As expected from this conformational integrity of the CD4 binding site of gp145, the antibodies VRC01 and NIH 45-46 G54W neutralized the 93IN101 pseudovirus at low concentrations (Table 1). As previously reported, most primary HIV-1 isolates from India are resistant to neutralization by b12 (32, 35–37). We also observed poor neutralization of HIV-1 93IN101 by b12. The gp41 directed monoclonal antibodies 2F5 and 10E8 did not bind to 93IN101 gp145. This is not surprising due to the absence of critical residues (K665S in 2F5 epitope and N671S for 10E8 epitope) in MPER of 93IN101. The 93IN101 virus was moderately neutralized by the broadly neutralizing antibody 4E10 (IC90 at 10 µg/ml). Additional evidence of conformational integrity of 93IN101 gp145 was provided by the strong binding of the quaternary epitope dependant broadly neutralizing antibody PG9 to gp145. This was observed both in ELISA and in Octet studies. The absence of ELISA reactivity of the closely related PG16 antibody may be due to rapid dissociation of PG16 compared PG9, as supported by Octet binding studies. A corresponding difference in the neutralization of the homologous 93IN101 pseudovirus was also observed between PG9 and PG16 (Table 1, IC90-1.6 µg/ml PG9 and 20.9 µg/ml PG16). All the above studies with ELISA, $K_d$ measurements with Octet and neutralization of the 93IN101 pseudovirus demonstrated that 93IN101 has properly folded structure to expose a variety of epitopes in the gp120 and gp41 domains to the host immune system.

The RV144 HIV-1 vaccine trial has also demonstrated that a prime boost regimen with a recombinant viral vector results in a protective immune response, since the proteins alone were not sufficient to prevent virus acquisition (22). The trial also showed that the protection correlated with the presence of non-neutralizing antibodies to the V1 and V2 domains of gp120. On the other hand, passive immunization with virus neutralizing sera or neutralizing monoclonal antibodies prevented SHIV infection in rhesus macaques (9, 12). Thus both neutralizing and non-neutralizing antibodies to the HIV-1 envelope proteins are essential for achieving significant protection against HIV-1 infection. In addition, we may have to consider the antigenic variations in the envelope proteins of HIV-1 isolates from different parts of the world. One of the goals of this study is to assess the immunogenicity of the gp145 protein from a chronic Indian isolate of HIV-1 since these viral isolates form a group by themselves and have unique antigenic properties (32). As described, we have demonstrated the conformational integrity of trimeric gp145 from an Indian isolate by its reactivity with HIV-1 neutralizing human monoclonal antibodies and thus may have exposed epitopes for generating the corresponding antibodies. The envelope proteins from these isolates may expose additional uncharacterized epitopes to the immune system. The immunogenicity studies in rabbits showed that the 93IN101 gp145 is highly immunogenic and generates strong response to the V2 and V3 domains of HIV-1 gp120. The rabbit sera also generated strong neutralizing antibodies against a number of clade B and African clade C viruses.
(Table 2 and 3). However, the Tier 1 neutralization is mainly due to the immune response against the V3 domain of gp120, similar to immunogenicity studies with other HIV-1 gp120 and gp140 (64, 65). The challenge is therefore to expand the breadth the neutralization by generating antibodies against other conserved domains of the gp120-gp41 spikes on HIV-1 virions.

The 93IN101 gp145 immunized rabbits were boosted with a heterologous clade B HIV-1 Bal gp145; we observed an expanded but weak neutralization of Tier 2 viruses in the TZM-bl assay. This has not been reported before with other HIV-1 gp140s. Also, potent neutralization of HIV-1 Bal pseudovirus was observed following boosting with Bal gp145 and this response was shown to be markedly higher than that was noticed in rabbits immunized with Bal gp145 only. These results suggest that 93IN101 gp145 might have primed epitopes involved in HIV-1 Bal neutralization. Together, these data provide evidence that a combination of HIV-1 gp145s from different clades and more importantly different serotypes might generate broadly neutralizing antibodies. Also, as suggested by the RV144 trial, priming with viral vectors or DNA followed by immunization using a mixture of envelope proteins from different serotypes may be essential to boost the host protective immune response in vaccinees. The present study demonstrates that such epitopes are exposed in the 93IN101 gp145 but have only weak recognition by the immune system for generating broadly neutralizing antibodies and this be enhanced with an appropriate envelope protein from different serotypes. One of the possible approaches is to test a heterologous mixture of envelope proteins of HIV-1 in combination with a prime boost regimen in the vaccine strategy. In any event the envelope proteins of HIV-1 are an essential component for future AIDS vaccine development. In view of the fact that many of these broadly neutralizing human monoclonal antibodies have undergone many somatic mutations from germ line sequences (66, 67), it is essential to identify envelope proteins that react with a broad spectrum of germ line sequences. The present study suggests that an appropriate Indian clade C envelope protein can be such a candidate in future HIV vaccine regimens.
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FOOTNOTES
The abbreviations used are: AIDS, acquired immune deficiency syndrome; bNAb, broadly neutralizing antibodies; Env, envelope; TPA, tissue plasminogen activator; GNL, galavantius nilvatis lectin; EGS, ethylene glycolbis(succinimidylsuccinate); CD, circular dichroism; ELISA, enzyme linked immune sorbent assay; TMB, 3,3',5,5'-tetramethylbenzidine; CNBr, cyanogen bromide; TCID, tissue culture infectivity dose; MPER, membrane proximal external region.

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FIGURE LEGENDS

TABLE 1: Neutralization sensitivity of 93IN101 pseudovirus to sCD4 and various known conformational broadly neutralizing antibodies to HIV-1. IC90- Concentration for 90% inhibition of infection.

TABLE 2: IC50 values for neutralization of various HIV-1 pseudoviruses as well as autologous pseudovirus by purified IgG from immunized rabbit sera.

TABLE 3: Summary of neutralizing antibody titers for various HIV-1 strains by immunized rabbit sera.

FIGURE 1. Design and purification of trimeric 93IN101 envelope immunogen. A. Schematic representation of 93IN101 full length gp160 showing presence of original signal peptide and intact 4E10 epitope. TM Transmembrane and CT Cytoplasmic Tail. Number corresponds to last amino acid as per HXB2 numbering. 93IN101 gp145 immunogen having Tissue Plasminogen Activator (TPA) signal peptide, abolished protease cleavage site and cytoplasmic tail truncation. Sequence alignment of 2F5-10E8 and 4E10 epitopes from HXB2 and 93IN101. B. Gel electrophoresis of purified gp120 and gp145 proteins. In Native PAGE, pooled trimeric fractions of gp145 after gel filtration (lane 2) compared to starting material (lane 3). 93IN101 gp145 (lane 2) and gp120 (lane 3) proteins were purified from stable HEK-293 cells and resolved in SDS PAGE. Purified gp145 was analyzed under reducing condition in SDS-PAGE after cross-linking with indicated concentrations of EGS ethylene glycolbis(succinimidylsuccinate). In all gels 5 µg protein was loaded per lane.

FIGURE 2. Far UV Circular Dichroism spectral analysis. Purified 93IN101 gp120 and gp145 trimers were analyzed by CD at room temperature in PBS.

FIGURE 3. Receptor and co-receptor binding site analysis using Octet Red-Bio Layer Interferometry. A. Octet sensograms generated by binding of serial dilutions of purified envelope gp120 and gp145 in solution to biotinylated sCD4 immobilized on streptavidin sensors. K_D- Dissociation constant, kon- rate of association and kdis- rate of dissociation. The red lines denote 1:1 global curve fits. B. Exposure of co-receptor binding site antibody 17b epitope upon CD4 binding to gp120 and gp145 proteins of 93IN101. Env concentrations were 100 nM and Env:sCD4 molar ratio of 1:2.

FIGURE 4. Binding of 93IN101 gp120 and gp145 to CD4 binding site antibodies (CD4bs Ab). A. Binding curves generated by coating ELISA wells using gp120 or gp145 and incubating with serial
dilutions of b6 (Non-neutralizing Ab) or b12, VRC01 and NIH 45-46 G54W (Neutralizing Abs). B. Table comparing kinetic parameters $K_D$-Dissociation constant, kon- rate of association and kdis- rate of dissociation for immobilized gp145 and gp120 binding to various CD4bs Ab in solution, using Octet Bio Layer Interferometry. $X^2$ and $R^2$ are measure of goodness of fit.

FIGURE 5. **93IN101 gp145 binding analysis to known broadly neutralizing human monoclonal antibodies.** A. ELISA binding curves generated by coating the plate with gp120 or gp145 and incubating with serial dilutions of various human monoclonal antibodies. B. Summary of kinetic parameters $K_D$-Dissociation constant, kon- rate of association and kdis- rate of dissociation calculated using Octet-Bio Layer Interferometry for immobilized gp145 binding to PG9, 4E10 and PG16 in solution. $X^2$ and $R^2$ are measure of goodness of fit.

FIGURE 6. **Protein immunization in New Zealand White Rabbits.** A. Schematic representation of immunization schedule showing time of immunization and bleed. B. Serial dilutions of rabbit sera were tested for reactivity by ELISA against 93IN101 gp145 protein bound on plate. Specific IgG quantification was calculated by generating standard curve.

FIGURE 7. **Linear epitope mapping of 93IN101 gp145 immunized rabbit sera by ELISA.** A. Total of 192 peptides 15 amino acid long with 11 amino acid overlapping sequence covering the entire 93IN101 gp160 protein were used. Sera from two weeks post final protein immunization were tested for reactivity at a dilution of 1:50. Rabbit identification numbers C2507, C2508, C2509 and C2510 are used in the study. B Titers to V1, V2 and V3 linear peptides of 93IN101 gp120.
Table 1

| Sample         | IC90 (µg/ml) |
|----------------|--------------|
| NIH45-46 G46W  | 0.38         |
| PG9            | 1.6          |
| VRC01          | 3.2          |
| 4 E 10         | 10.3         |
| PG16           | 20.9         |
| B12            | 41.4         |
| 2G12           | 43.5         |
| 1.7b           | 52.3         |
| VRC03          | 52.8         |
| sCD4           | 57.2         |
### Table 2

| Animal Number | Bleed Week | IC50 (µg/ml) | Tier 1 | Autologous pseudovirus |
|---------------|------------|--------------|--------|-------------------------|
|               |            |              | Clade B | Clade C | Clade B | Clade C |
| 93IN101 gp145 only |           |              | NL4-3  | SF162   | GS015   | BaL     | 93IN101 |
| C2507         | 13         | >250 (12%)   | >250 (43%) | 0.0007 | 215     | 30      |
| C2508         | 13         | >250 (11%)   | >250 (36%) | 0.31   | >250 (40%) | >250 (35%) |
| C2509         | 13         | >250 (22%)   | 30      | 0.0002  | 35      | >250 (42%) |
| C2510         | 13         | 103          | 40      | 0.13    | 20      | >250 (18%) |

| 93IN101 gp145 + Bal gp145 |           |              | NL4-3  | SF162   | GS015   | BaL     | 93IN101 |
| C2507         | 35         | 262          | 2      | 0.22    | 0.1     | >250 (38%) |
| C2508         | 35         | >250 (41%)   | 0.05   | 0.15    | 0.001   | >250 (40%) |
| C2509         | 35         | 50           | 5.8    | 0.01    | 0.92    | 267     |
| C2510         | 35         | 19           | 3      | 0.03    | 0.06    | 200     |

| Bal gp145 Only |           |              | NL4-3  | SF162   | GS015   | BaL     | 93IN101 |
| L50680        | 13         | >250 (48%)   | 31     | 12      | 34      | >250 (19%) |
| L50681        | 13         | 239          | 4      | 5       | 0.00006 | >250 (17%) |
| L50682        | 13         | 100          | 1      | 0.36    | 4.7     | >250 (26%) |
| L50685        | 13         | >250 (44%)   | 0.98   | 1.7     | 4.5     | >250 (18%) |

IC50- The IgG concentration at which relative luminescence units (RLUs) were reduced 50% compared to pre-bleed IgG. If >250µg/ml then, percentage neutralization at 250µg/ml has been mentioned within bracket.

|       |       |       |       |       |       |       |
|-------|-------|-------|-------|-------|-------|-------|
| >250  | 270-50| 50-1  | <1    |       |       |       |

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Table 3

| Animal   | Bleed Week | ID#177 | ID#641 | ID#135 | ID#645 | ID#772 | ID#544 | ID#250 | ID#271 | ID#6 | ID#2 | ID#5 | ID#645 |
|-----------|------------|--------|--------|--------|--------|--------|--------|--------|--------|------|------|------|--------|
| C2507     | 0          | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | <20    |
|           | 13         | 89     | 2307   | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | <20    |
|           | 35         | 2240   | 2505   | 24     | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 62     |
| C2508     | 0          | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | <20    |
|           | 13         | 3937   | 6582   | 21     | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 20     |
|           | 35         | 2550   | 3686   | 34     | 23     | 25     | 27     | 24     | 22     | 34   | 26   | <20  | 83     |
| C2509     | 0          | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 21     |
|           | 13         | 85     | 2316   | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 22     |
|           | 35         | 3306   | 7199   | 105    | 23     | 23     | <20    | <20    | <20    | <20  | 28   | <20  | 57     |
| C2510     | 0          | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 23     |
|           | 13         | 2414   | 6220   | 41     | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 23     |
|           | 35         | 3146   | 6364   | 100    | <20    | <20    | <20    | <20    | <20    | <20  | <20  | <20  | 45     |

Values are the sample dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).
Figure 1

A

![Chart showing the structure of Indian clade C HIV-1 Env gp145 based Immunogen](chart.png)

B

![Images of gel electrophoresis showing gp145 Trimer](gel_images.png)
Figure 3

A

$K_o = 30.73$ nM  
$kon = 2.13 \times 10^4$ 1/MS  
$kdis = 6.62 \times 10^{-4}$ 1/s

B

$K_o = 0.133$ nM  
$kon = 1.797 \times 10^5$ 1/MS  
$kdis = 2.41 \times 10^{-5}$ 1/s

17b binding
Indian clade C HIV-1 Env gp145 based Immunogen

Figure 4

| Ligand         | Sample ID | KD (nM) | kon(1/Ms) | kon Error | kdis(1/s) | kdis Error | Full X^2 | Full R^2 |
|----------------|-----------|---------|-----------|-----------|-----------|------------|----------|----------|
| 93IN101 gp145  | B6        | 0.057   | 3.95E+05  | 1.94E+03  | 2.24E-05  | 1.39E-06   | 0.025    | 0.996    |
|                | B12       | 0.233   | 1.81E+06  | 1.59E+04  | 4.22E-04  | 2.51E-06   | 0.031    | 0.984    |
|                | VRC01     | 0.022   | 4.88E+04  | 4.95E+02  | 1.10E-06  | 3.10E-06   | 0.035    | 0.987    |
|                | NIH45-46W | 0.549   | 2.51E+05  | 2.93E+03  | 1.38E-04  | 2.48E-06   | 0.022    | 0.99     |
| 93IN101 gp120  | B6        | 0.293   | 2.61E+05  | 3.52E+03  | 7.65E-05  | 2.82E-06   | 0.044    | 0.985    |
|                | B12       | 1.225   | 1.07E+06  | 1.49E+04  | 1.31E-03  | 7.12E-06   | 0.066    | 0.928    |
|                | VRC01     | NB      | -         | -         | -         | -          | -        | 0        |
|                | NIH45-46W | 0.510   | 1.43E+05  | 2.49E+03  | 7.29E-05  | 2.36E-06   | 0.066    | 0.992    |
Figure 5

![Graph showing absorption at 450nm against concentration of IgG](image)

| Epitope     | Sample ID | KD (nM) | Kon (1/Ms) | kon Error | Kdis (1/s) | kdis Error | Full X^2 | Full R^2 |
|-------------|-----------|---------|------------|-----------|------------|------------|----------|----------|
| gp120-V2/V3 | PG9       | 8.086   | 9.98  E+04 | 2.18  E+03 | 8.07  E-04 | 1.33  E-05 | 0.007    | 0.944    |
| gp41-MPER   | 4E10      | 57.8    | 1.94  E+04 | 2.86  E+02 | 1.12  E-03 | 7.45  E-06 | 0.065    | 0.937    |
| gp120-V2/V3 | PG16      | 34.820  | 1.99  E+05 | 6.87  E+03 | 6.94  E-03 | 6.59  E-05 | 0.021    | 0.933    |
Figure 6
Figure 7

A

Rabbit sera PepScan ELISA

B

Variable region peptide reactivity

Absorbance (450nm)

Inverse of Dilution

Absorbance (450nm)

Variable region peptide reactivity

V1

V2

V3
Antigenicity and Immunogenicity of a Trimeric Envelope protein from an Indian clade C HIV-1 isolate
Rangasamy Sneha Priya, Menon Veena, Irene Kalisz, Stephen Whitney, Dhopeshwarkar Priyanka, Celia C. LaBranche, Mullapudi Sri Teja, David C. Montefiori, Ranajit Pal, Sundarasamy Mahalingam and Vaniambadi S. Kalyanaraman

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