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Mapping of Neutralizing Antibody Epitopes on the Envelope of Viruses Obtained from Plasma Samples Exhibiting Broad Cross-Clade Neutralization Potential Against HIV-1

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Abstract

Several broadly neutralizing antibodies (bNAbs) that can target HIV strains with large degrees of variability have recently been identified. However, efforts to induce synthesis of such bNAbs that can protect against HIV infection have not met with much success. Identification of specific epitopes encoded in the HIV-1 envelope (Env) that can direct the host to synthesize bNAbs remains a challenge. In a previous study, we identified 12 antiretroviral therapy-naive HIV-1-infected individuals whose plasma exhibited broad cross-clade neutralization property against different clades of HIV-1. In this study, we sequenced the full-length HIV-1 gp160 from 11 of these individuals and analyzed the sequences to identify bNAb epitopes. We identified critical residues in the viral envelopes that contribute to the formation of conformational epitopes and possibly induce the production of bNAbs, using *in silico* methods. We found that many of the sequences had conserved glycans at positions N160 (10/11) and N332 (9/11), which are known to be critical for the binding of PG9/PG16-like and PGT128-like bNAbs, respectively. We also observed conservation of critical glycans at positions N234 and N276 critical for the interaction with CD4 binding site bNAbs in 8/11 and 11/11 sequences, respectively. We modeled the three-dimensional structure of the 11 HIV-1 envelopes and found that though each had structural differences, the critical residues were mostly present on the surface of the Env structures. The identified critical residues are proposed as candidates for further evaluation as bNAb epitopes.

Keywords: HIV-1, subtype C envelope, broadly neutralizing antibodies, epitope specificity, HIV-1 Env structure

Introduction

THREE AND HALF DECADES of intense research since the discovery of the human immunodeficiency virus (HIV) has not brought us anywhere close to the eradication of HIV infection, largely due to the genetic and immunological complexity of the virus.[1] Since the identification of HIV infection in India, every year ~0.1 million people are being newly infected with the virus (www.naco.gov.in). The ultimate goal for prevention and control of infection is the development of an effective vaccine. The envelope glycoprotein (Env) of HIV-1 is essential for infecting host cells. Env is also the only target on the surface of the virus for antibody-mediated neutralization. However, high levels of glycosylation and great sequence diversity of the Env enable the virus to evade the human immune response.[2] This offers a major challenge to the host immune system to develop sufficient quantities of antibodies that can neutralize the wide variety of viral variants at the appropriate time.

In recent years, a large number of broadly neutralizing antibodies (bNAbs) that are capable of binding to several strains of the virus and preventing infection have been identified from some HIV-1-infected individuals.[3] These antibodies bind primarily to five vulnerable sites on the HIV

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Env, namely, the CD4 binding site (CD4BS), V1V2-glycans, V3-glycans, membrane proximal external region (MPER), and gp120–gp41 interface.[4] Two bNAbs, VRC01 and 3BNC117, which target the CD4BS, are currently being tested for their potential as therapeutic tools in clinical trials.[5,6] However, studies from India in this line have been very limited.[7,8] Recently, we reported the identification of broad and potent cross-clade neutralization activity in plasma of HIV-1 subtype C-infected individuals from India.[9] To enable the host immune system to produce antibodies of this nature, it is essential that the appropriate epitopes are presented to the immune system. Hence, in the context of vaccine design, it is important to identify epitopes that can elicit the production of broad and potent neutralizing antibodies in the human host.

A total of 117 critical amino acids have been identified as potential epitopes on the Env of HIV by *in vitro* testing, this list is provided in the HIV database (https://www.hiv.lanl.gov/components/sequence/HIV/featuredb/search/env_ab_search_pub.comp). The list categorizes the critical amino acids on the basis of the class of antibodies they bind to, namely, CD4BS (63), N160 (13), and N332 (41) class-specific epitopes. There are several online bioinformatics tools and methods that can be used to identify potential epitopes from protein sequence and structure. However, the challenge in identifying neutralizing antibody-specific epitopes is that these are most commonly discontinuous/conformational epitopes comprising amino acids present at different locations on the HIV-1 envelope. Although there is a scope for improvement of the sensitivity and specificity of the different bioinformatics methods for identifying epitopes, they can still be used for this purpose. Since usage of more than one bioinformatics method can improve the reliability of the prediction,[10,11] use of combinations of methods is generally recommended.

Our recent study identified 12 HIV-1-infected individuals whose plasma exhibited BCN property against HIV.[9] In this study, we analyzed the sequence of the full-length HIV-1 Envs obtained from these individuals to identify neutralizing antibody-specific epitopes responsible for the production of bNAbs in these individuals.

Materials and Methods

Ethics statement

The study was approved by the Institutional Ethics Committee of the National Institute for Research in Tuberculosis, Chennai, India (NIRT IEC No: 2011001) and all experiments were performed in accordance with relevant guidelines and regulations. Sample collection was done after obtaining written informed consent from the study participants.

Amplification of HIV-1 subtype C env gene

Full-length HIV-1 subtype C gp160 *env* gene was amplified from 11 of the 12 BCN plasma samples and sequenced as previously described.[12] In brief, viral RNA (vRNA) was extracted from plasma using the QIAamp vRNA mini kit (Qiagen, Valencia, CA). For Env amplification, vRNA was reverse transcribed using SuperScript III according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). RNA, deoxynucleoside triphosphates (0.5 mM each), and 0.25 μM primer OFM19 (5-GCACTC AAGGCAAGCT TTATTGAGGCTTA-3; nucleotides (nt) 9,604–9,632 of the HXB2 sequence) were first incubated for 5 min at 65°C to denature the secondary structure of the RNA. First-strand cDNA synthesis was carried out in 60 μL reaction mixture with 1× reverse transcriptase buffer containing 5 mM dithiothreitol, 2 U/μL RNase inhibitor (RNaseOUT), and 10 U/μL SuperScript III, at 50°C for 60 min, followed by an additional 1 hour at 55°C. After this step, the reaction mixture was inactivated at 70°C for 15 min and RNase H digestion was performed at 37°C for 20 min (Life Technologies). The resulting cDNA was used immediately for polymerase chain reaction (PCR) or kept frozen at −80°C until further use. Full-length *rev/env* cassettes were amplified by nested PCR from plasma-derived viral cDNA. In brief, 1 μL of bulk cDNA was subjected to first-round PCR in a volume of 20 μL using Platinum Taq High Fidelity polymerase enzyme (Life Technologies) in 1× HiFi buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 0.2 μM Vif1 (5-GGGTTTATTACAGGGACAGCAGAG-3; nt 4,900–4,923) and OFM19 primers. PCR conditions included denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 4 min, with a final extension at 68°C for 10 min. Second-round PCR was performed using 1 μL of the first-round PCR product and primers EnvA* (*indicates forward primer carries 'CACC' overhang for cloning purpose) (5-CACC GGCTTAGGCATCTCCTAT GGCAGGAAGAA-3; nt 5,954–5,982) and EnvN (5-CTGC CAATCAGGGAAGTAGCCTTGTGT-3; nt 9,145–9,171) under the same conditions used for the first-round PCR. The final PCR products were analyzed using a 1% agarose gel, and products of predicted size ($\sim$3.0 kb) were used for sequencing.

DNA sequencing and sequence analysis

Sequencing of amplicons was performed using 20 HIV-1 subtype C Env sequencing primers[13] on an ABI 3100 Genetic Analyzer (model 3100; Applied Biosystems, Inc.) following the procedure recommended by the manufacturer. Nucleotide and deduced amino acid sequence of full-length gp160 were formatted using SeqScape v2.5 (Applied Biosystems, Inc.), aligned using clustal X and imported into MEGA 6.06 for molecular phylogenetic tree construction using maximum likelihood algorithm. Reference sequences for HIV-1 subtypes A to K were obtained from the HIV database and included with the 11 experimental sequences for phylogenetic analysis. Neutralizing antibody-specific epitopes were highlighted manually with respect to HXB2 sequence position.

Building of consensus sequence

Multiple sequence alignment was performed with MUSCLE using MEGA (version 6.06). Consensus sequence was established using Consensus Maker available at (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). Sequence of one of the infectious HIV-1 subtype C clones from India, pIndie (GenBank accession number AB023804), was included along with the envelope sequences obtained from this study, while establishing the consensus sequence. The *Consensus Maker* tool uses the most frequent residue (occurring at a frequency of >50%) to automatically generate the consensus sequence. However, we manually relaxed the cutoff value to 36.36%, so as to set the limit to

one-third instead of half, in which case if a residue was present in at least 4 out of the 11 sequences, then that particular residue was chosen to represent the consensus sequence. For the remaining positions, an amino acid present at the corresponding position in the reference sequence (pIndie) was used.

To validate the representativeness of the consensus sequence generated (NABCon), this sequence was included in the original data set and the phylogenetic tree was reconstructed using MEGA. Furthermore, to determine the quantity of relatedness between the individual envelopes and the consensus sequence, pairwise alignment was performed using *BLAST* Global Align (Needleman-Wunsch alignment of two sequences available in NCBI at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=blastn&BLAST_SPEC=GlobalAln&LINK_LOC=BlastHomeLink).

Prediction of conformational epitopes

The three-dimensional structure of the gp160 sequences obtained from the BCN samples and that of NABCon were modeled using SWISS-MODEL homology-modeling server (https://swissmodel.expasy.org).[14] The modeled structures were validated using Ramachandran plot[15] and used for the prediction of conformational epitopes critical for the synthesis/binding of bNAbs. Conformational epitopes may be spread across the HIV-1 envelope and are, therefore, also referred to as discontinuous conformational epitopes. Two different bioinformatics tools, namely, ElliPro[10] and DiscoTope 2.0,[11] were used to identify conformational epitopes from the protein sequences. To improve the value of the *in silico* prediction, we selected only epitopes that were consistently identified by both DiscoTope and ElliPro algorithms. The conformational epitope repertoire presented in the HIV LANL database was used as the reference data set for comparing/validating the results of the prediction.

Enzyme-linked immunosorbent assay

V1, V2, V3, and MPER peptides of 93IN101 gp160 (V1peptide: RNVSRNVSSYNTYNGSVEEIKNC, V2peptide: SFNATPEVRDRKQRMYALFYGLDIVPLNKKNSSENSSEYRLINC, V3peptide: TRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC, and MPER peptide: LALDSWKNLWSWFDITNWLWYIK) were commercially synthesized (Infinity Biotech and Resource, Inc., PA). HIV-1 subtype C (C.1048) V1V2 tag (Cat. no. 12568), D7gp120 (Cat. no. 12582), and gp140C trimer (Cat. no. 12581) proteins were obtained from the AIDS Research Reagent Program, Division of AIDS, NIAID, NIH. Enzyme-linked immunosorbent assay (ELISA) was performed as previously described.[9] The peptides were adsorbed onto 96-well ELISA immunomaxisorp plates (Thermo Fisher) at a concentration of 5 μg/mL in 100 mM $NaHCO_3$, pH 9.6, by overnight incubation at 4°C. The plates were washed four times with PBST (1× phosphate-buffered saline [PBS] containing 0.05% Tween 20) and blocked for 2 h at room temperature with PBS containing 1% bovine serum albumin and 0.05% Tween 20. The plates were washed four times, and heat-inactivated plasma samples diluted serially with the diluent (ABL, Inc., MD) from 1:20 to 1:43,740 were added to the respective wells. A single dilution (1:200) was used for the whole protein ELISA. Plates were incubated at 37°C for 1 h. After another four washes, goat antihuman IgG horse radish peroxidase conjugated secondary antibody (Thermo Fisher) at 1:120K dilution was added and incubated for 45 min at 37°C. Plates were washed four times with wash buffer and developed using One-STEP TMB (Thermo Fisher) substrate. After 30 min of incubation, the reaction was stopped with 1 N H_2SO_4 and the plates were read using a microplate reader (ELx808-BioTek). Each sample was tested in triplicate and the mean value was used to represent the binding reactivity. Healthy human plasma pool was used as the negative control.

Statistical analysis

All statistical analyses were performed using the software Graph pad prism 5.0. Experiments were performed two or more times and values obtained from three replicate samples were averaged in each experiment. Statistical significance was tested using *t*-test. Two-way analysis of variance (ANOVA) was used to test the significance of the difference in binding to the monomeric D7gp120 and trimeric gp140 protein. Differences were considered significant at $p < .05$.

Results

Envs from the BCN samples show distinct clustering with subtype C sequences

The clinical, immunological, and demographic details of the individuals from whom HIV-1 envelope sequences were obtained are provided in Table 1. Full-length HIV-1 gp160 *env* gene was successfully amplified and sequenced from the plasma of 11 of the 12 BCN samples by RT-PCR. The nucleotide and deduced amino acid sequence of full-length gp160 were obtained using SeqScape v2.5 software (Applied Biosystems, Inc.). The sequences were deposited in GenBank (Accession numbers: KY929360–KY929370) and the details are provided in Table 1. A phylogenetic tree was constructed with reference sequences representing each of the major HIV-1 subtypes available in the HIV database (www.hivlanl.gov), using the maximum likelihood method (Supplementary Fig. S1); another phylogenetic tree was constructed including the consensus sequence for validating the representativeness of this sequence (Fig. 1 and Table 2).

Phylogenetic analysis revealed that the majority of the sequences belonged to subtype C ($n = 10$, 90.9%), with only one isolate from sample NAB001 belonging to subtype B. *In silico* analysis for viral tropism and coreceptor usage revealed that the only subtype B isolate used CXCR4 as the coreceptor, whereas all the rest used CCR5 (Table 3). Upon subsequent discussion with the subject who was identified to have HIV-1 subtype B virus infection (NAB001), we came to know that the individual had lived overseas for a significant period of time and, thus might possibly have picked up the infection from outside India.

Natural development of bNAbs in HIV-1-infected individuals majorly depends on the native structure of the Env trimeric spikes on the virion, epitope exposure, and the position of N-linked glycosylation sites.[16,17] The length and sequence of the variable loops (V1–V5) of the HIV Env also play a critical role in influencing exposure of epitopes[18] to the host immune system. Analysis of the variable regions of the Env genes sequenced in the study showed that the V3 region

Table 1. Clinical, Immunological, and Demographic Profile of 11 HIV-1-Infected Individuals with Broad Cross-Clade Neutralization Property[9]

Sample ID	*Gender*	*Age*[a]	*Mode of transmission*	*Date of diagnosis (month/date/year)*	*Date of collection (month/date/year)*	*CD4 cells/mm*^{3} *At the time of samples collection*	*Viral load (RNA copies/mL) At the time of samples collection*	*Neutralization profile*	*Accession no.*
NAB001	F	37	M-F	2/20/2007	4/11/2011	487	61,600	NP	KY929360
NAB016	M	40	M-M	9/2/2010	4/19/2011	290	188,000	Multiple specific	KY929361
NAB033	M	33	F-M	6/4/2006	4/27/2011	521	97,600	Multiple specific	KY929362
NAB046	F	50	M-F	1/5/2011	5/9/2011	292	107,000	V3-glycan (N332)	KY929363
NAB059	M	47	F-M	10/2/2007	5/9/2011	507	380,000	CD4BS	KY929364
NAB062	F	26	M-F	3/4/2010	5/23/2011	666	3,520	Multiple specific	KY929365
NAB063	M	30	F-M	3/4/2010	5/23/2011	417	33,800	CD4BS	KY929366
NAB065	M	37	NA	3/2/2011	5/26/2011	207	83,400	V3-glycan (N332)	KY929367
NAB069	M	32	M-M	4/20/2011	5/30/2011	210	94,500	Multiple specific	KY929368
NAB120	F	30	M-F	7/1/2007	4/3/2012	435	218,816	NP	KY929369
NAB122	F	47	M-F	4/9/2009	4/3/2012	477	3,074	MPER	KY929370
Mean ± SD	5F:6M	37.18 ± 7.61		Median (IQR)		435 (290–507)	88,950 (11,090–167,750)		

[a]Age at the time of sample collection.
CD4BS, CD4 binding site; MPER, membrane proximal external region; NA, not available; NP, not performed; SD, standard deviation.

was of invariable length (35 aa), whereas each of the remaining variable loops (V1/V2, V4, and V5) had some amount of variation in length and sequence (Supplementary Table S1). However, it was observed that the cysteine residues that were involved in the formation of the V1/V2, V3 and V4 loops were conserved in all the sequences despite the existing variations (Fig. 2). A moderate amount of variation was also observed in the number and position of potential N-linked glycosylation sites in gp120, which ranged from 20 to 30 (Supplementary Table S2), and these were heavily clustered in the V1/V2 and V4 regions. All Env sequences contained four highly conserved potential N-linked glycosylation sites in the gp41 ectodomain (Fig. 2).

Nature and representativeness of the consensus sequence

A consensus sequence representing the 11 experimental sequences was generated with an initial cutoff value of 50% for each position; the consensus sequence had a total of 37 positions as inconclusive at this cutoff. When the cutoff was relaxed to 36.36% (i.e., conserved in at least 4 strains or more out of 11 clinical strains), 21 positions were resolved and the remaining 16 positions were filled using the corresponding amino acids from the HIV-1C reference sequence, pIndie. It was found that except for one envelope sequence (NAB001), all the remaining sequences shared 83%–88% sequence identity and 88%–91% sequence similarity with that of the consensus sequence (Table 2 and Supplementary Fig. S2). The lone NAB001 envelope sequence had only 76% identity and 85% similarity with that of the consensus. pIndie shared 90% sequence identity and 93% sequence similarity with the consensus sequence, whereas HXB2 shared 75% identity and 85% similarity with the consensus. The higher level of similarity between the consensus sequence and the majority of the experimental envelope sequences (10 out of 11) enhanced our confidence to use the consensus sequence as the representative sequence for the study samples. Previous studies have reported amino acid sequences of HIV-1 proteins to differ by 8%–17% within a particular clade and up to 35% between clades depending on the subtypes and genomic regions examined.[16] Similar observations were made in our study. Interestingly, when the reference sequences of clades B (HXB2) and C (pIndie) were compared, it was found that they had only 73% identity. In contrast, when one of the representative samples, NAB062, was compared with the clade B and clade C reference sequences, there was an identity of 81% with pIndie but only 73% with HXB2 (Table 2). All these pieces of evidence suggest that the consensus sequence generated in this study could serve as a reasonably good experimental material for further studies.

Conformational epitopes in the envelope of HIV strains from BCN samples

About 38–73 amino acids were predicted to form conformational epitopes from each of the 11 experimental envelope sequences analyzed (Table 4 and Supplementary Fig. S3). The neutralizing antibody epitopes were highlighted manually in the experimental sequences as well as in the consensus, keeping HXB2 as the reference. Interestingly, we found that about one-fifth of the predicted epitopes were already reported critical residues of known conformational epitopes

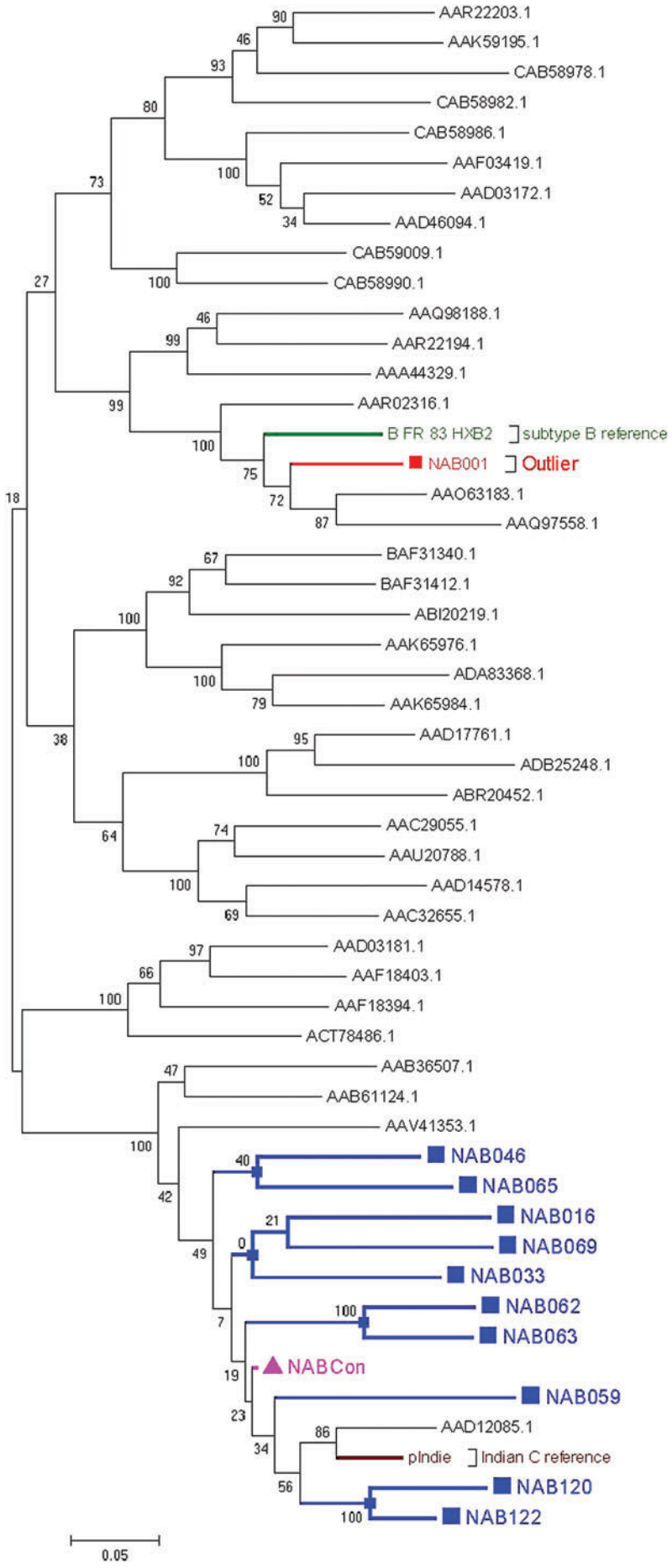

FIG. 1. Phylogenetic analysis using maximum likelihood method. The evolutionary history was inferred using the maximum likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (−22281.2994) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured based on the number of substitutions per site. The analysis involved 49 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 750 positions in the final data set. Evolutionary analyses were conducted using MEGA6.06. *Green*, HIV-1 subtype B reference strain, HXB_2. *Brown*, HIV-1 subtype C reference strain from India, pIndie. *Blue*, sequences generated in this study. *Red*, the one experimental sequence which belongs to subtype B (NAB001). *Pink*, the consensus sequence generated in this study. JTT, Jones, Taylor and Thornton model (1992). Color images are available online.

Table 2. Extent of Relatedness Between the Consensus Sequence (NABCon) and the 11 Envelope Sequences from Clinical Samples, and 2 Reference Sequences (HXB2 and pIndie)

S. no.	Consensus sequence vs. clinical samples	Identity (%)	Positives (%)	Gaps (%)
1	NAB001	661/873 (76)	741/873 (84)	26/873 (2)
2	NAB016	728/864 (84)	776/864 (89)	24/864 (2)
3	NAB033	739/875 (84)	792/875 (90)	22/875 (2)
4	NAB046	745/872 (85)	789/872 (90)	23/872 (2)
5	NAB059	719/863 (83)	775/863 (89)	8/863 (0)
6	NAB062	735/862 (85)	781/862 (90)	14/862 (1)
7	NAB063	732/861 (85)	777/861 (90)	12/861 (1)
8	NAB065	730/872 (84)	777/872 (89)	17/872 (1)
9	NAB069	725/874 (83)	778/874 (89)	14/874 (1)
10	NAB120	742/870 (85)	785/870 (90)	21/870 (2)
11	NAB122	763/868 (88)	799/868 (92)	8/868 (0)
12	pIndie	775/864 (90)	808/864 (93)	11/864 (1)
13	HXB2	654/871 (75)	743/871 (85)	26/871 (2)
14	HXB2 vs. pIndie	630/867 (73)	730/867 (84)	21/867 (2)
15	NAB062 vs. pIndie	700/859 (81)	758/859 (88)	11/859 (1)
16	NAB062 vs. HXB2	630/860 (73)	727/860 (84)	14/860 (1)

(www.hivlanl.gov). The surface area of the envelope and the location of the prioritized amino acids for the representative consensus sequence are shown in Figure 3, and for the experimental sequences, this information is provided in Supplementary Figures. S4–S14. The amino acids identified in this study but not reported previously are listed in Table 5. Interestingly, for samples NAB0122 and NAB033, 73 and 70 amino acids were predicted as critical residues in our analysis, of which, 20 and 23 residues were already reported (Table 4). For three other samples (NAB062, NAB120, and NAB001), 38, 38, and 39 critical amino acid residues were predicted, of which 11, 8, and 8 amino acid residues, respectively, were already reported by others as conformational epitopes.[19,20] Conservation of glycans at position 160 (N160) was observed in 10/11 BCN Envs, and at positions N301 and N332 were seen 10/11 and 9/11env sequences, respectively. Glycans at positions N234 and N276 were found to be conserved in 8/11 and 11/11 env sequences, respectively (Fig. 2).

Conformational epitopes were also predicted from the NABCon. A total of 52 amino acids were identified to form conformational epitopes, of which 10 amino acids were found to be already reported and the remaining (42) were novel (Table 4). Of the 10 already reported amino acids, residues at positions 165, 167, and 168 are known to be specific to the N160 class of bNAbs, whereas those at positions 459, 460, 461, 462, 463, 464, and 465 are critical for CD4BS-specific antibodies.

Characterization of binding specificities of the BCN samples

We previously performed overlapping PepScan ELISA[9] and now extended ELISA with V1, V2, V3, and MPER full-length peptides to determine the binding reactivity of the samples to the full-length peptides (Fig. 4 and Table 6). All the plasma samples ($n=11$) demonstrated strong binding reactivity to the V3 full-length peptide with binding titers ranging between 4,860 and 43,740. Five of the BCN samples (NAB001, NAB016, NAB059, NAB063, and NAB069) showed binding to the full-length MPER peptide with binding titer >180. Sample NAB069, which showed strong binding in the overlapping gp160 PepScan ELISA, gave the highest binding titer of 542.5 to the MPER peptide. We infer that these five plasma samples probably contain antibodies such as 2F5, Z13, 4E10, and 10E8, which recognize linear epitopes of MPER. Two other samples (NAB120 and NAB122), which showed weak reactivity in the overlapping peptide ELISA,[9] failed to demonstrate binding to the MPER full-length peptide. Six plasma samples (NAB001, NAB046, NAB059, NAB062, NAB063, and NAB069) gave low antibody titers (60–62.5) with V1 and V2 peptides; the binding of the other five plasma samples to V1 and V2 peptides was even weaker. Three samples (NAB033,

Table 3. HIV-1 Subtypes and Coreceptor Tropism

Sample ID	Subtype prediction tools				Coreceptor prediction tools					Conclusion Subtype/ coreceptor
	RIP	REGA	jpHHH	COMET	Geno2Pheno	Phenotype	Dskernel	WebPSSM	AUTO-MUTE	
NAB001	B	B	B	B	CXCR4	CXCR4	CXCR4/CCR5	CXCR4	CXCR4	B/CXCR4
NAB016	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB033	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB046	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB059	C	C	C	C	CCR5	CXCR4	CCR5	CCR5	CCR5	C/CCR5
NAB062	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB063	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB065	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB069	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5
NAB120	C	C	C	C	CCR5	CXCR4	CCR5	CCR5	CCR5	C/CCR5
NAB122	C	C	C	C	CCR5	CCR5	CCR5	CCR5	CCR5	C/CCR5

One BCN plasma sample alone was identified as HIV-1 subtype ''B'' and the rest as subtype ''C''.
All samples except one showed CCR5 coreceptor usage, whereas one alone showed CXCR4 usage.
BCN, broad cross-clade neutralization.

NAB065, and NAB120) showed binding response to the V3 peptide only. Two other samples (NAB016 and NAB122) showed binding to V3 and MPER peptides.

A recent study reported that antibodies against the V1–V2 region could play a major role in decreasing the risk of infection.[21] HIV-1 subtype C V1–V2 tag, C.1086 D7gp120, and C.1086 gp140C (Env subunit protein) representing the subtype C.1086 envelope of a South African clade C-transmitted founder virus[22] were used for this part of the analysis. We performed ELISA to look for the presence of V1–V2 targeting antibodies, as well as antibodies to the gp120 monomeric and gp140 trimeric proteins. All the plasma samples showed weak binding to the V1–V2 tags. In contrast, all samples, except NAB069, showed similar levels of reactivity with both D7gp120 and gp140 of C.1086. Sample NAB069 alone bound more strongly to gp140 than to D7gp120 (Fig. 5). We performed two-way ANOVA to test the significance of difference in binding to the monomeric D7gp120 and trimeric gp140 protein, and found that the binding of NAB069 to gp140 protein was significantly greater ($p < .001$) than the binding to the monomeric protein (Fig. 5).

Sample NAB069 exhibited multiple specificities, such as CD4BS (+++), N160 (++), and N332 (+++) glycan-specific neutralization properties. The *in vitro* results obtained were duly supported by the *in silico* predictions. Eight CD4BS-specific amino acid residues (at positions 467, 468, 471–476 of NAB069 corresponding to positions 458–465 in HXB2) were identified (Table 4 and Supplementary Fig. S12); these amino acid residues were tested experimentally and confirmed to be present in the bNAb epitopes by other investigators.[19,23] Furthermore, conservation was noted at position 141THR (which corresponds to N137 in HXB2), which is again a known critical amino acid residue in the conformational epitope. Three amino acids at 166, 168, and 169 of NAB069 corresponding to 165ILE, 167GLY, and 168LYS of HXB2 (Table 5), also known to be essential for interaction with N160-specific bNAbs, were found to be present in the NAB069 env.[24,25] In addition to these 12 amino acids, 53 more residues were consistently predicted as critical residues for conformational epitopes, which are novel and their ability to induce/bind to bNAbs is yet to be evaluated (Table 5). Interestingly, 10 of the 12 amino acids in the conformational epitope positions were also predicted as epitopes in the NABCon (Table 4).

Another sample that showed strong neutralization specificity to the N160 and N332 glycans was NAB062. The *in silico* analysis identified two known critical amino acid residues for the conformational epitopes, namely 165ILE and 167GLY, in this sample. However, we did not find any known critical residues in the epitopes associated with N332 specificity. Nine of the 11 amino acids for the conformational epitopes identified in this sample through bioinformatics analysis at positions 273, 450, 451, 454–459, corresponding to positions 278 and 458–465 in HXB2, are known to confer CD4BS specificity; however, only 4 of the 9 amino acids at these positions (278THR, 458GLY, 459GLY, and 463ASN) were conserved whereas the remaining 5 had substitutions with other amino acids, but still predicted as conformational epitopes in this analysis. The difference between the *in vitro* observations and the *in silico* prediction needs to be further evaluated, although it may be attributed to the presence of the five mutations at the predicted locations in the clinical sample. Furthermore, 27 novel residues were predicted as important amino acids for conformational epitopes, which could also contribute to the NAb specificities seen in this sample (Table 5 and Supplementary Fig. S9).

Sample NAB063 was found to possess strong CD4BS-specific neutralization activity (++). A total of 41 different amino acids were predicted consistently to make conformational epitopes in this sequence using bioinformatics methods. Of these, 11 amino acids were previously reported; 8 of the 11 reported residues at positions 458, 459, 460, 461, 462, 463, 464, and 465 are known CD4BS-specific epitopes. Among these eight, three residues (458GLY, 459GLY, and 463ASN) were conserved between NAB063 and HXB2, whereas the remaining five were not (Table 4 and Supplementary Fig. S10).

NAB033 was found to exhibit *in vitro* neutralizing activity specific to the CD4BS (+), N160 (+), and N332 (++) glycans. The *in silico* results correlated well with the *in vitro* results of this sample. Six different residues at positions 308, 318, 460, 461, 463, and 464, known to be CD4BS-specific conformational epitopes, and another six residues at positions 156, 160, 161, 162, 163, and 164, known to be N160-specific epitopes, were identified in this sample. Furthermore, a total of 14 amino acids known to contribute to specificity to the N332 class of bNAbs were also identified (Table 4 and Supplementary Fig. S6).

NAB046 was found to exhibit strong specificity (+++) to the N332 glycans in the *in vitro* experiments. A total of 54

FIG. 2. Alignment of deduced amino acid sequences of the HIV-1 Envs obtained from the 11 samples exhibiting BCN property. Nucleotide sequences were translated, aligned, and compared with a consensus of the 11 sequences using clustal; the consensus sequence was generated using Consensus Maker (www.hivlanl.gov). Numbering of amino acid residues begin with the first residue of Env sequence according to the consensus sequence. *Dots* denote sequence identity, whereas dashes represent gaps introduced to optimize alignments. *Triangles* above the consensus sequence denote cysteine residues (*solid triangles* indicate sequence identity, whereas *open triangles* indicate sequence variation). V1, V2, V3, V4, and V5 regions denote hypervariable HIV-1 gp120 domains, as previously described. The signal peptide and Env precursor cleavage sites are indicated; ''MPER'' denotes the membrane proximal external region in gp41. Potential N-linked glycosylation sites (NXYX motif, where X is any amino acid other than proline and Y is either serine or threonine) conserved in at least 8 of the 11 clones are *shaded gray*. Positions of N-linked glycans that are part of broadly neutralizing epitopes are indicated: N156 and N160 (e.g., PG9), N234 and N276 (e.g., 8ANC195), and N301 and N332 (e.g., PGT128). Seven BCN Env sequences (7/11) possess lysine (K) residue at position 169 in the V2 region, which was identified as a conserved site of immune pressure in the RV144 vaccine trail. *Asterisks* (*blue*) are used to denote positions associated with resistance to broadly neutralizing antibodies targeting the CD4BS (HXB2 positions 121, 179, 202, 279, 280, 304, 420, 423, 424, 435, 456,458, 459, 471, and 474).[65] BCN, broad cross-clade neutralization; CD4BS, CD4 binding site. Color images are available online. Figure 2 can be viewed in greater detail online.

amino acids were consistently predicted as critical amino acid residues for conformational epitopes from the viral env sequence, of which, 11 were already reported and 43 were novel (Table 4 and Supplementary Fig. S7). However, none of the known amino acid residues were specific to the N332 class of bNAbs, implying that novel determinants for this specificity could be found among the 43 novel amino acids in the conformational epitopes predicted by the bioinformatics tools (Table 5).

NAB065 also demonstrated strong binding specificity (++) similar to the N332 class of bNAbs in *in vitro* analysis. A total of 45 amino acid residues were identified as critical for

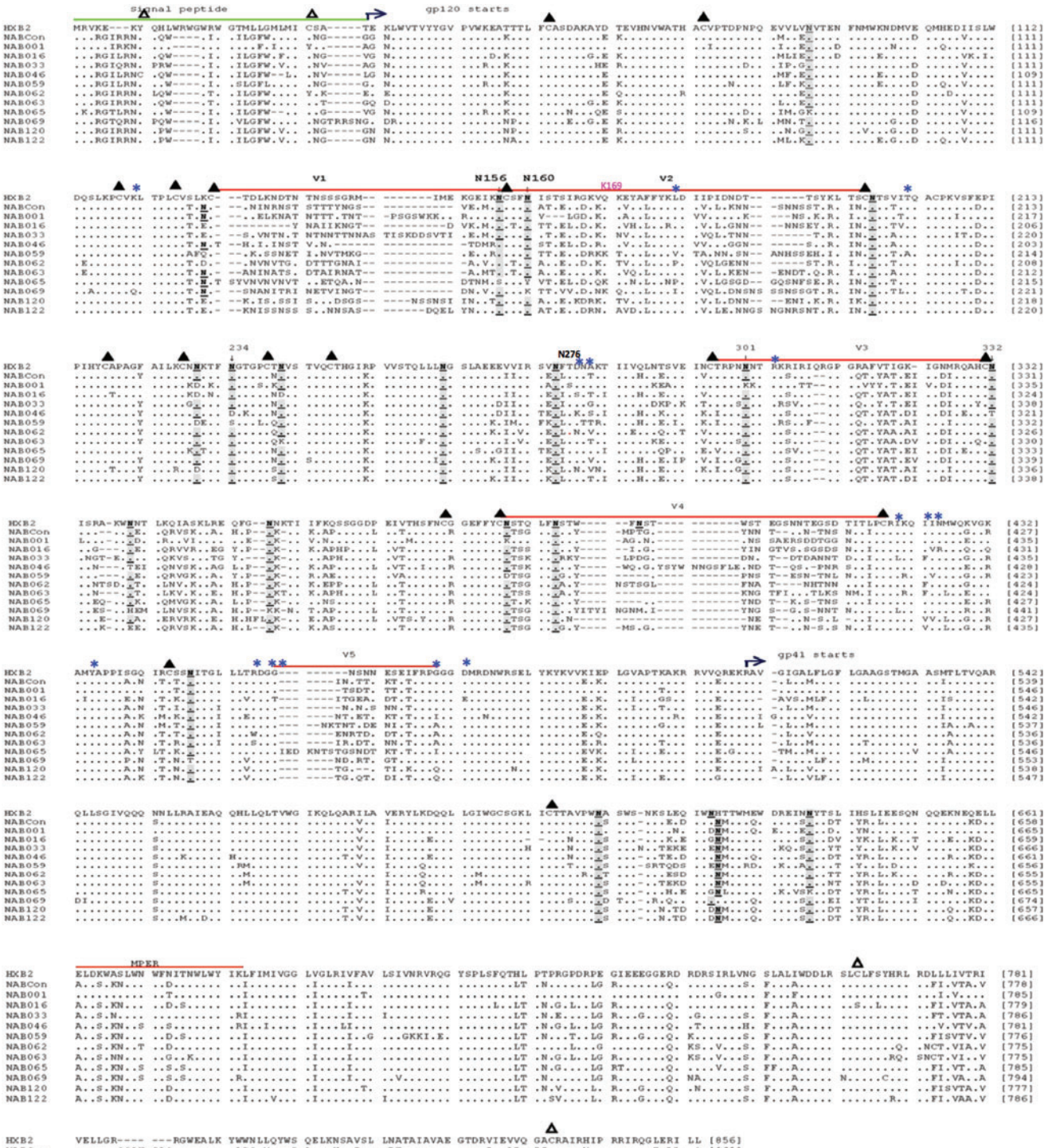

Table 4. Neutralization Epitopes Predicted Using Bioinformatics Tools

S.No	Sample ID	Total no. of amino acids	Reported	Novel	Neutralization property				Amino acid residues predicted using in silico methods and also already reported			
					CD4BS	N160	N332	MPER	Residue number in the clinical sample	Residue name in the clinical sample	Residue number in the HXB2	Residue name in the HXB2
1	NAB001	39	8	31	–	ND	ND	ND	169, 462, 464, 465, 466, 467, 468, 469	ASP, GLY, SER, ASP, THR, ASN, THR, THR	167, 460, 461, 462, 463, 464, 465, 466	GLY, GLY, ASN, SER, ASN, ASN, GLU, SER
2	NAB016	49	18	31	–	+	+	ND	146, 150, 151, 153, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 451, 454, 455	ASN, ASN, THR, THR, ARG, ILE, GLY, PRO, GLY, GLN, THR, PHE, TYR, ALA, THR, GLY, ASN, ASP	156, 160, 161, 163, 308, 309, 312, 313, 314, 315, 316, 317, 318, 319, 320, 460, 463, 464	ASN, ASN, ILE, THR, ARG, ILE, GLY, PRO, GLY, ARG, ALA, PHE, VAL, THR, ILE, ASN, ASN, GLU
3	NAB033	70	23	47	+	+	++	ND	136, 164, 168, 169, 170, 171, 172, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 412, 464, 465, 467, 468	ASN, ASN, ASN, ILE, THR, THR, GLU, ARG, ILE, GLY, PRO, LY, GLN, ALA, PHE, TYR, THR, THR, ALA, ASN, ASN, SER, ASN	137, 156, 160, 161, 162, 163, 164, 308, 309, 312, 313, 314, 315, 316, 317, 318, 319, 320, 409, 460, 461, 463, 464	ASP, ASN, ASN, ILE, SER, THR, SER, ARG, ILE, GLY, PRO, GLY, ARG, ALA, PHE, VAL, THR, ILE, GLU, ASN, SER, ASN, GLU
4	NAB046	54	11	43	–	–	+++	ND	155, 157, 158, 453, 454, 457, 458, 459, 460, 461, 462	LEU, ASP, LYS, GLY, GLY, ASN, GLU, THR, SN, LYS, THR	165, 167, 168, 458, 459, 460, 461, 462, 463, 464, 465	ILE, GLY, LYS, GLY, GLY, ASN, SER, ASN, ASN, GLU, SER
5	NAB059	65	23	42	+	–	+	ND	136, 152, 156, 157, 158, 159, 160, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 455, 456, 457, 458, 459	ASN, ASN, ASN, THR, THR, THR, GLU, ARG, PHE, GLY, PRO, GLY, GLN, ALA, PHE, TYR, ALA, THR, THR, SER, ASP, GLU, ASN	137, 156, 160, 161, 162, 163, 164, 308, 309, 312, 313, 314, 315, 316, 317, 318, 319, 320, 460, 461, 462, 463, 464	ASP, ASN, ASN, ILE, SER, THR, SER, ARG, ILE, GLY, PRO, GLY, ARG, ALA, PHE, VAL, THR, ILE, ASN, SER, ASN, ASN, GLU
6	NAB062	38	11	27	–	+++	+++	ND	160, 162, 273, 450, 451, 454, 455, 456, 457, 458, 459	ILE, ASP, THR, GLY, GLY, ARG, THR, ASP, ASN, ASP, THR	165, 167, 278, 458, 459, 460, 461, 462, 463, 464, 465	ILE, GLY, THR, GLY, GLY, ASN, SER, ASN, ASN, GLU, SER
7	NAB063	41	11	30	++	–	–	ND	160, 162, 163, 450, 451, 454, 455, 456, 457, 458, 459	ILE, GLY, LYS, GLY, GLY, ASN, ASP, THR, ASN, ASN, ASN	165, 167, 168, 458, 459, 460, 461, 462, 463, 464, 465	ILE, GLY, LYS, GLY, GLY, ASN, SER, ASN, ASN, GLU, SER

(*continued*)

TABLE 4. (CONTINUED)

S.No	Sample ID	Total no. of amino acids	Reported	Novel	Neutralization property				Amino acid residues predicted using in silico methods and also already reported			
					CD4BS	N160	N332	MPER	Residue number in the clinical sample	Residue name in the clinical sample	Residue number in the HXB2	Residue name in the HXB2
8	NAB065	45	10	35	–	–	++	ND	137, 162, 164, 454, 464, 465, 466, 467, 468, 469	ASN, LEU, ASP, GLY, SER, ASN, ASP, THR, LYS, THR	137, 165, 167, 459, 460, 461, 462, 463, 464, 465	ASP, ILE, GLY, GLY, ASN, SER, ASN, ASN, GLU, SER
9	NAB069	65	12	53	+++	++	+++	++	141, 166, 168, 169, 467, 468, 471, 472, 473, 474, 475, 476	THR, VAL, ASP, LYS, GLY, GLY, ASN, ARG, THR, ASN, GLY, THR	137, 165, 167, 168, 458, 459, 460, 461, 462, 463, 464, 465	ASP, ILE, GLY, LYS, GLY, GLY, ASN, SER, ASN, ASN, GLU, SER
10	NAB120	38	8	30	–	ND	ND	–	167, 169, 170, 454, 457, 458, 459, 460	ILE, ASP, ARG, GLY, ASN, ASN, THR, ILE	165, 167, 168, 459, 460, 463, 464, 465	ILE, GLY, LYS, GLY, ASN, ASN, GLU, SER
11	NAB122	73	20	53	–	ND	ND	++	136, 156, 160, 161, 162, 163, 164, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 465, 468	ASN, ASN, ASN, ALA, THR, THR, GLU, ARG, ILE, GLY, PRO, GLY, GLN, THR, PHE, TYR, ALA, THR, ASN, ASN	137, 156, 160, 161, 162, 163, 164, 308, 309, 312, 313, 314, 315, 316, 317, 318, 319, 320, 460, 463	ASP, ASN, ASN, ILE, SER, THR, SER, ARG, ILE, GLY, PRO, GLY, ARG, ALA, PHE, VAL, THR, ILE, ASN, ASN
12	NABCon	52	10	42	–	–	–	–	161, 163, 164, 454, 457, 458, 459, 460, 461, 462	ILE, ASP, LYS, GLY, ASN, THR, THR, ASN, LYS, THR	165, 167, 168, ***459, 460, 461, 462, 463, 464, 465***	ILE, GLY, LYS, **GLY, ASN, SER, ASN, ASN, GLU, SER**

Bold indicates amino acids within the CD4BS.
Bold italics indicates amino acid positions responsible for CD4 BS-specific antibody specificity.
– indicates <1-fold; + indicates 1 to 1.9-fold; ++ indicates 2 to 2.9-fold; +++ indicates >3-fold increase in neutralization activity.
Amino acid positions 165, 167, and 168 are N160 Class specific, while positions 459, 460, 461, 462, 463, 464, and 465 are CD4BS-specific antibodies.
ND, not done.

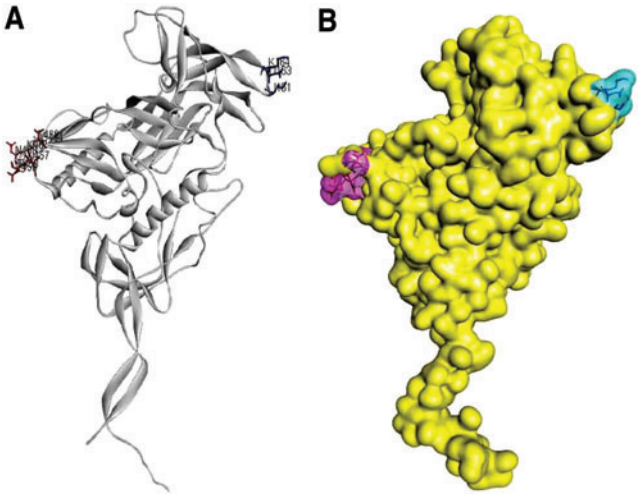

FIG. 3. Mapping of conformational epitopes on the envelope structure of NABCon. **(A)** The envelope structure is shown in the form of solid *gray ribbon*. CD4BS-specific neutralizing antibody epitopes are displayed as *red sticks* and N160-neutralizing antibody epitopes are displayed as *blue sticks*. Position and amino acid residue indicated in the figure pertain to the sequence of the respective clinical sample. **(B)** The surface representation of the envelope structure is shown in *yellow*; CD4BS-neutralizing antibody-specific epitopes are in *pink*, and N160-neutralizing antibody-specific epitopes are in *cyan*. Color images are available online.

conformational epitopes in this sample: of these, 10 were already reported and the remaining 35 were novel (Tables 4 and 5; and Supplementary Fig. S11). Among the reported amino acid residues, however, only one was found to be specific for N332, again prompting us to look for novel amino acid residues in the conformational epitopes identified for the first time in this sample.

Discussion

The HIV envelope is known to be the sole target for neutralizing antibodies, and molecular characterization of the envelope will help in the identification of epitopes capable of catalyzing the synthesis of/or binding to bNAbs, and provide critical information for vaccine design. India is reported to be home to 7.6% of the world's population living with HIV-1.[26] Studies from different parts of India, including Chennai, found that subtype C viruses are the most predominant in the Indian epidemic.[27–32] Hemelaar *et al.* reported that 97% of HIV-1 infections in India are caused by subtype C viruses.[33] However, sporadic cases of subtypes A, B, E, and recombinant forms have also been reported.[31,32,34–40] In this study too the majority of the HIV-1 sequences analyzed belonged to clade C. The sporadic occurrence of clade B infection in one sample was noticed.

A consensus sequence, which we call NABCon, representing the 11 experimental sequences was generated in this study. Although the consensus sequence is essentially a theoretical sequence, we were able to confirm through further sequence analysis that the vast majority of the clinical strains shared 83%–88% sequence identity and 88%–91% sequence similarity with that of the consensus sequence, except the sole subtype B sequence that had 76% identity and 85% similarity

Table 5. Neutralization Epitope-Specific Amino Acid Residues Identified Using *In Silico* Methods from the 11 BCN and NABCon Env Sequences

S. no	*Sample ID*	*Novel amino acid residues predicted using* in silico *methods*
1	NAB001	D61, T138, N139, T140, N188, D189, K190, N191, E357, N358, K359, T360, W397, A398, G399, N400, N404, S405, S406, A407, E408, S410, T413, T468, R508, V509, V510, Q511, R512.
2	NAB016	G31, N32, L33, T139, D140, V141, K142, E143, M144, K145, N146, C147, T148, F149, N150, T151, T153, F166, N179, N180, S181, K298, S299, I300, I302, G303, P304, G305, Q306, T307, A310, T311, G387, T388, N391, S395, G451, N454.
3	NAB033	G31, N32, L33, V133, N134, T135, N136, T138, N139, T140, N141, N142, T143, N145, A147, S148, T149, I150, S151, K152, D153, D154, S155, T157, I158, K159, S166, E172, L183, R311, A321, F322, I328, P401, D402, G403, N406, A412, N413, N468.
4	NAB046	G29, N30, D131, D133, S136, L155, D157, D158, P341, N342, D343, T344, W382, Q383, G385, Y387, S388, W390, N391, G393, S394, F395, L396, E397, W398, N399, D400, T401, Q402, S403, T404, P405, N406, G453, G454, D497, R499, R500, R501, V502, V503.
5	NAB059	E31, N32, L33, K132, L133, S134, S135, N136, T138, I139, N140, N141, M144, K145, G146, K147, E148, E149, I150, R151, N152, C153, S154, F155, T157, T158, T159, F172, S188, S189, E190, Y192Y, I193, R305, R306, S307, F310, G311, I322, P394, N395, S396, E398, K452.
6	NAB062	E31, E32, E61, P349, N350, K351, T352, N390, S391, G394, F396, N397, T399, N400, G451, E452, N453, R454, K494, K496, R497, R498, V499, V500, E501, R502.
7	NAB063	Q31, D32, E61, N138, D139, T140, E346, P350, N351, K352, T353, T354, N393, G394, T395, F396, I397, N399, T400, G451, I452, R453, N454, R497, R498, V499, V500, E501, R502.
8	NAB065	G29, N30, E59, N135, V136, V138, T139, T140, N141, P355, N356, K357, T358, S396, T397, Y398, N399, D400, T401, K402, N403, G454, K468, E504, K506, R507, R508, V509, V510, E511, R512.
9	NAB069	D37, R38, W39, V40, V42, I143, N144, E145, V166, R167, D168, K169, S189, N190, S191, K362, I402, T403, Y404, I405, N406, G407, N408, M409, S410, I411, Y412, N413, G414, S415, G416, D470, N471, G475, P509, T510, K511, A512, K513, R514, R515, V516, V517, E518, R519.

(*continued*)

Table 5. (Continued)

S. no	*Sample ID*	*Novel amino acid residues predicted using* in silico *methods*
10	NAB120	N31, N32, L33, N140, N147, N189, N190, H358, F359, L360, N361, K362, T363, E401, T402, N403, N404, N405, S406, G454, K495, A496, K497, R498, R499, V500, V501, E502.
11	NAB122	G30, N31, N32, L33, W34, V35, N132, I133, S134, S135, N136, S138, S139, N140, S141, N142, S144, A145, S146, D147, Q148, E149, L150, Y151, N152, E153, I154, K155, C157, S158, F159, Y177, N196, R199, R311, T321, F322, I328, M401, S402, N403, G404, N407, N412, S413, N468, P503, E505.
12	NABCon	G31, N32, L33, S137, S139, T140, T141, Y143, R162, N184, S185, N187, P352, N353, K354, T355, M393, P394, T395, G396, T397, Y398, N399, N400, T401, N402, N403, N404, T405, I455, N456, K497, A498, K499, R500, R501, V502, V503, E504, R505, E506, K507

with the consensus. Furthermore, we found very close relatedness between the NABCon and the subtype C reference sequence, pIndie, and remote relatedness between the NABCon and the clade B reference strain HXB2: these observations provide encouraging evidence for the usefulness of the generated consensus sequence for further research. It was interesting to note that the two reference sequences of clade C and clade B also shared only 73% identity, whereas the NABCon shared >90% sequence identity with pIndie. These findings are in line with previous studies that found that a theoretical consensus sequence generally differs from the natural sequences by ~17% (4%–30%) in the Env.[41] These findings suggest that the consensus sequence generated in this study is in fact central and representative of the HIV-1C strains circulating in India.

Of the 52 amino acids predicted to form conformational epitopes using *in silico* methods, 10 were found to have already been identified by other investigators as critical residues of conformational HIV-1 epitopes. We, therefore, believe that the remaining 42 amino acid residues identified as components of conformational epitopes for the first time in this study have a fairly high chance of being important residues within the conformational neutralizing antibody epitopes. We used two different antibody-specific epitope prediction servers namely ElliPro[10] and DiscoTope 2.0[11] to improve the likelihood of our prediction. Discotope algorithm was reported by its developers to fail in identifying conformational epitopes from HIV antigen–antibody complexes.[11] However, in this study, we used the antigen (protein) structure alone (not the antigen–antibody complex) to predict epitopes and this could be the probable reason for the prediction of large number of amino acids forming conformational epitopes successfully. To verify our results and increase the accuracy of our prediction, we used an additional algorithm (ElliPro) and report only epitopes identified by both tools as potential epitopes. Since one-fifth of the predicted amino acids in this study were previously identified to be residues within antibody-specific epitopes by other investigators using experimental studies, the value of the prediction gains significance and suggests that the amino acids identified as novel in this study could be potentially critical residues of conformational epitopes targeted by bNAbs, although the prediction needs to be experimentally verified.

Numerous studies[19–23,42] have contributed to the identification of essential amino acids required for the binding of CD4BS, PG9/PG16-like, and PGT series of bNAbs on the HIV-1 Env. We observed a fair amount of correlation

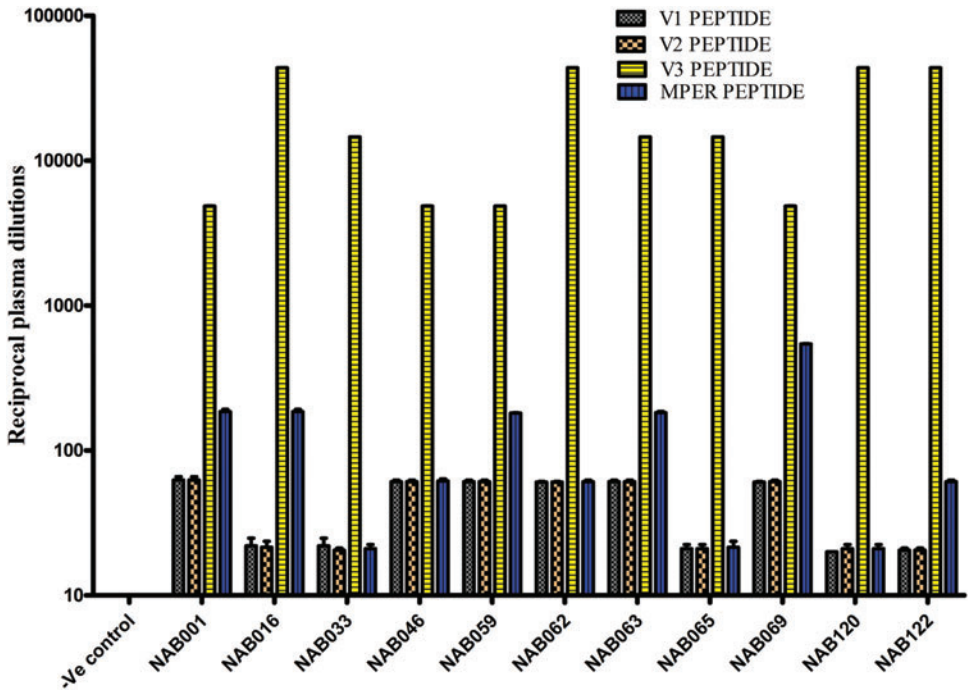

FIG. 4. ELISA with full-length variable loop (V1, V2, and V3) and MPER peptides. Full-length peptides V1, V2, V3, and MPER of 93IN101 envelope protein were used. Plasma samples ($n = 11$) from BCN were tested at dilutions 1:20 to 1:43,740 for the identification of binding reactivity. HHP was used as the negative control. Mean absorbance was calculated from each experiment performed in triplicate and on two independent occasions. HHP, healthy human plasma; MPER, membrane proximal external region. Color images are available online.

TABLE 6. BINDING TITER ANALYSIS WITH V1, V2, V3, AND MEMBRANE PROXIMAL EXTERNAL REGION PEPTIDES

Peptide	Sequence	Peptide binding titers (reciprocal plasma dilutions)										Control plasma (HHP)
		NAB001	NAB016	NAB033	NAB046	NAB059	NAB062	NAB063	NAB065	NAB069	NAB120	NAB122	
V1	RNVSRNVSSYNTYNGSVEEIKNC	62.5	22	22	61	61	60.5	61	21	60.5	20	20.5	<20
V2	SFNATPEVRDRKQRMYALFYGLD IVPLNKKNSSENSSEYRLINC	62.5	21.5	20.5	61	61	60.5	61	21	61	21	20.5	<20
V3	CTRPNNNTRKSIRIGPGQTFYATG DIIGDIRQAHC	4865	43740	14580	4863	4861	43730	14580	14580	4863	43740	43740	<20
MPER	LALDSWKNLWSWFDITNWLWYIK	181	180	21	61.5	181	61	182.5	21.5	542.5	21	61	<20

ELISA for binding titer analysis of 11 BCN plasma samples: the binding of plasma samples to V1, V2, V3, and MPER peptides tested by ELISA at dilutions ranging from 1:20 to 1:43,740 (8 dilutions). HHP was used as negative control. The maximum binding titers to the peptides are indicated as color less if <50, *light gray* if 50–100, *dark gray* if 101–1000, and *gray* if >1000. This experiment was performed in triplicate on two independent occasions.

<50 50–100 101–1000 >1000

HHP, healthy human plasma.

between the *in vitro* results and *in silico* predictions of conformational NAb epitopes. Based on both *in vitro* and *in silico* findings, we surmise that NAB069 is one of the most promising samples identified in this study, showing multiple neutralization specificities resembling that of the CD4BS, N160, and N332 class of bNAbs. The second best sample appeared to be NAB062, which demonstrated *in vitro* specificity to the N160 and N332 class, but no specificity to the CD4BS. In contrast, in the *in silico* analysis, nine amino acids were identified as critical amino acid residues for conformational epitopes specific to the CD4BS. However, when the sequence alignment was examined, it was found that only four of these nine amino acids were conserved as in HXB2, whereas the remaining five amino acid positions had substitutions, prompting us to hypothesize that these mutations could be responsible for lack of production of this class of neutralizing antibodies in this individual. However, this hypothesis needs to be further investigated and validated using experimental studies. Several important correlations and also some differences were observed between the *in vitro* results and *in silico* predictions: 8/11 and 11/11 BCN samples showed conservation (>70%) of glycans at position N234 and N276, respectively, which are thought to be critical for CD4BS specificity. The PG9/PG16 and PGT class specificities depend largely on glycans at positions N160 and N332, respectively, for neutralization activity. We found that 10/11 and 11/11 BCN samples showed conservation (>90%) at glycan positions N160 and N332, respectively, suggesting the presence of PG9/PG16-like and PGT series of bNAbs in these samples.

It is well known that the CD4BS and coreceptor binding site (CoRS) of HIV-1 gp120 form critical interactions with the host cell surface proteins, which are essential for initiating infection. The CoRS comprises of regions in the V3 loop and the bridging sheet, which includes the β20 and β21 strands of C4 and the β2 and β3 strands of the V1V2 stem.[43–46] Infectivity is completely abrogated with deletion of the V3 region of gp120.[47] The essential role played by the V3 region makes it a popular target for the development of antibodies. Most HIV vaccines induce production of antibodies against the V3 region.[48–50] In natural infection, V3-specific antibodies are known to develop early during HIV-1 infection. In subtype C HIV-1, the GPGQ linear motif in the V3 region is believed to play a major role in the binding of V3-specific antibodies. It has been observed that the V3-binding antibodies generally neutralize tier-1 HIV-1 pseudoviruses, but rarely neutralize tier-2 or tier-3 pseudoviruses.[51,52] Although anti-V3 antibodies are largely non-neutralizing in nature, a very small percentage of these types of antibodies are capable of mediating antibody-dependent cell-mediated cytotoxicity by binding to virions.[53] Hence, antibodies that bind to the V3 region can potentially neutralize HIV-1 or block the virus from interacting with coreceptors. All the samples tested in this study showed strong binding specificity to V3 full-length peptide, indicating the presence of V3-specific antibodies. In contrast, none of the samples showed significant binding to the V1/V2 peptides.

The MPER that is present on the transmembrane region of gp41 is known to be critical for fusion and cellular entry, implying that a large degree of conservation is required in this region to maintain its function.[54–56] Previous studies have reported that antibody responses to MPER were detectable

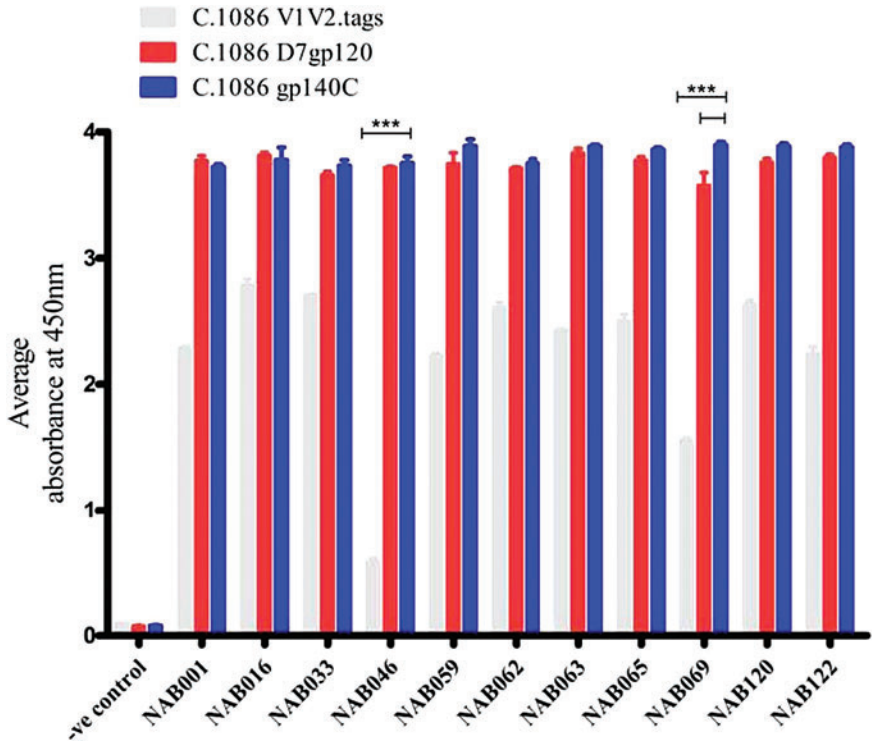

FIG. 5. ELISA with HIV-1 subtype C V1V2 tag, D7gp120, and gp140 proteins. Three forms of Env proteins (V1V2 tagged, gp120 monomer, and gp140 trimer) from HIV-1 subtype C strain were used for the identification of antibodies specific to conformational epitopes in BCN samples. A single dilution of 1:200 was used for plasma. HHP was used as the negative control. Mean absorbance was calculated from each experiment performed in triplicate on two independent occasions. Color images are available online.

within 40 to 70 days of infection[57]; however, only a few individuals may develop broad neutralization activity.[58,59] In our study, three BCN samples (NAB069, NAB120, and NAB122) showed antibody binding response to the overlapping peptides of MPER. However, only one of the three samples (NAB069) exhibited strong binding when tested subsequently with full-length MPER peptide, with a titer >500. The remaining four samples exhibited moderate reactivity with the full-length MPER peptide (titer 180–500).

It is now widely accepted that the antibody response to HIV is diverse and that only antibodies binding to the functional envelope spikes are able to directly neutralize the virus.[3] The HIV-1 Env functional spike is a trimeric heterodimer formed by three molecules of gp120 and three molecules of gp41. The functional spike of HIV-1 Env has certain conserved conformational epitopes essential for viral entry into $CD4^+$ target cells.[60] Most of the well-characterized bNAbs have been shown to bind to conformational epitopes present on the Env of HIV-1.[8,61] In our experiments as well, we found that the BCN samples reacted better with the conformational Env proteins than with the linear peptides. One BCN sample (NAB069) showed significantly stronger binding to gp140 trimer (full gp120+a part of gp41) than to the other two proteins, implying the predominant presence of antibodies that target conformational epitopes in the native form of the protein in this individual. Our observations are in agreement with previous studies,[62–64] which also reported that antibodies in HIV-1-infected individuals show stronger binding to natural forms of the HIV-1 Env protein than to the linear peptides.

To summarize, all the BCN Env sequences had conserved residues at the major glycans positions critical for the development of bNAbs. Furthermore, all sequences had conserved cysteine residues involved in the formation of the variable loops V1, V2, V3, and V4. In the *in vitro* binding assay, sample NAB069 showed significant difference in binding to the gp120 monomer and gp140 trimer, strongly indicating the presence of conformational antibodies, besides having conserved glycans critical for binding of glycan-dependent antibodies. The next big challenge after successful identification of critical residues of conformational bNAb epitopes is to apply this information to guide the synthesis of neutralizing antibody epitopes that can elicit a protective antibody response in the human to develop a successful HIV-1 vaccine. One of the encouraging findings of this study is that the consensus sequence, NABCon, which we generated based on the experimental sequences obtained in this study, though theoretical, is truly representative, and contains 52 amino acids forming conformational epitopes, of which 10 are already reported. This sequence can, therefore, serve as a good resource for further work in this line. Furthermore, we strongly propose that the novel amino acid residues in the conformational epitopes identified in this study could be highly potential candidates that deserve further testing and evaluation *in vitro* for their role in neutralizing antibody production.

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Authors Disclosure Statement

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