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Neutralization Potential of the Plasma of HIV-1 Infected Indian Patients in the Context of Anti-V3 Antibody Content and Antiretroviral Theraphy

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We assessed the anti-V3 antibody content and viral neutralization potential of the plasma of 63 HIV-1-infected patients (antiretroviral naïve=39, treated=24) against four primary isolates (PIs) of clade C and a tier 1 clade B isolate SF162. Depletion and inhibition of anti-V3 antibodies in the plasma of five patients with high titers of anti-V3 antibodies led to modest change in the neutralization percentage against two PIs (range 0–21%). The plasma of antiretroviral-treated patients exhibited higher neutralization potential than that of the drug-naïve plasmas against the four PIs tested which was further evidenced by a follow-up study.

Keywords: HIV-1, India, neutralizing antibodies, anti-V3 antibodies, antiretroviral therapy

Introduction

The relative resistance of primary isolates (PIs) of human immunodeficiency virus (HIV-1) to neutralization by a wide range of antibodies (Abs) remains a theoretical and practical barrier to the development of an effective HIV vaccine. During the course of HIV-1 infection, neutralizing antibody (NAb) response is an important component of the host immune response (Richman *et al.*, 2003; Wei *et al.*, 2003; Frost *et al.*, 2005). The viral envelope (env) glycoprotein (gp120 and gp41) appears to be the sole target for NAbs. The presence of broadly cross-reactive anti-V3 Abs suggests that the third variable (V3) domain of gp120 is a key immunogenic epitope (Stanfield *et al.*, 2006). Further, the extended nature and accessibility of the V3 region on the trimeric envelope explain its immunodominance (Huang *et al.*, 2005). Recent studies on the immune response against HIV-1 have been focused on the V3 loop of the gp120 env region and have found that the neutralizing activity is primarily due to the V3-specific Abs (Zolla-Pazner *et al.*, 2008).

In a study conducted in 35 Indian HIV-1 patients, Lakhashe *et al.* (2007) have shown that 29% of the patient plasma have effective NAbs to HIV-1 at a tenfold dilution. In a recent report, Kulkarni *et al.* (2008) observed that, of 33 HIV-1 infected plasma samples tested against two HIV-1 subtype C isolates, 21% of the plasma samples exhibited 65–100% neutralization potential against both isolates. Based on the sequence diversity of the V3 region of the viruses infecting these patients, they predicted the V3 region may not play an important role in eliciting a neutralization response in these patients (Kulkarni *et al.*, 2008). The precise status of anti-V3 Abs in the plasma of Indian HIV-1 infected patients and their role in virus neutralization needs to be assessed.

Antiretroviral therapy (ART) effectively reduces the viral load in HIV-1 infected patients (Morris *et al.*, 1998). The neutralization potential of the plasmas of antiretroviral-treated patients has not been studied in detail. In the present study, we have characterized the plasma of HIV-1-infected Indian patients for their anti-V3 antibody content and potency of viral neutralization against four PIs (clade C) raised in our laboratory and one clade B, tier 1 isolate SF162 (obtained from the NIH Reference and Reagent Program).

Sixty-three HIV-1-seropositive patients were recruited for this study, including both antiretroviral-naïve and treated patients. We further followed up seven drug-naïve patients after 1 month of initiation of ART. The study was approved by the institute ethics committee and informed consent was obtained from all the participants. Whole blood was collected in EDTA vacutainers. The plasmas of HIV-1 seropositive patients ware aliquoted and stored at -70°C until tested. The plasma was heat inactivated for 30 min at 56°C before being used in the experiments.

Viruses were isolated from the blood of HIV-1-infected patients by standard co-culture techniques using peripheral blood mononuclear cells (PBMCs) (Schuitemaker and Kootstra, 2005). Four PIs (AIIMS 151, AIIMS 126, AIIMS 70, and AIIMS 65) were raised exclusively in PBMCs obtained from healthy donor blood.

To obtain high titers of viruses in culture, we again passaged the viruses in donor PBMCs. Viral envelope sequences from the partial C2 region to the partial C4 region were determined using nested PCR. Viral RNA was isolated from the culture supernatant using a viral RNA purification kit (QIAGEN, Germany). First round forward and reverse pri-

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mers were *Env*-1 gp120 5′TCAGCACAGTACAATGTAC ACATGGAAT3′ (HXB2 6949–6976) and *Env*-2 gp120 5′ GTGCTTCCTGCTCCCAAGAACCCA (HXB2 7810–7784). The second round primers were *Env*-3 gp120 5′TGTTAAA TGGCAGTCTAGCAGAA (HXB2 7003–7025) and *Env*-4 gp120 5′TTATATAATTCACTTCTCCAATT3′ (HXB2 7678–7656). Sequencing of the purified PCR products was done commercially at Lab India (India). Phylogenetic analysis was done by the neighbor joining method using MEGA4 software with 1,000 bootstrap replicates.

Neutralizing activity against PIs was measured as the reduction in luciferase reporter gene expression after a single round of virus infection in JC53-BL cells as described previously (Li *et al.*, 2005). The patients plasmas were tested against each PI at different dilutions from 1:100 to 1:20000. The controls for background detection contained cells only while the virus control contained cells and the viruses. For all dilutions of plasma, the percent neutralization was calculated based on the relative luminescence units (RLU) in the presence of plasma divided by the virus control. Cell control value was subtracted from the plasma RLU value as the background cut-off. The 50% neutralizing titer (ID50 titer) was determined from the linear portion of the titration curve using the method of least squares. HIV-1 seronegative

Table 1. Neutralization titer (ID50) and anti-V3 antibody content of the plasma of antiretroviral naive patients (n=39)

Plasma ID	Sex	Age	AIIMS 65	AIIMS 70	AIIMS 126	AIIMS 151	SF 162	CD4 Count (cells/μl)	Clinical status	OD 405
Healthy Donor	M	30	NN	NN	NN	NN	NN	ND	-	0.168
102	M	32	ND	ND	< 100	< 100	ND	55	S	0.428
107	M	38	ND	ND	> 20000	< 100	ND	110	S	0.7
104	M	31	< 100	221	< 100	< 100	1530	263	AS	2.842
111	M	32	< 100	145	3800	< 100	930	102	S	1.544
112	M	27	< 100	< 100	< 100	< 100	4300	300	AS	3.325
118	M	35	ND	ND	> 20000	8050	ND	220	S	2.39
125	F	48	< 100	< 100	162	< 100	290	70	S	3.264
137	M	27	< 100	261	1410	< 100	2360	132	S	3.462
147	M	42	ND	ND	372	< 100	ND	28	S	0.334
148	M	40	< 100	443	1980	280	1160	53	S	1.982
149	M	38	1470	3090	> 20000	442	3680	163	S	1.476
150	M	34	< 100	242	314	< 100	300	147	S	3.197
151	M	36	428	< 100	178	< 100	3580	112	S	2.842
154	M	37	ND	ND	1590	< 100	ND	4	S	0.59
155	F	32	236	1195	2330	262	1800	5	S	1.07
203	F	25	< 100	< 100	< 100	< 100	840	402	AS	1.249
205	M	24	< 100	< 100	< 100	< 100	850	350	AS	2.877
206	M	34	15583	1615	> 20000	3800	5050	583	AS	3.552
207	F	28	< 100	< 100	< 100	< 100	930	413	AS	2.937
208	M	25	< 100	< 100	< 100	< 100	810	246	S	3.577
210	M	32	5250	< 100	< 100	< 100	1830	213	S	0.95
211	F	31	< 100	< 100	410	< 100	1890	278	AS	0.51
212	F	38	< 100	< 100	278	< 100	1380	78	S	2.917
213	M	35	208	< 100	< 100	101	2210	332	AS	1.449
216	M	45	1400	1470	170	< 100	1600	284	AS	2.69
218	F	36	1620	1410	224	101	1260	361	AS	2.006
219	F	26	< 100	< 100	< 100	< 100	1420	579	AS	2.563
220	F	45	358	1870	< 100	101	1980	515	AS	1.274
223	F	20	< 100	< 100	< 100	< 100	< 100	450	AS	0.679
224	M	45	< 100	413	< 100	< 100	< 100	60	S	0.608
225	F	29	180	231	< 100	< 100	356	161	S	3.011
226	F	28	< 100	< 100	180	< 100	1190	109	S	3.379
231	M	37	300	470	690	< 100	2940	416	AS	ND
232	F	35	374	< 100	458	< 100	3480	213	S	ND
233	F	28	< 100	100	700	< 100	980	385	AS	ND
237	M	27	120	186	< 100	240	1270	521	AS	ND
239	M	25	388	2660	2020	112	1060	366	AS	ND
242	M	42	< 100	1280	< 100	< 100	1280	226	S	ND
248	F	36	440	< 100	830	< 100	4020	303	AS	ND

ID50 neutralization titers of the plasma of antiretroviral naïve patients against the primary isolates tested. OD405 refers to the anti-V3 antibodies at absorbance 405 nm. M, male; F, female; NN, non-neutralizing; ND, not determined; S, Symptomatic; AS, Asymptomatic.

Table 2. Neutralization titer (ID50) and anti-V3 antibody content of the plasma of ART treated patients (n=24)

Plasma ID	Sex	Age	Duration of ART (in days)	AIIMS 65	AIIMS 70	AIIMS 126	AIIMS 151	SF 162	CD4 count (cells/μl)	OD405
Healty Donor	M	30	-	NN	NN	NN	NN	NN	ND	0.168
123	M	35	ND	< 100	< 100	266	< 100	1000	ND	2.044
153	F	30	ND	1620	1350	2920	< 100	3000	212	1.934
156	M	28	ND	236	1205	5050	< 100	3100	ND	1.703
191	M	35	ND	282	1060	1080	< 100	1110	ND	0.564
192	M	29	ND	< 100	< 100	1640	< 100	380	ND	0.56
202	M	48	394	100	277	1600	< 100	1220	353	3.15
204	M	31	223	1120	1345	6900	262	3850	216	0.693
209	M	35	ND	290	365	1550	100	1180	297	0.389
214	M	32	228	236	225	1010	1250	1750	192	0.517
215	F	25	170	2880	910	890	312	7400	288	3.245
217	M	35	14	264	265	1110	176	1320	285	0.334
221	M	40	426	8000	7300	11917	2340	4860	460	0.363
222	F	35	523	1220	462	1370	< 100	1120	576	0.257
230	M	30	ND	186	432	3960	< 100	240	357	ND
234	M	28	ND	378	356	< 100	218	6200	255	ND
236	M	41	84	204	1500	436	338	1540	162	ND
238	F	38	548	368	1310	1980	168	3980	271	ND
240	M	40	185	168	186	< 100	166	2980	405	ND
241	M	28	ND	< 100	1120	< 100	< 100	1480	175	ND
243	M	20	134	< 100	1100	< 100	< 100	870	223	ND
244	M	25	ND	246	1060	< 100	272	870	89	ND
245	M	38	207	< 100	338	310	134	1120	126	ND
246	M	38	387	7700	6075	> 20000	5800	9950	83	ND
247	F	42	295	312	457	1750	220	394	682	ND

ID50 neutralization titers of the plasma of ART treated patients against the primary isolates tested. OD405 refers to the anti-V3 antibodies at absorbance 405 nm. M, male; F, female; NN, non-neutralizing; ND, not determined.

healthy donor plasma was used as negative control.

The anti-V3 Ab content in the HIV-1 seropositive plasma was determined using V3 peptide ELISA. The sequence of the synthesized V3 peptide (CTRPNNNTRKSIRIGPGQTF YATGDIIGDIRQAHC) (Sigma Aldrich, USA) was based on the consensus clade C V3 sequence. We coated the V3 peptide at a concentration of 1 μg/ml. PBS/0.2% Tween 20 (PBST) was used for washing the plates. Blocking was done with PBS containing 5% BSA. Heat inactivated plasma (100 μl, 1:2500 dilution) was added to each well and incubated for 1.5 h at 37°C. Following three washings with PBST, the bound V3 specific Abs were detected by addition of 100 μl of alkaline-phosphatase conjugated anti-human IgG Fc. Immune complexes were revealed with AP-Substrate in 10% diethanolamine buffer and the colorimetric reaction was stopped by the addition of 6 N NaOH. The optical density was read at 405 nm against 650 nm as reference wavelength (OD 405–650). The cut-off value was defined as three times the OD 405–650 of uninfected plasma tested seronegative for HIV-1 infection. The assay was repeated three times and the results were found to be consistent.

The depletion and inhibition assay to determine the role of anti-V3 antibodies in neutralization was performed as previously described (Spenlehauer *et al.*, 1998). For the inhibition assay, V3 peptide was pre-incubated for 30 min at a final concentration of 100 μg/ml with HIV-1 positive test plasma to inhibit the anti-V3 Abs present in the plasma. The rest of the assay was as described for the neutralization assay.

To remove V3-specific Abs from the plasma, six to eight passages on V3 peptide-coated wells (20 μg/ml) were carried out using the ELISA binding protocol.

Statistical analyses were performed using Graph Pad Prism 5 for Windows (USA). Median ID50 neutralization titers of the antiretroviral drug-naïve and treated patients were compared by the Mann-Whitney test. A paired t test was done to check the change in neutralization potential of plasma and the CD4 cell count before and after the drug treatment. An unpaired t test was done to compare the anti-V3 antibody content between antiretroviral-naïve and treated patients. A p-value of < 0.05 was considered significant.

A total of 63 HIV-1-infected patients, 43 males and 20 females of median age 34 years, were recruited and categorized into two groups; drug naïve (n=39) and treated (n=24) (Tables 1 and 2). The majority of the patients were infected through heterosexual transmission. Follow-up of seven drug naïve patients after initiation of ART revealed a significant increase in their CD4 counts (p=0.03).

Sequence analysis of all four of the primary isolates revealed that they were of subtype C (Fig. 1) (AIIMS65 GU057986, AIIMS70 GU057985, AIIMS126 FJ940736, and AIIMS151 FJ940737). The viral neutralization potential of the HIV-1-infected patient plasma samples was tested against the four PIs and one tier 1 clade B virus SF162. The neutralization titers (ID50) of both naïve and treated patients are presented in Table 1 and 2 respectively.

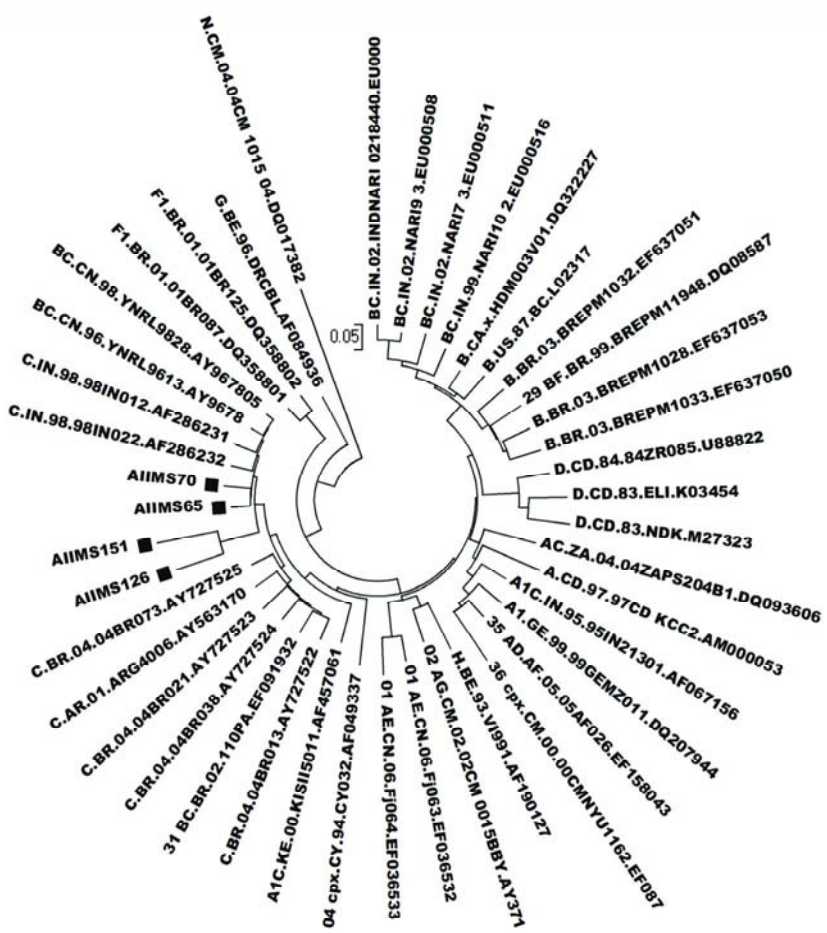

Fig. 1. Phylogenetic analysis of the envelope sequence (partial C2-partial C4) of HIV-1 primary isolates from Indian patients using MEGA4 software. The primary isolates used in this study are prefixed by shaded squares.

A total of 40 patient plasma samples (27 drug naïve and 13 patients on ART) and 10 HIV-1 seronegative plasma samples were tested for the presence of anti-V3 Abs. The median OD405 of the drug treated plasma (0.564) was significantly less than that of the drug naive plasma (2.198) (Fig. 2, p= 0.0252). The neutralization potential of five selected patient plasma samples with high anti-V3 antibody titers showed only a modest change (0–21%) in their neutralization percentage against the PIs AIIMS 126 and AIIMS 151 after depleting their plasma of anti-V3 antibodies (Table 3). No change in viral neutralization potential of the plasma was observed after the inhibition assay (data not shown).

Drug-treated plasma exhibited significantly higher neutralization potential as compared to the drug-naïve plasma against the PIs AIIMS126[AIIMS126=180_{Naive}/$1240_{Treated}$ (p= 0.03)], AIIMS151 [AIIMS151=100_{Naive}/$150_{Treated}$, p=0.02] and AIIMS70 [AIIMS70=122_{Naive}/$686_{Treated}$ (p=0.005)]. A similar trend was observed against the other PIs although not statistically significant [AIIMS65=100_{Naive}/$255_{Treated}$, p=ns]. The efficiency of neutralization of the treated and naïve plasmas was similar to the tier 1 SF162 isolate (SF162=1330_{Naive}/$1400_{Treated}$, p=ns). To further assess the role of ART in viral neutralization, we undertook a minimal follow up of seven drug-naïve patients. Plasma of these patients was tested for anti-V3 antibody content and neutralization potential against AIIMS 126 and AIIMS 151 in their drug-naïve status and after one month of initiation of ART. Antiretroviral-treated plasma of these patients exhibited significantly higher neutralization efficiency for AIIMS 126 (p=0.03) and AIIMS 151 (p=0.03) isolates than in their respective drug-naïve states (Table 4).

Our observation that viral neutralization did not correlate with anti-V3 Ab titers by inhibition and depletion of anti-V3 Abs from selected plasmas with high V3 Ab content

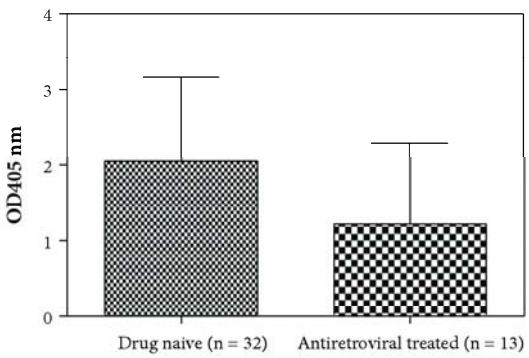

Fig. 2. Comparison of the anti-V3 antibody content (OD405 nm) between antiretroviral naïve and treated HIV-1 infected Indian patients (*P* value = 0.0252).

Table 3. Role of anti-V3 Abs in the neutralization potential against two HIV-1 primary isolates

Plasma ID	% Depletion of the V3 Abs[a]	% Increase (↑) or decrease (↓) in neutralization of AIIMS126 after depletion of V3 Abs[b]	% Increase (↑) or decrease (↓) in neutralization of AIIMS151 after depletion of V3 Abs[b]
118	53.11	3(↑)	2(↓)
150	59.14	4(↓)	3(↑)
151	100	↔	4(↓)
202	92.87	8(↓)	21(↑)
206	80.48	↔	2(↓)

Anti-V3 antibody-depleted plasma was tested for neutralization against two primary isolates AIIMS126 and AIIMS151.
[a] Percentage depletion of V3 antibodies indicates the final depletion achieved after the 6th cycle.
[b] Numbers represent the change [(↑) increase; (↓) decrease; (↔) no change] in the percent neutralization against the primary isolate after the depletion of anti-V3 Abs.

suggests that V3 Abs may not be the major determinants of viral neutralization (Spenlehauer *et al.*, 1998). One possible explanation for the above finding could be that the amount of peptide was not adequate to inhibit all of the anti-V3 Abs present in the plasma. Secondly, the anti-V3 Abs were inhibited/ depleted, using a linear V3 peptide. The V3 Abs against conformationally non-linear native epitopes (that have not been depleted) may be responsible for viral neutralization. Many studies (Fouts *et al.*, 1997; Parren *et al.*, 1997; Nandi *et al.*, 2010) have shown that nonlinear epitopes on the native, intact, trimeric envelope are responsible for eliciting NAbs. However, antibodies against the conformational epitopes are relatively few as compared to those elicited against the monomeric gp120.

An interesting observation of this study is that the plasma of patients on ART for variable periods of time showed significantly higher neutralization potential as compared to the drug-naïve patients which was further confirmed in follow up patients after initiation of ART. This was in agreement with the observations made earlier correlating higher neutralizing antibody titers in antiretroviral-treated patients (Sarmati *et al.*, 1997; Kim *et al.*, 2001) and a recent study in cynomolgus macaques (Ozkaya Sahin *et al.*, 2010). The higher neutralization potential found in the antiretroviral-treated patients could not be an artifact caused by the presence of the antiretroviral drugs in their plasma, as it is well documented that the presence of these drugs in the plasma of treated patients does not influence viral neutralization at plasma dilutions >1:40 in the viral neutralization assays (Dreyer *et al.*, 1999). To eliminate any possibility of the antiretroviral drugs in the plasma of the treated patients contributing to the improvement in viral neutralization, we tested the plasma of patients on ART starting from a dilution of 1:100.

The efficacy of antiretroviral therapy may be established by the status of viremia in these patients, which, however, was not determined and is a limitation of this study.

Further follow-up studies need to be carried out in a large cohort of patients to confirm the beneficial role of ART in viral neutralization.

Table 4. Neutralization titer (ID50) of the plasma of patients before (N) and after 1 month of ART (T)

Plasma ID	N/T	OD405	CD4 count (cells/μl)	AIIMS 126	AIIMS 151
102	N	0.428	55	< 100	< 100
	T	0.351	112	1090	330
107	N	0.7	110	> 20000	< 100
	T	0.538	210	> 20000	2260
112	N	3.325	225	< 100	< 100
	T	1.617	300	1660	< 100
147	N	0.334	28	372	< 100
	T	0.153	86	1360	226
150	N	3.197	147	314	< 100
	T	1.71	250	2480	214
151	N	2.842	112	178	< 100
	T	2.476	307	4800	118
154	N	0.59	4	1590	< 100
	T	0.445	33	2460	146

Seven HIV-1 infected patients were followed up after one month of initiation of ART and their plasma were tested for the neutralization potential against two primary isolates AIIMS126 and AIIMS151. OD405 refers to the anti-V3 antibodies at absorbance 405 nm. ND, not determined.

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