

## Reduced Capacity of Antibodies from Patients Infected with Human Immunodeficiency Virus Type 1 (HIV-1) Group O to Neutralize Primary Isolates of HIV-1 Group M Viruses

Phillipe N. Nyambi, John Nkengasong, Martine Peeters, François Simon, Josef Eberle, Wouter Janssens, Katrien Franssen, Betty Willems, Katleen Vereecken, Leo Heyndrickx, Peter Piot, and Guido van der Groen

*Institute of Tropical Medicine, Antwerp, Belgium; Retrovirus Laboratory, ORSTOM, Montpellier, and Hôpital Claude Bernard, Paris, France; Max v. Pettenkofer-Institute for Hygiene and Medical Microbiology, Ludwig-Maximilians-University, Munich, Germany*

Neutralizing antibody patterns in sera of persons infected with human immunodeficiency virus type 1 (HIV-1) groups M and O to their homologous and heterologous primary isolates were determined in a peripheral blood mononuclear cell-based neutralization assay and correlated with their ability to bind to V3 loop synthetic peptides. Most HIV-1 group M sera (9/16) neutralized HIV-1 group O viruses, whereas fewer group O sera (3/13) only weakly neutralized HIV-1 group M viruses. Group M sera neutralizing HIV-1 group O viruses neutralized other HIV-1 group M viruses with titers of 1:10–1:1280. V3 loop binding capacity of sera did not reflect their neutralizing capacity of the homologous isolate. Despite the reduced neutralizing capacity of group O-infected patients' sera to group M viruses, some group M-infected patients' sera neutralized both HIV-1 group M and O isolates, suggesting that they share some conserved neutralizing epitopes.

Nucleic acid sequence analysis of the envelope gene of human immunodeficiency virus type 1 (HIV-1) isolates has thus far distinguished at least 8 subtypes, A–H [1–3], which together are referred to as HIV-1 group M (for major). However, the relevance of these genetic subtypes in terms of neutralization serotypes is not known. Recently, more divergent HIV-1 strains, temporarily classified as HIV-1 group O (for genetic outliers), have been described and sequence information made available [4–7]. Even though their genomic organization is similar to that of HIV-1 group M viruses, their envelope gene products show a 50% restriction homology with HIV-1 reference strains of group M [7]. The amino acid sequence at the tip of the V3 loop (the principal neutralizing domain) for these group O viruses is quite distinct (GPMAYW for HIV-1<sub>ANT70</sub> and VAU [4, 7] and GPMRW for MVP5180 [6]) from those frequently found in European/American (GPGRAF) and African (GPGQAL) HIV-1 isolates [8, 9].

Given the observed genetic diversity between HIV-1 group M and O viruses, a key question relevant to vaccine design

is whether this genetic diversity reflects antigenic diversity. Independent studies have shown that sera from HIV-1-infected humans can neutralize a broad spectrum of HIV-1 isolates [10–12]. However, these studies lacked information on the genetic subtypes to which the virus isolates or sera belonged. In a recent study, Moore et al. [13] demonstrated that some primary HIV-1 isolates of genetic subtype B were sensitive to neutralization by some monoclonal antibodies, even though most of the isolates were resistant to neutralization. This neutralization by monoclonal antibodies may not reflect the neutralization capacity of sera *in vivo*. However, in a previous study, Mascola et al. [14], using a peripheral blood mononuclear cell (PBMC)-based neutralization assay with plasma and viruses representing 2 distinct HIV-1 subtypes (B and E), demonstrated that HIV-1 viral genotypes predict neutralization serotypes. If these observations are confirmed for other HIV-1 subtypes of group M and O viruses, then an effective vaccine for humans against HIV-1 may have to contain neutralizing epitopes representing distinct genetic subtypes.

In this study, we report on the capacity of sera of HIV-1 group M- and group O-infected persons to neutralize their homologous and heterologous primary and laboratory HIV-1 isolates in a PBMC-based neutralization assay. The HIV-1 isolates and sera from group M (subtypes A–H) and group O were chosen on the basis of phylogenetic classification of the *env* or *gag* gene (or both). We also examined the relationship between the presence of neutralizing antibodies in sera and their ability to bind to the homologous V3 synthetic peptide of the isolate.

### Materials and Methods

*Patient sera.* A total of 29 sera obtained from patients from whom HIV-1 had been isolated were tested for the presence of

Received 5 May 1995; revised 10 July 1995.

Presented in part: Conference on Advances in AIDS Vaccine Development: Seventh Annual Meeting of the National Cooperative Development Groups for AIDS, November 1994, Reston, Virginia (poster 27); VIII Mediterranean Symposium on HIV Infection, March 1995, Toulon, France.

Informed consent was obtained from all subjects.

Financial support: National Fonds voor Wetenschappelijk Onderzoek (grants 3.3025.91 and 3.0137.94) and Fonds voor Geneskundig Wetenschappelijk Onderzoek. Algemeen Bestuur voor Ontwikkelingssamenwerking (doctoral training fellowship to P.N.N.).

Reprints or correspondence: Prof. Guido van der Groen, Dept. of Infection and Immunity, Division of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B2000 Antwerp, Belgium.

*The Journal of Infectious Diseases* 1995;172:1228–37  
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0022-1899/95/7205-0008\$01.00



neutralizing antibodies. Of these, 16 were from patients infected with group M (subtype A–H) and 13 from patients with group O viruses. Sera and viruses were collected at the same time after infection, except for sera VI918 and VI498, which were collected 4 and 3.5 years after virus isolation, respectively. Sera were given the same identification numbers as the corresponding isolates. The corresponding viruses of the group M sera classified into genetic subtypes A–H and clinical stages, based on the World Health Organization (WHO) clinical AIDS definition [15], are as follows: subtype A: CA1 (symptomatic), CA18 and VI191 (asymptomatic); B: CA5 (asymptomatic), CI22 (symptomatic); C: VI313 (asymptomatic); D: CI13 and VI918 (symptomatic); E: CA10 (symptomatic); F: CA4 (symptomatic), CA16 and CA20 (asymptomatic); G: VI525 and VI526 (symptomatic); H: CA13 (symptomatic), VI557 (asymptomatic).

The group O sera were VI498, FAN, MVP6116, MVP7852, and MVP8166 from asymptomatic and CA9, VI686, DUR, MAA, ESS, LOB, MVP7851, and MVP8161 from symptomatic persons. The classification into genetic subtypes is based on sequence information obtained from the *env* or *gag* genes [1, 3, 16, 17] (unpublished data). Sera designated by code CA or MVP, CI, and VI, respectively, were obtained from seropositive persons in Cameroon, Ivory Coast, and Belgium. VI sera were obtained from persons consulting at the Institute of Tropical Medicine in Antwerp. Specimens VI686, VI525, and VI526 were from Gabonese living in Gabon. Four group O sera were obtained from Cameroonians (FAN, ESS, MAA, LOB) living in France and 1 (DUR) from a French citizen.

**Virus isolates.** Fifteen HIV-1 primary isolates of group M were classified into subtypes A–H on the basis of phylogenetic analysis of the *env* (V3–V5 and beginning of gp41) or *gag* genes [1, 3, 16, 17]. Two primary HIV-1 group O isolates, CA9 and VI686, were genetically characterized on the basis of the *pol* and *env* (gp160) sequences [18] (unpublished data). The HIV-1<sub>ANT70</sub> (group O) isolate has recently been completely sequenced [7]. The strains had been cultured only once or twice in PBMC. In addition, 3 HIV-1 laboratory strains were used: HIV-1<sub>IIB</sub> and HIV-1<sub>MN</sub> (provided by R. Gallo, National Cancer Institute, Bethesda, MD) and HIV-1<sub>MAL</sub> (provided by L. Montagnier, Institut Pasteur, Paris). These isolates, provided as chronically infected H9 cells, were passaged in PBMC to eliminate the influence of any host cell-dependent epigenetic factors on virus neutralization [19]. Virus stocks were prepared on phytohemagglutinin (PHA)-stimulated primary human PBMC and titrated essentially as described [10].

**Neutralization assay.** Neutralization was done as described [10] with some modifications. Tissue culture supernatants of PBMC infected with virus (50 TCID<sub>50</sub>) and 2-fold serial dilutions of heat-inactivated (56°C for 30 min) serum were incubated for 1 h at 37°C in a 5% CO<sub>2</sub> atmosphere before addition of 5 × 10<sup>4</sup> 3-day PHA-stimulated PBMC for 2 h. The cells were washed and incubated in RPMI 1640/interleukin-2 medium (GIBCO, Paisley, UK) supplemented with 10% fetal calf serum, 0.03% L-glutamine, (Janssen Chimica, Geel, Belgium), and antibiotics. Viral replication was assessed after 7 days by an in-house p24 antigen–capture ELISA.

A series of experiments was done to ensure the validity of the neutralization assay. In each neutralization assay on PBMC, virus was titrated again to check the titer. If the titer differed by >3-fold dilutions, neutralization assays were considered invalid and repeated. To ensure the reproducibility of our method, 25% of sera

Peptides	Amino acid sequences
<b>Subtype B</b>	
MN	CNKRKRIHIGPGRAFYTTKN
HXB2	CNTRKRIRIQRGPGRAFVTI
<b>Subtype D</b>	
MAL	CNTRRGIHFGPGQALYTTGI
<b>Group O</b>	
ANT70	CDIQEMRIGPMAWYSMGIG

**Figure 1.** Reactivity of both HIV-1 group M– and O–infected sera were tested on 3 HIV-1 group M (MN, HXB2, and Mal) and 1 HIV-1 group O (ANT70) V3-loop synthetic peptides.

were randomly retested. In each neutralization assay, the last wash fluid obtained after washing away the nonabsorbed serum-virus mixture was carefully monitored for the presence of residual p24 antibody by use of a commercial HIV-1 and -2 antibody ELISA (Vironostika HIV Uni-Form II; Organon Teknika, Boxtel, Netherlands) as described [10]. Such a control is important since p24 antibody could complex with the p24 antigen released in culture and interfere with p24 antigen detection. The ability of our in-house p24 antigen–capture ELISA to equally detect p24 antigen of both group M and O viruses was also confirmed. Neutralizing titers were defined as the reciprocal of the highest serum dilution giving a 90% reduction in absorbance in the HIV antigen assay compared with negative controls. HIV-1 serum neutralizing titers of ≥1:10 were considered positive.

**Peptides and peptide ELISA.** All peptides used were synthesized by Neosystem Laboratoire (Strasbourg, France). A total of four peptides corresponding to the gp120 V3 region of HIV-1 were evaluated: of subtype B, 2 strains of North American origin (HIV-1<sub>MN</sub> and HXB2 [HIV-1<sub>IIB</sub>]), of subtype D, 1 African strain (HIV-1<sub>MAL</sub>), and of group O, HIV-1<sub>ANT70</sub> (subtypes according to the classification of Myers et al. [2]). The amino acid residues of these peptides covered the principal neutralization domain containing the V3 loop of gp120 (figure 1).

Micro-ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 ng of peptide/well in 200 μL of 0.05 mol/L carbonate buffer (pH 9.6) overnight and stored at –20°C until use. The assay was done essentially as described [10].

**Western blot.** Western blots were prepared from HIV-1<sub>ANT70</sub> as described [20] with some minor modifications. In brief, supernatant of virus grown on Molt-4 clone 8 cells in RPMI 1640 supplemented with 1% nutridoma (Boehringer, Mannheim, Germany), 0.03% glutamine, gentamicin, and 0.1% fetal calf serum was pelleted by centrifugation. Virus pellets were resuspended in an elec-

Table 1. Neutralizing antibodies to diverse HIV-1 isolates by sera of HIV-1 group O- and group M-infected persons.

Subtype, serum	Reciprocal of neutralizing antibody titer against HIV isolate of group																						
	O			A			B			C			D			E		F			G		H
	ANT70	CA9	VI686	CA1	CA18	VI191	MN	III B	CI22	CA5	VI313	CI13	VI205	MAL	CA10	CA4	CA16	CA20	VI525	VI526	CA13		
Group O																							
VI498	160	40	—	—	—	—	—	—	10	—	—	—	—	—	10	—	—	—	—	—			
CA9	10	40	10	—	—	—	—	—	—	20	—	—	—	—	—	—	—	—	—	—			
VI686	—	—	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
DUR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
FAN	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
MAA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
ESS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Group M																							
A																							
CA1	—	—	—	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
CA18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
VI191	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
B																							
CA5	640	640	>1280	640	—	80	>1280	320	—	160	640	320	—	20	80	1280	—	640	160	160	40		
CI22	320	80	160	640	—	—	>1280	—	—	40	160	80	—	20	—	—	—	—	—	—			
C, VI313																							
CA5	20	10	10	20	—	80	10	—	—	20	80	20	—	—	20	—	160	40	40	—			
D																							
CI13	20	10	20	—	—	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—			
VI918	—	—	—	—	—	10	10	—	—	—	—	10	—	—	—	—	—	—	—	—			
E, CA10																							
CA10	40	40	40	80	—	160	10	—	—	10	40	20	—	20	40	—	80	—	—	—			
F																							
CA4	80	40	40	160	—	10	640	40	—	40	160	80	—	10	20	80	—	160	10	10	20		
CA16	—	—	—	10	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—	—			
CA20	20	40	20	160	—	320	40	10	—	20	80	20	—	80	—	160	—	80	10	—			
G																							
VI525	—	—	—	20	—	10	10	—	—	20	80	80	—	10	10	80	—	160	40	20	—		
VI526	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
H																							
CA13	80	40	40	160	—	—	320	20	—	40	40	80	—	—	10	320	—	160	20	20	40		
VI557	10	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			

NOTE. Empty space indicates test not done due to limited amount of serum. Sera and isolates indicated with same identity indicate same time of collection. Sera VI498 and VI918, respectively, were collected from patients infected with ANT70 and VI205 isolates, respectively, 3.5 and 4 years after virus collection. Genetic subtypes of HIV-1 group M isolates are based on *env* gene. Isolate VI313 in subtype C is based on total *gag* gene sequence. Primary isolates with total gp160 sequence information include CA9, VI686, ANT70, VI191, and VI525; isolates with V3-V5 and start of gp41 sequence include CA1, CA18, CA5, CA10, CA4, CA16, CA13, CI22, CI13, and VI526; and with total *gag* sequence include VI313, VI205, VI191, and VI525. —, neutralizing antibody titer <1:10.

trophoresis buffer for 3 min at 100°C and electrophoresed on a 10% SDS-polyacrylamide slab gel, and proteins were transferred to nitrocellulose paper. Blotting was done in a Multiphor II Novablot (LKB, Bromma, Sweden) electrophoretic unit for 1 h as described [21].

## Results

*Capacity of HIV-1 group O-infected patients' sera to neutralize groups O and M viruses.* The ability of group O sera to neutralize their autologous contemporaneous (serum collected at the same time point as virus isolation) or heterologous group O viruses was absent or weak (titers ranging from <1:10 to 1:40; table 1). A higher titer was observed for the VI498 serum to its homologous virus (HIV-1<sub>ANT70</sub>) probably because the serum was collected 3.5 years after the virus used in the neutralization assay was isolated.

To determine the presence of neutralizing antibodies in HIV-1 group O sera to HIV-1 group M isolates, 13 group O sera (first

7 sera, table 1, and 6 others [MVP6116, MVP7851, MVP7852, MVP8161, MVP8166, and LOB] not shown in table 1) were challenged with 4 group M viruses including 3 field isolates, CA1 (subtype A), CA16 (subtype F), and CI13 (subtype D), and a laboratory strain, HIV-1<sub>MN</sub> (grown in PBMC). None of these sera neutralized any of the isolates tested, except for MVP7852, which had weak neutralizing antibody titers of 1:10 and 1:20 to CA16 and CI13, respectively. To further explore these observations, 7 of the 13 HIV-1 group O sera were tested for their capacity to neutralize a wider range of group M isolates. With rare exceptions, these group O sera failed to neutralize the group M isolates (table 1).

*Capacity of HIV-1 group M-infected patients' sera to neutralize groups O and M viruses.* In contrast to group O sera, which had very weak or no neutralizing antibodies to the group M viruses, some group M sera neutralized the group O isolates. Of 16 sera from HIV-1 group M-infected patients, 9 (56.3%) had neutralizing antibodies to all 3 HIV-1 group O viruses (table 1). At least 1 serum each of subtype B-F and H had

neutralizing antibodies to group O viruses. However, the 3 subtype A sera and 2 of subtype G did not neutralize any of the HIV-1 group O isolates.

We hypothesized that HIV-1 group M sera capable of cross-neutralizing the very divergent HIV-1 group O isolates may also neutralize HIV-1 group M field and laboratory isolates with even higher titers. To verify this, 7 sera that initially neutralized the 3 group O viruses in table 1 were tested for their capacity to neutralize 11 field (subtype A-H) and 3 laboratory isolates. Six sera that did not neutralize any of the group O isolates in table 1 were also included. The 7 sera initially neutralizing the group O viruses (table 1) were capable of neutralizing a majority of the isolates of subtypes A-H, with neutralization titers ranging from 1:10 to >1:1280. Among the laboratory isolates, HIV-1<sub>MN</sub> was the most sensitive to neutralizing antibodies, with titers ranging from 1:10 to >1:1280. The field isolates, CA1 (subtype A) and VI313 (subtype C), were most sensitive, with titers ranging from 1:40 to 1:640. Two sera, CA5 and CA4, neutralized all isolates (subtype A-H), with titers ranging from 1:10 to >1:1280. In contrast, 5 of the 6 sera (table 1) that initially did not neutralize the HIV-1 group O isolates failed to neutralize the majority of the other group M isolates of subtype A-H. With rare exceptions, very weak titers of 1:10 were observed among these 5 sera.

No correlation was observed between residual antibody levels present in the final washing fluid of the neutralization assay of sera and their neutralizing titers (data not shown). We interpret this lack of correlation as a lack of any influence of residual antibodies on the neutralizing effect of the sera.

*Correlation between the neutralizing capacity of sera and clinical stage.* We compared the cross-neutralizing capacity to HIV-1 group M and group O viruses with disease stages at which the sera were obtained. Neutralization of group M or group O isolates by homologous or heterologous sera was not related to the clinical stage. Sera from asymptomatic (CA5, CA20, VI313) and symptomatic (CA4, CA10, CA13, CI22) patients had neutralizing antibodies to group M (subtypes A-H) and group O viruses (table 1). In addition, similar numbers of sera lacking neutralizing antibodies to both group M and group O viruses were from asymptomatic (CA16, CA18) and symptomatic (CA1, VI918, VI526) persons.

*Serum neutralization of virus grown on different host cells.* Several studies [13, 19, 22] have shown that neutralization sensitivity of HIV-1 is due in part to the host cell in which the virus is propagated. To verify the effect of the host cells used on the neutralizing antibody titers, we prepared stocks of HIV-1<sub>ANT70</sub> (a field isolate) and HIV-1<sub>IIIB</sub> (a laboratory strain) in both PBMC and CEMSS cells. These isolates were tested for their capacity to become neutralized by 7 broadly cross-neutralizing and 4 nonneutralizing sera. Overall, the nonneutralizing sera did not neutralize both isolates, while the broadly cross-neutralizing sera neutralized both isolates in both PBMC and CEMSS cell lines (table 2). HIV-1<sub>IIIB</sub> was more sensitive to neutralization on CEMSS cells than on PBMC. In particular,

**Table 2.** Neutralization of HIV-1<sub>ANT70</sub> and HIV-1<sub>IIIB</sub> on CEMSS cell and PBMC assay.

Serum	CEMSS		PBMC	
	ANT70	IIIB	ANT70	IIIB
CA5	320	1280	640	320
CI22	>80	>80	320	ND
CA10	40	20	40	10
CA4	320	160	80	40
CA20	10	20	20	10
CA13	20	80	80	20
VI557	10	20	10	10
CA1	—	—	—	—
CA18	—	—	—	—
CA16	—	—	—	—
CA9	—	—	10	—

NOTE. Neutralization on CEMSS cell line was done essentially as described [10]. Data are neutralizing antibody titers expressed as reciprocals. PBMC, peripheral blood mononuclear cell; —, titer <1:10.

3 of 7 broadly cross-neutralizing sera had a 4-fold rise in neutralizing antibody titer to HIV-1<sub>IIIB</sub> on CEMSS cells compared with neutralization on PBMC.

*Effect of PBMC from different donors on infectious HIV.* PBMC from different donors have been shown to vary in their susceptibility to HIV infection [23-25]. This variation may lead to fluctuations in virus titration on different donor PBMC, which might subsequently play a role in neutralization. Figure 2 shows the effect of PBMC from different donors on virus titers of some of the isolates during neutralization. A 3-fold difference in virus titer on different donor PBMC showed no significant effect on the neutralizing antibody titer of sera (figure 3). When the virus titer differed by >3-fold, in some cases there was a significant difference in neutralizing antibody titer. In particular, when the titer of virus CI22 dropped 5-fold, the neutralizing antibody titer of serum CA9 increased from <1:10 to 1:40 (figure 3A). As a consequence, we considered a neutralization assay valid if the virus titer (simultaneously titrated on the same donor PBMC as used in the neutralization assay) was the same or differed by only 3-fold to the initial (input virus) titer.

*Sensitivity of ELISA to detect p24 antigen from both group M and O virus supernatants.* To ensure that our in-house p24 antigen ELISA could efficiently detect p24 antigen of both group M and O viruses, diluted culture supernatants with similar TCID<sub>50</sub> of 2 group M (CA1 and CA13) and 3 group O (HIV-1<sub>ANT70</sub>, VI686, and CA9) viruses were tested and optical densities were compared. Detection of p24 in both group M and O diluted culture supernatants were similar, as indicated by optical densities (figure 4). These results indicate that our in-house p24 antigen-capture ELISA detects p24 in group M and O virus supernatants with a similar sensitivity.

*Relationship between the neutralization ability of antibodies and binding capacity on V3 loop peptides and Western blot.* The relationship between the V3 loop peptide-binding capacity

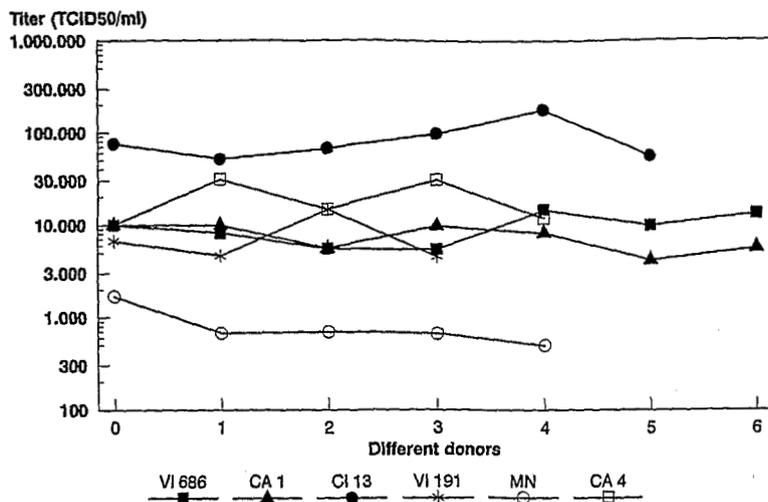


Figure 2. Effect of different donor peripheral blood mononuclear cells on HIV-1 titers: 0 on x axis, reference virus titer.

of sera and their functional activity as measured in a neutralization assay was determined for homologous and heterologous isolates. In most cases, high V3 loop peptide-binding capacity of sera did not reflect their capacity to neutralize the homologous isolate (figure 5). In particular, of 10 sera with high reactivity to the peptide of HIV-1<sub>MN</sub>, only 4 neutralized the homologous isolate, with a neutralizing antibody titer of  $\geq 1:320$ , while 5 lacked neutralizing antibodies (titer  $\leq 1:10$ ). Of more interest, of 9 group M sera that lacked reactivity for HIV-1<sub>ANT70</sub> V3 loop peptide, 6 neutralized the homologous (HIV-1<sub>ANT70</sub>) virus, with titers ranging from 1:20 to 1:640. Conversely, 2 group O sera, CA9 and VI686, which were highly reactive with HIV-1<sub>ANT70</sub> V3 loop peptide, had very weak (titer, 1:10) or no neutralizing antibody to HIV-1<sub>ANT70</sub>. However, reactivity of 2 subtype B sera with HIV-1<sub>MN</sub> but not HIV-1<sub>IIIIB</sub> V3 peptide and 1 group O serum (VI498) with HIV-1<sub>ANT70</sub> V3 peptide correlated with neutralization of the homologous isolate (figure 5). These data demonstrate the lack of correlation between neutralizing activity and reactivity to V3 loop peptide in ELISA.

When we compared the reactivity of group M sera to HIV-1<sub>ANT70</sub> gp41 on Western blot with the presence of neutralizing antibodies to HIV-1<sub>ANT70</sub>, only 4 of 8 sera that neutralized HIV-1<sub>ANT70</sub> reacted with gp41 on Western blot. Three of 5 sera that lacked neutralizing antibodies to HIV-1<sub>ANT70</sub> also reacted with gp41 on Western blot. None of the group M sera reacted with HIV-1<sub>ANT70</sub> gp120, irrespective of their neutralizing capacity, whereas the group O sera did.

## Discussion

A successful vaccine against HIV-1 must elicit an immune response that protects against infection by numerous genetic variants that characterize this virus. To our knowledge, this is the first study in which genetically characterized primary HIV-1 group M isolates, representing 8 different genetic subtypes, and HIV-1 group O isolates have been used in a PBMC-based

neutralization assay with homologous and heterologous polyclonal sera.

A significantly higher number of sera (9/16) from patients infected with different genetic subtypes of group M neutralized the 3 HIV-1 group O isolates than did group O sera (3/13) which only sporadically had weak neutralizing antibodies (titer, 1:10–1:20) to 5 of 18 HIV-1 group M viruses of 8 different genetic subtypes. This inability of sera of HIV-1 group O-infected persons to cross-neutralize HIV-1 group M viruses could be attributed first to the genetic diversity (50%) in the *env* gene product that distinguishes both groups [7]. Second, antibodies induced by HIV-1 group O viruses perhaps fail to recognize the equivalent HIV-1 group M antigens or the counterpart group M antigens may not be accessible to HIV-1 group O antibodies because of differences in glycosylation patterns that might mask the epitopes [26]. Last, it is possible that HIV-1 group O viruses lack strong immunogenic epitopes necessary to initiate production of neutralizing antibodies to group M viruses. This possibility is in line with the weak cross-neutralization observed within the group O viruses compared with the group M viruses. However, the number of group O sera and isolates analyzed may be too limited for a final conclusion.

Of interest, the HIV-1 group M-infected sera neutralized all of the group O isolates with a conspicuous similarity in titer. Furthermore, these group M sera consistently neutralized other group M isolates of diverse genetic subtypes. The possibility exists that sera of some HIV-1-infected persons contain mixtures of antibodies induced at different moments after infection against different HIV-1 isolates. Consequently, broadly cross-neutralizing sera may contain a population of antibodies directed against a single or multiple conserved epitopes present in all 8 subtypes of HIV-1 group M and in group O viruses.

Our results clearly demonstrate that the ability of sera to neutralize isolates or for isolates to be neutralized by sera did not correlate with the genetic subtype. These results do not confirm the findings of Mascola et al. [14], who used primary HIV-1 isolates representing 2 distinct genetic subtypes (B and

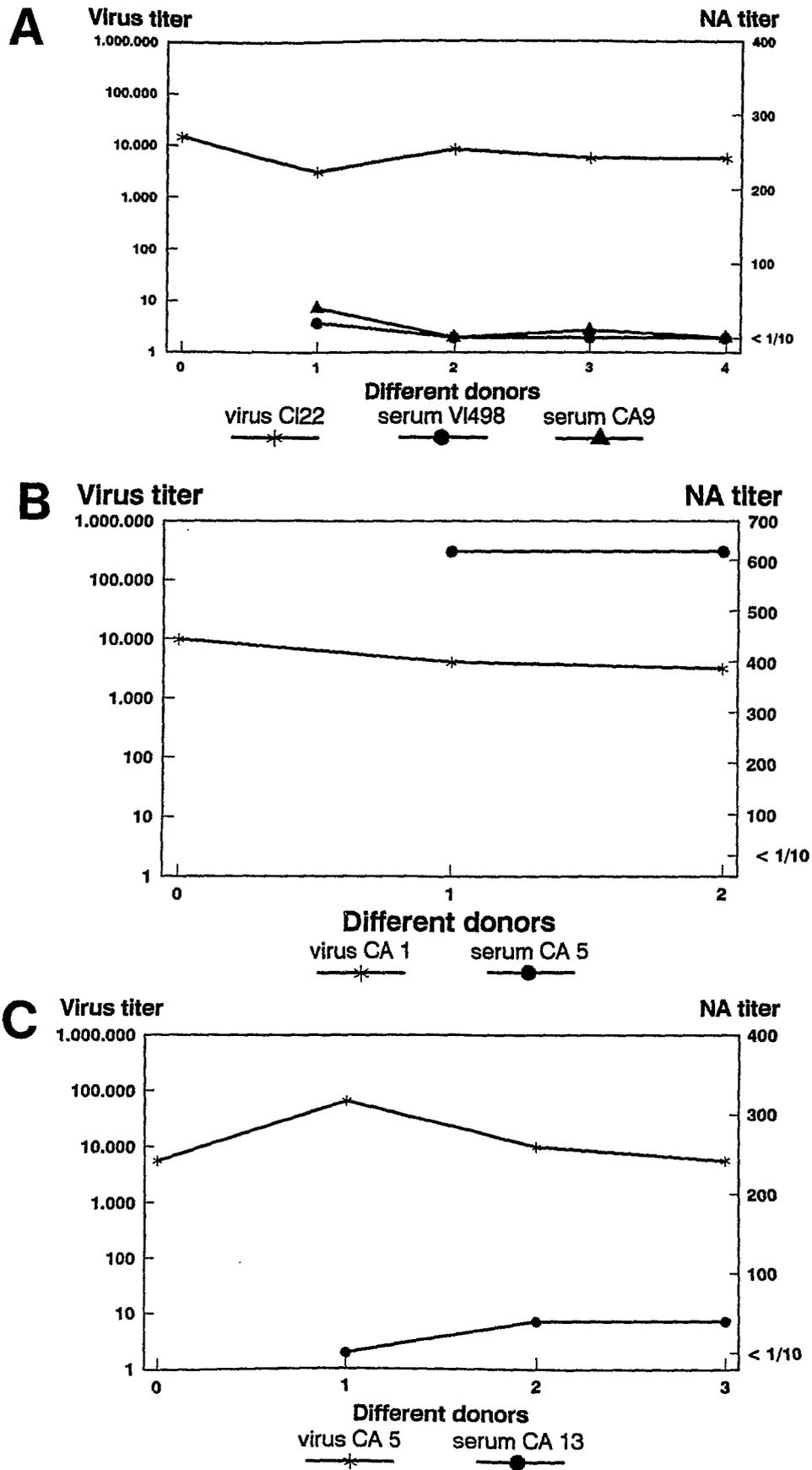


Figure 3. Effect of different donor peripheral blood mononuclear cells on HIV-1 titers and neutralizing antibody titers of sera: 0 on x axis, reference (input) virus titer; NA, neutralizing antibody. Drop (>3-fold, A) and increase (C) in titer results in corresponding increase and decrease, respectively, in NA titer.

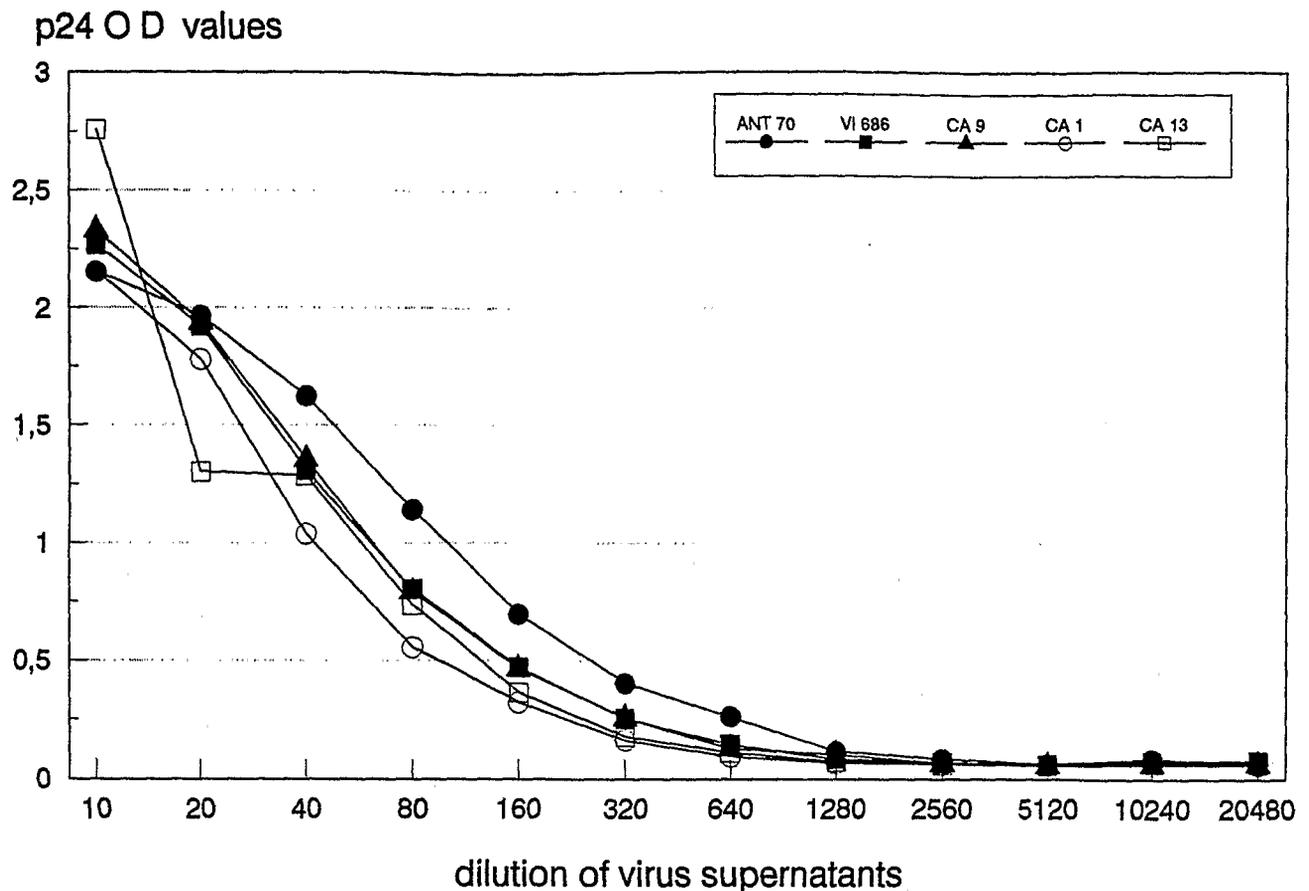


Figure 4. Sensitivity of HIV-1 p24 antigen-capture ELISA to detect p24 antigen in culture supernatants of HIV-1 group M and O viruses. OD, optical density. Note similarity in ODs for both groups.

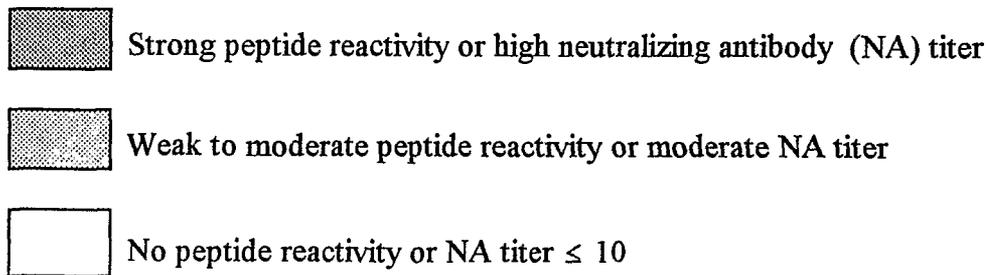
E) to demonstrate that they represent distinct neutralization serotypes. Similar to our findings, studies by others using monoclonal antibodies mostly directed against CD4 conformational epitopes [13, 27] or anti-HIV-1-positive human sera [10, 28] demonstrated broad cross-neutralizing antibodies to distinct genetic subtypes of HIV-1 group M or group O viruses (or both). In particular, serum CA5 (subtype B) and CA4 (subtype F; table 1) neutralized all isolates of group M and group O tested. Taken together, these results indicate that the considerable genetic diversity observed within HIV-1 isolates does not reflect a similar diversity in neutralization serotypes. Furthermore, the cross-neutralizing capacity of sera was not related to the clinical stage of disease, since similar numbers of asymptomatic and symptomatic patients' sera had equivalent capacity.

Thus far, HIV-1 isolates have been phylogenetically classified into different subtypes on the basis of sequence information. The data in table 1 suggest that both sera and HIV-1 isolates can also be grouped by the homology of their neutralization spectrum (serotype). Each row in table 1 can be regarded as a serum neutralization spectrum and each column as an isolate neutralization spectrum. The serum neutralization spectrum of HIV-1 group O-infected persons was remarkably different from HIV-1 group M-infected persons. In particular,

the neutralization spectra of CA5 and CA4 look similar, in that both sera were capable of neutralizing (with high neutralizing antibody titers) all isolates (table 1) representing HIV-1 group O and 8 different genetic subtypes of group M. Conversely, a close homology in the neutralization spectra of group O sera was observed, whereby most of them failed to neutralize the group M isolates. Therefore, the possibility exists that if sera and HIV-1 isolates are classified into neutralization serotypes, conserved epitopes involved in neutralization may be identified in some key isolates in each group.

Although this study was not designed to address the issue of reproducibility of neutralization assays, two observations are of note. First, neutralization sensitivity of HIV has been reported to be due in part to the cell in which the virus is propagated [13, 19]. The neutralizing antibody titers of sera to HIV-1<sub>ANT70</sub> and HIV-1<sub>IIIIB</sub> on PBMC and CEMSS cells were comparable, even though not reproducible; however, neutralization of HIV-1<sub>IIIIB</sub> was relatively higher on CEMSS cells than on PBMC. Second, there has been considerable concern regarding the validity of PBMC-based neutralization assays with primary viruses [29, 30]. In particular, it is wise to note that variation in PBMC infectability of different donors will certainly have implications in neutralization assays. However,

Virus Subtype, Serum	MN (B)		IIIb (B)		MAL (D)		ANT 70 (O)	
	OD V3	NA	OD V3	NA	OD V3	NA	OD V3	NA
A, CA1	2.999	<10	0.057	<10	1.050	<10	0.079	<10
B, CA5	2.999	1280	0.066	320	0.072	20	0.060	640
B, CI22	2.999	1280	0.312	ND	0.071	20	0.060	320
D, VI918	2.423	10	0.519	10	2.999	<10	0.073	<10
E, CA10	0.078	160	0.063	10	0.054	<10	0.066	40
F, CA16	2.047	<10	0.060	<10	0.059	<10	0.085	<10
F, CA4	2.999	640	0.056	40	0.127	10	0.078	80
F, CA20	2.999	40	0.079	10	0.062	20	0.062	20
H, CA13	2.999	320	0.060	20	0.062	<10	0.063	40
O, VI498	1.886	<10	0.127	ND	0.061	<10	2.999	160
O, CA9	1.498	<10	0.086	<10	0.057	<10	2.999	10
O, VI686	0.068	<10	0.056	ND	0.061	<10	1.833	<10



**Figure 5.** Relationship between neutralizing and V3 loop peptide-binding antibodies in sera of persons infected with HIV-1 group M (subtypes shown in parentheses) and O viruses: ND, not done; OD V3, optical density of V3 loop peptide ELISA. OD of 0.5 was considered positive; <0.5, negative.

in a recent study addressing the issue of PBMC infectability by primary HIV-1 isolates, Spira and Ho [24] demonstrated a 40-fold range of infection of different donor PBMC, which is less than the 1000-fold previously reported by others [23, 25], and that no donor cells totally resisted infection. In our study, the acceptable variation range of virus titer considered valid

for a neutralization assay was a 3-fold difference. We observed that neutralizing antibody titers of sera were similar as long as the variation in titer was within the acceptable range of 3-fold difference. These results indicate that using different donor PBMC with less variation of infectivity by primary HIV-1 for neutralization assays yields valid results.

Several determinants of the HIV-1 envelope have previously been suggested to be involved in virus neutralization [31–38]. Most of these studies have focused on the third variable region (V3) of gp120, and because of its importance as the principal neutralization epitope, it has become a favored target for serology aimed at characterizing virus isolates. Reactivity with peptides was compared with the presence of neutralizing antibodies to the homologous isolates. With rare exceptions, reactivity with V3 peptide did not correlate with neutralization of the homologous isolate (figure 5) nor with HIV-1<sub>ANT70</sub> gp41 on Western blot. These results provide indirect evidence that the cross-neutralizing activity of human sera may be due in part to antibodies directed to conformational epitopes in HIV-1 gp120 or gp41. Mapping of such conserved cross-neutralizing epitopes is invaluable for the development of a polyvalent HIV vaccine.

In conclusion, these results demonstrate that despite the reduced capacity of sera of HIV-1 group O-infected persons to neutralize group M viruses, both HIV-1 group M (subtype A–H) and group O viruses are sensitive to neutralization by anti-HIV-1 group M-positive sera from patients infected with different genetic subtypes. A method to classify sera and HIV-1 isolates of diverse genetic subtypes by the homology of their neutralization spectra is therefore needed, which may lead to the identification and characterization of some key epitopes present in a large set of genetically diverse HIV-1 isolates that, in a suitable vaccine preparation, can help to induce protective immunity in a naive person. Studies similar to ours, aimed at determining the antigenic relationship of HIV-2 and simian immunodeficiency virus, are invaluable.

#### Acknowledgments

We thank R. Gallo and L. Montagnier for HIV isolates and P. Nys and C. Maeckelbergh for illustrations.

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