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Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization

(envelope glycoproteins/human immunity/serotypes)

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ABSTRACT **The human immunodeficiency virus (HIV) type 1 displays a high degree of genetic variation, especially in the glycoprotein (gp120) domain of the envelope gene. To determine whether this genomic heterogeneity leads to the expression of independent HIV subtypes, 12 sera from HIV type 1 antibody-positive individuals were tested for their ability to neutralize 20 HIV isolates of various origins. Four distinct HIV subtypes with different sensitivity to serum neutralization were identified. These results suggest that a finite number of HIV subtypes exist and that the combined use of selected HIV isolates representing several subtypes may be necessary for the development of an effective vaccine.**

The human immunodeficiency virus (HIV) type 1 is etiologically associated with the acquired immunodeficiency syndrome (AIDS) (1–3). The virus preferentially replicates in T helper/inducer lymphoid cells and usually causes cytopathic effects in these cells (4–8). Isolates of HIV-1 have been molecularly cloned and sequenced (9–12). Analyses of the genomic structure of these cloned viruses reveal substantial genetic variabilities, particularly in the NH_2-terminal region of the envelope glycoprotein (gp120). These changes can result in up to 25% differences in the predicted amino acid sequence (13). In other retroviral systems, the envelope glycoprotein has been shown to be the target of neutralizing antibodies (14–16). Likewise, gp120 purified from HIV-1, as well as various segments of the viral gp120 expressed in mammalian and bacterial cells, elicit the production of neutralizing antibodies in experimental animals (17–19). For the future development of a vaccine, it is therefore important to determine whether the genomic heterogeneity of the HIV envelope gene results in immunologically different HIV subgroups.

Twelve sera from HIV-1 antibody-positive individuals were screened against 20 HIV isolates for their ability to neutralize viral infectivity *in vitro*. The HIV used were obtained from the United States, Dominican Republic, and Africa, and some of these viruses were isolated from nerve tissues. Results showed that the isolates studied can be grouped into four classes according to their degree of sensitivity to serum neutralization. The data indicate the presence of distinct subtypes of HIV-1 and suggest that the use of representative viruses in combination should be considered for development of an effective vaccine.

MATERIALS AND METHODS

Cells and Viruses. Human peripheral blood mononuclear cells (PBMC) from HIV-1-seronegative individuals were prepared on Ficoll/Hypaque gradients as described (6) and propagated in RPMI 1640 medium containing 10% heat-inactivated (56°C for 30 min) fetal calf serum, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% interleukin 2 (Electronucleonics, Silver Spring, MD).

All 20 HIV isolates were initially recovered by cocultivation of patient's PBMC or central nervous system tissues with normal PBMC as described (6, 20). Only six isolates were subsequently found to replicate also in the HUT-78 cell line (HIV_{SF2}, HIV_{SF4}, HIV_{SF33}, HIV_{SF66}, HIV_{SF117}, and HIV_{SF301A}). Culture supernatants were assayed every 3–4 days for particle-associated reverse transcriptase activity (21). When high titers of virus were obtained (reverse transcriptase $\geq 10^6$ cpm/ml), aliquots of filtered virus stocks were frozen (−70°C). All viral fluids used in this study were from infected PBMCs except for HIV_{SF2}, HIV_{SF4}, HIV_{SF33}, and HIV_{SF66}, where supernatants from infected HUT-78 cells were used. Results were similar when supernatants from either HIV-infected PBMC or HUT-78 cells were used.

HIV_{SF2}, HIV_{SF4}, HIV_{SF13}, HIV_{SF33}, and HIV_{SF66} were recovered from PBMC of patients with AIDS; HIV_{SF113} and HIV_{SF117} were from PBMC of patients with Kaposi sarcoma; and HIV_{SF97} and HIV_{SF315} were from PBMC of seropositive healthy individuals. HIV_{SF128A} was obtained at autopsy from spinal cord tissue of an AIDS patient who died with neurologic disease. HIV_{SF98}, HIV_{SF178}, and HIV_{SF185} were recovered from the cerebrospinal fluid of patients with neurologic symptoms. HIV_{SF161B} and HIV_{SF162} were obtained from the cerebrospinal fluid of HIV-infected individuals without neurologic symptoms, and HIV_{SF301A} was from the cerebral cortex tissue of a patient with AIDS. Three isolates were obtained from the PBMC of AIDS or AIDS-related complex (ARC) patients living in Kigali, Rwanda (HIV_{SF153}, HIV_{SF170}, and HIV_{SF171}), and one isolate was from a child with AIDS living in the Dominican Republic (HIV_{SF247}).

Sera. All sera used came from HIV-1 antibody-positive individuals, except for serum 3, which was obtained from a chimpanzee inoculated with HIV_{SF2}; the sera were collected in 1985 and 1986. Sera 10, 11, and 12 were from Kigali, Rwanda.† The reactivity of the sera to individual HIV-1 viral proteins was determined by immunoblot analysis (22). Titers of these sera were determined by an indirect immunofluorescence assay (IFA) using HIV-1-infected cells (23) and by an indirect ELISA assay.

For the indirect ELISA, 96-well plates were coated with either semi-purified preparations of HIV_{SF2} or recombinant envelope protein. HIV_{SF2} preparations were obtained by centrifugation (16,500 × g for 2 hr) of infected culture

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PBMC, peripheral blood mononuclear cells; IFA, immunofluorescence assay.

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†Ntahorutaba, M., Allen, S., Evans, L., Levy, J. A., Rouvroys, D. & LePage, P., Second International Conference on AIDS, June 23–25, 1986, Paris, p. 146 (abstr.).

Table 1. Anti-HIV serum antibody titers

Serum	Source	IFA	ELISA	
			Anti-HIV	Anti-*env*
1	HH (SF)	640	16,000	16,000
2	HH (SF)	320	2,000	1,000
3	HC	160	2,000	4,000
4	PCP (SF)	160	1,000	1,000
5	PCP (SF)	40	4,000	2,000
6	KS (SF)	40	4,000	2,000
7	PCP (SF)	320	4,000	16,000
8	HH (SF)	1280	32,000	8,000
9	LAN (SF)	2560	512,000	8,000
10	LAN (Af)	640	64,000	32,000
11	LAN (Af)	160	16,000	16,000
12	LAN (Af)	80	16,000	2,000

The anti-HIV and anti-*env*-2-product antibody titers of the 12 sera used for typing HIV were determined by an indirect immunofluorescence assay (23) and by an indirect ELISA procedure as described. Figures represent the reciprocal of the end-dilution giving a positive result. HH, healthy homosexual; HC, healthy chimpanzee inoculated with HIV_{SF2} (formerly AIDS-associated retrovirus, ARV-2); PCP, *Pneumocystis carinii* pneumonia patient; LAN, lymphadenopathy syndrome patient; KS, Kaposi sarcoma patient; SF, San Francisco; Af, Africa.

supernatants over a 20% (vol/vol) glycerol cushion. Bacterially expressed recombinant envelope protein (*env*-2 product), representing the NH_2-terminal half of gp120, was provided by the Chiron Corporation (Emeryville, CA) (24). The sera were tested for reactivity against both antigens using standard ELISA procedures (25). Serum titers are expressed as the reciprocal of the last dilution giving a signal-to-noise ratio greater than or equal to four relative to control sera.

HIV Neutralization. For virus neutralization assays, 100 μl of 10-fold and 2-fold dilutions of the heat-inactivated (56°C for 30 min) serum were incubated with an equal volume of virus-containing fluid (reverse transcriptase activity, $\approx 10^6$ cpm/ml) for 1 hr at room temperature. The highest serum dilution used for this study was 1:1000. The mixture was then inoculated onto PBMC as described (6). Briefly, 3×10^6 PBMC were pretreated with Polybrene (2 μg/ml) for 30 min at 37°C and infected with virus for 2 hr at 37°C. The cells were subsequently pelleted and resuspended in medium, and culture supernatants were assayed for reverse transcriptase activity 4 and 7 days postinfection (21). Control cultures received virus incubated with sera from seronegative healthy individuals. A two-third (67%) or greater reduction in reverse transcriptase activity at both time points was considered indicative of HIV neutralization. Each experiment was repeated at least three times.

RESULTS

Characterization of Sera Used in Neutralization. Twelve HIV-1 antibody-positive sera were used for the neutralization studies. All sera had IFA titers in the range of 1:40–1:2560 (Table 1). Anti-HIV-1 and anti-*env*-2-product ELISA titers ranged between 1:1000–1:512,000 and 1:1000–1:32,000, respectively (Table 1). In several instances, the anti-*env*-2-product titers differed substantially from the anti-HIV-1 titers (e.g., sera 7, 8, 9, and 12).

All sera, except serum 4, showed reactivity to gp160 (Fig. 1). Six of the 12 sera showed reactivity to gp120 and only three showed reactivity to gp41. Serum 4 reacted with a single band of M_r 95,000 that was present only in HIV-1-infected cell lysates (Fig. 1, lanes I, J).

Kinetics of Serum Neutralization. Serum neutralization was indicated by a reduction of virus infectivity as measured by reverse transcriptase activity in culture supernatants. To rule out the possibility of nonspecific viral aggregation in this procedure, the kinetics of serum neutralization was determined (26). Dilutions of serum 1 (1:10, 1:50, and 1:100) were incubated with HIV_{SF33} (reverse transcriptase activity, $\approx 10^6$ cpm/ml). At various time points after mixing (0, 2, 5, 10, 20, and 30 min) 0.2-ml aliquots of the mixture were assayed for residual viral infectivity in PBMC (Fig. 2). Data showed that the neutralization of HIV was a linear function of the incubation time of the virus with immune serum. Further-

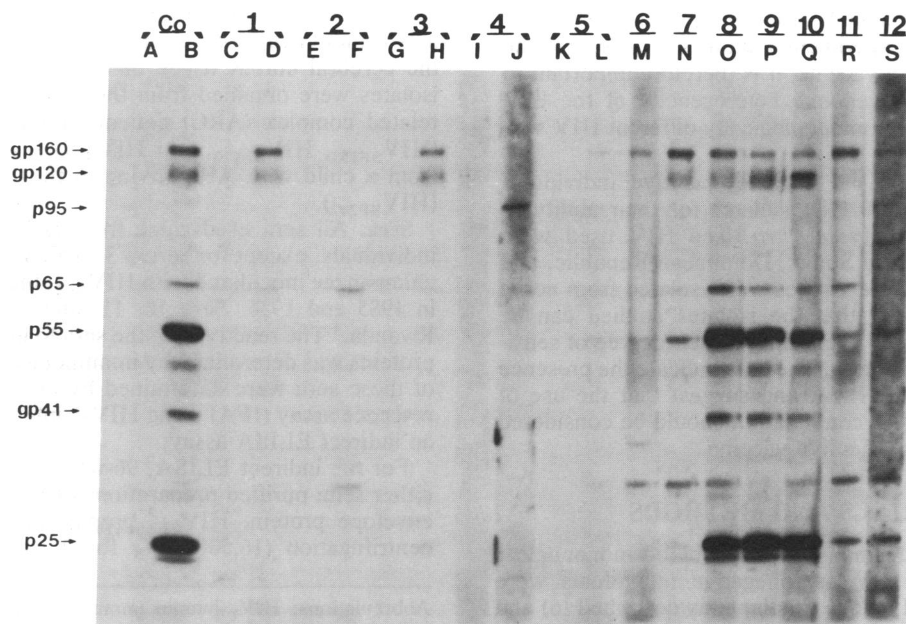

FIG. 1. Immunoblot analyses of anti-HIV-1 sera. The patterns of reactivity of the 12 sera to HIV-1 viral proteins were determined as described (22). Molecular sizes of the major viral proteins are indicated. Uninfected HUT-78 lysates (lanes A, C, E, G, I, K); and HIV_{SF2}-infected HUT-78 cell lysates (lanes B, D, F, H, J, L, M–S) were treated with the indicated sera (sera 1–12). Co, control positive serum for HIV-1 antibodies.

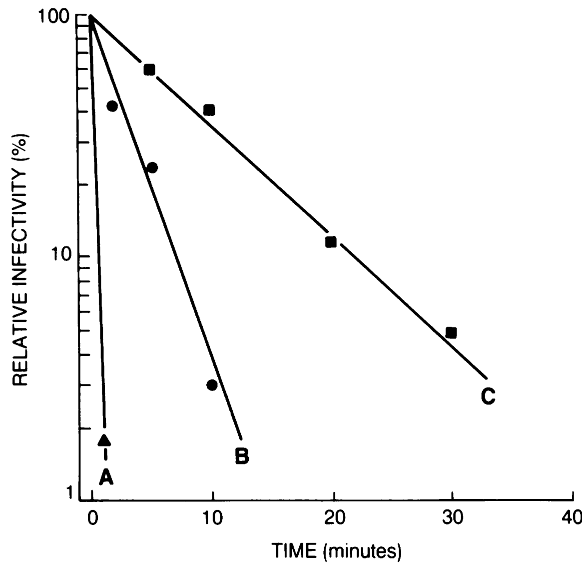

FIG. 2. Kinetics of neutralization. Kinetic curves of neutralization of HIV_{SF33} with serum 1 diluted at 1:10 (A), 1:50 (B), and 1:100 (C). Residual virus infectivity was expressed as % of initial infectious titer.

more, rate of neutralization (as reflected by the slope of the curves) changed as serum concentration changed.

Neutralization of HIV Isolates. The ability of three of the HIV-1 antibody-positive sera to neutralize infection of normal PBMC by 20 different HIV isolates was tested (Table 2). Serum 1 showed reactivity primarily to gp160/gp120 (Fig. 1) and had antibody titers that were 4- to 8-fold higher than sera 2 and 3 (Table 1). The neutralization titers are the results of tri- or quadruplicate experiments. Furthermore, neutralization of HIV_{SF2} by a serum of known titer was always included in each experiment as a control for variability of either the PBMC, antibody reactivity, or virus infectivity. The results suggest the presence of four distinct HIV subtypes. Nine HIV isolates were neutralized by all three sera (group A); two isolates (group B) were neutralized by two sera; eight isolates (group C) were neutralized by serum 1 only, and none of the three sera tested neutralized HIV_{SF170}, an isolate from Kigali, Rwanda (group D). In addition, an immunoglobulin G preparation obtained from serum 1 by protein A chromatography neutralized HIV_{SF2} infection at a concentration as low as 10 μg/ml (data not shown).

To further evaluate the presence of HIV subtypes, nine additional HIV-1 antibody-positive sera (listed in Table 1) were tested for their ability to neutralize five representative HIV isolates selected from the four groups described in Table 2. Because the inability of some sera to neutralize independent HIV isolates could be the result of low antibody titers, some of the sera were chosen for their high anti-HIV-1 and anti-*env*-2-product antibody titers—e.g., sera 8, 9, and 10 (Table 1). Results again showed that HIV_{SF2} and HIV_{SF4} (group A) were neutralized by all sera. Titers of neutralizing antibodies to HIV_{SF4}, however, were lower in at least five of the nine sera (Table 3). Two of seven tested sera neutralized HIV_{SF97} (group B) at low titers. In contrast, only one serum of the nine sera neutralized HIV_{SF128A} (group C); this serum came from a patient who died with *P. carinii* pneumonia. Serum 9, with the highest anti-HIV-1 antibody titer (1:512,000) as measured by ELISA, did not neutralize HIV_{SF128A}. None of the nine sera neutralized HIV_{SF170} (group D) at the lowest serum dilution used (1:10).

Characterization of HIV Isolates. To determine whether isolates in groups C and D (Table 2), similar to HIV type 2

Table 2. Neutralization of HIV infectivity by anti-HIV-1 sera

Proposed subtype	HIV_{SF}	Source	Serum 1	Serum 2	Serum 3
A	2	PBMC (SF, 1983*)	≥1000	100	10
	4	PBMC (SF, 1984)	≥1000	100	100
	13	PBMC (SF, 1984)	≥1000	100	10
	33	PBMC (Ph, 1984)	≥1000	100	10
	66	PBMC (SF, 1984)	≥1000	100	10
	113	PBMC (SF, 1985)	≥1000	100	100
	117	PBMC (SF, 1985)	≥1000	100	10
	301A	CNS (SF, 1986)	≥1000	100	100
	315	PBMC (SF, 1986)	100	10	10
B	97	PBMC (SF, 1985)	80	20	–
	171	PBMC (Af, 1985)	20	10	–
C	98	CNS (SF, 1985)	20	–	–
	128A	CNS (SF, 1985)	20	–	–
	161B	CNS (SF, 1985)	100	–	–
	162	CNS (SF, 1985)	100	–	–
	178	CNS (SF, 1985)	20	–	–
	185	CNS (SF, 1985)	20	–	–
	153	PBMC (Af, 1985)	20	–	–
	247	PBMC (DR, 1985)	100	–	–
D	170	PBMC (Af, 1985)	–	–	–

Ten-fold and two-fold dilutions of anti-HIV-1 sera (1, 2, and 3) were tested for their ability to neutralize infectivity of PBMC by 20 different HIV isolates. Figures represent the reciprocal of the highest serum dilution (up to 1:1000), causing a reduction greater than or equal to 67% in reverse transcriptase activity in the culture fluid (21). A negative (–) indicates no virus neutralization at a 1:10 serum dilution. CNS, central nervous system; SF, San Francisco; Ph, Philadelphia; DR, Santo Domingo, Dominican Republic; Af, Kigali, Rwanda; *, figures in this column represent year of isolation.

(27), represent new HIV, six isolates from groups A, C, and D were treated by immunoblot with a pool of HIV-1 antibody-positive sera from the United States. The data showed that all the major viral proteins of HIV_{SF128A}, HIV_{SF170}, and HIV_{SF247}, similar to those of isolates in group A (HIV_{SF2}, HIV_{SF4}, and HIV_{SF33}), reacted with the antibody-positive serum (Fig. 3). The molecular size of gp120 varied among the different HIV-1 isolates. Whether this difference in size of the major envelope protein plays a role in determining the immunologic properties of the different HIV remains to be determined. Finally, HIV-1 antibody-positive sera from the United States and Africa were tested for their reactivity with

Table 3. Differences in susceptibility of proposed HIV subtypes to neutralization by human sera

Serum	A: HIV_{SF2}	A: HIV_{SF4}	B: HIV_{SF97}	C: HIV_{SF128A}	D: HIV_{SF170}
4	100	100	–	–	–
5	≥1000	100	–	–	–
6	100	100	–	–	–
7	≥1000	≥1000	–	10	–
8	100	100	10	–	–
9	≥1000	100	20	–	–
10	≥1000	100	–	–	–
11	≥1000	100	20	–	–
12	100	10	–	–	–

Column headings: Subtype, neutralizing titer.

Neutralization of HIV infectivity by nine anti-HIV human sera was done as described in *Materials and Methods* and the legend for Table 2. A minus sign (–) indicates no virus neutralization at a 1:10 serum dilution.

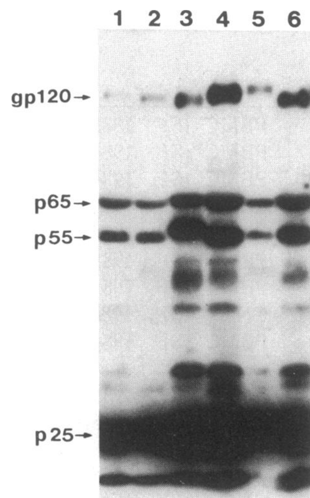

FIG. 3. Viral protein patterns of HIV isolates. Preparations of HIV were analyzed by immunoblot with a pool of HIV-1 positive sera. Lanes: 1, HIV_{SF2}; 2, HIV_{SF4}; 3, HIV_{SF33}; 4, HIV_{SF128A}; 5, HIV_{SF247}; and 6, HIV_{SF170}. Molecular sizes of the major viral proteins are indicated.

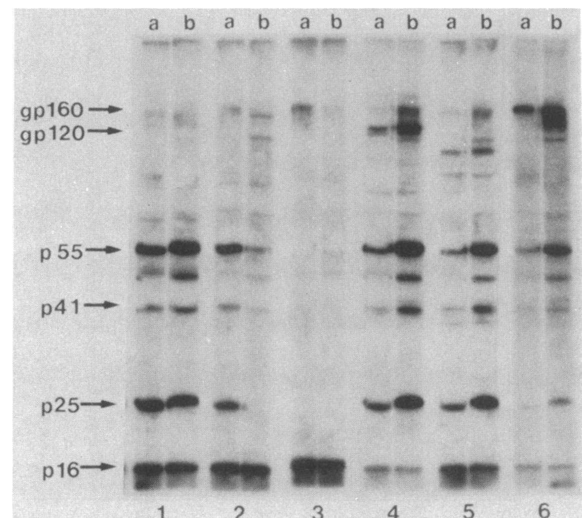

FIG. 4. Reactivity of HIV_{SF2}- and HIV_{SF170}-infected cell lysates with HIV-positive sera. Approximately 10^7 cell equivalents of HIV_{SF2}- and HIV_{SF170}-infected PBMC lysates (lanes a and b, respectively) were treated with anti-HIV-1 sera from the United States (lanes 1–3), Rwanda, Africa (lanes 4 and 5), and Uganda, Africa (lane 6). Molecular sizes of the major viral proteins are indicated.

lysates of cells infected with the United States isolate HIV_{SF2} and the African isolate HIV_{SF170}. In general, sera from the United States reacted more strongly with lysates from HIV_{SF2}-infected cells, whereas sera from Africa showed stronger reactivity with lysates from HIV_{SF170}-infected cells (Fig. 4).

DISCUSSION

Our results show that HIV isolates can be grouped into distinct subtypes with different sensitivity to serum neutralization (Table 2). The neutralization assay used reflected the reaction of specific antibody with virus as demonstrated by the kinetics of neutralization (Fig. 2) and by the neutralizing ability of purified IgG. Data show that viruses recovered from PBMC of individuals living in the United States are highly sensitive to neutralization and form one group (A). In particular, the prototype virus (HIV_{SF2}) was neutralized by all 12 sera at high titers. In fact, serum 1 at 1:20,000 still neutralized this isolate (data not shown). A similar finding has been reported by others (28). Interestingly, HIV recovered from cerebrospinal fluid or brain tissues of individuals with neurologic symptoms were characteristic of a separate group (group C, Table 2). Finally, HIV_{SF170}, an isolate from Kigali, Rwanda, was resistant to neutralization by all 12 sera tested (group D, Table 2). Nevertheless, all the major viral proteins of HIV_{SF170} reacted with HIV-1 antibody-positive sera (Fig. 3), although these proteins were recognized more strongly by sera from Africa than from the United States (Fig. 4). These findings suggest that HIV_{SF170} is a distinct variant of HIV-1, which may represent a subtype from Africa.

Previous studies showed that diverse HIV isolates exhibit similar susceptibility to neutralization by HIV-1 antibody-positive sera (28–30). These reports suggested that neutralizing antibodies are directed at conserved domains on the viral envelope. The HIV isolates used in these other studies, however, all replicate in established T-cell lines (e.g., H9 and HUT-78 cell lines). Note that while most HIV isolates (6/8) in group A replicate in HUT-78 cells, the isolates in the other groups (B, C, and D) do not replicate in HUT-78 cells (refs. 31, 32; C.C-M., unpublished observation). Therefore, the use of HIV that replicate in established T-cell lines for neutralization studies may be limited to defining neutralizing epitopes shared by a subset of similar HIV isolates.

Our results, similar to those reported by others (28–30, 33, 34), suggest that neutralizing antibodies to HIV are directed against the viral envelope glycoproteins (Table 2, Fig. 2). For one serum (serum 4), antibody reactivity was directed only to a protein of 95 kDa present only in HIV-1-infected cells. Because this serum also reacted by ELISA with semi-purified HIV_{SF2} virus and the recombinant envelope protein encoded by *env*-2 (Table 1), p95 may represent a partially glycosylated form of gp160/gp120 (35) or a degradation product of gp120 (17). No correlation between anti-*env*-product antibody titers and the ability to neutralize HIV infectivity was noted. This observation indicates that not all immunodominant envelope epitopes are neutralizing epitopes. Finally, as recently reported by some (28, 29), but not other (30, 33, 34) investigators, there is a lack of correlation between disease stage and the ability of a patient's serum to neutralize HIV infection *in vitro* (Tables 1–3). Even individuals with far advanced disease (e.g., serum 7, *P. carinii* pneumonia) have high serum-neutralizing antibodies.

In summary, the data indicate variations of neutralization antigens among HIV-1 isolates. Studies with other sera will undoubtedly identify further serologic subtypes, but the data obtained from these 20 HIV isolates suggest that a finite number of antigenic subgroups exist. Our findings imply that for vaccine development using the envelope antigen, the combined use of HIV isolates representing various subtypes may be necessary to obtain polyspecific immunoprotection. Finally, the preparation of antisera directed against representatives of the various HIV subtypes should help establish further the serotype classification proposed and recognize additional HIV subtypes.

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