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NOTES

Human Immunodeficiency Virus Neutralizing Antibodies Recognize Several Conserved Domains on the Envelope Glycoproteins

DAVID D. HO,[1]†* M. G. SARNGADHARAN,[2] MARTIN S. HIRSCH,[1] ROBERT T. SCHOOLEY,[1] TERESA R. ROTA,[1] RONALD C. KENNEDY,[3] TRAN C. CHANH,[3] AND VICKI L. SATO[4]

Infectious Disease Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114[1]; Bionetics Research, Inc., Rockville, Maryland 20850[2]; Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78284[3]; and Biogen Research Corporation, Cambridge, Massachusetts 02142[4]

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Serum neutralizing antibodies against the human immunodeficiency virus were frequently detected in infected individuals, and low or absent serum neutralizing titers correlated with poor prognosis. Multiple diverse human immunodeficiency virus isolates were found to exhibit similar susceptibility to neutralization by a panel of human seropositive sera, suggesting that neutralizing antibodies are largely directed against conserved viral domains. Furthermore, utilizing antisera raised against a library of synthetic *env* peptides, four regions which are important in the neutralization process have been identified within both human immunodeficiency virus envelope glycoproteins (gp41 and gp120). Three of these are in conserved domains and should be considered for inclusion in a candidate vaccine.

Human immunodeficiency virus (HIV) (12), previously known as lymphadenopathy-associated virus (1) or human T-cell lymphotropic virus type III (HTLV-III) (19), is the etiologic agent for the acquired immunodeficiency syndrome (AIDS), which has now reached epidemic proportions. A vaccine for HIV, as well as effective treatment, is urgently needed. Better understanding of the relationship between serum antibodies and HIV would aid the design and subsequent development of prophylactic programs. Several investigators have previously shown that HIV-neutralizing antibodies can be detected in the serum of most infected individuals (7, 21, 29). We now report findings from studies conducted to further characterize serum HIV-neutralizing antibodies, to assess the significance of HIV strain variability in virus neutralization, and to define the viral domains important in the neutralization of HIV.

Sera from healthy seropositive homosexual men and seronegative individuals, as well as from patients with AIDS or AIDS-related complex (ARC), were evaluated for HIV-neutralizing activity. Unless otherwise stated, HIV neutralization assays were performed using the H9 clone as the target cells and the HTLV-IIIB isolate as the virus inoculum (19). Cell-free HIV was harvested from a chronically infected H9 culture and titrated on uninfected H9 cells, and the titer was expressed as 50% tissue culture infective dose per milliliter. Each virus inoculum (100 μl, 50 50% tissue culture infective doses) was preincubated with test serum or immunoglobulin preparation (100 μl, serial twofold dilutions) for 1 h at 37°C before inoculation onto 2.0×10^6 H9 cells in 5 ml of RPMI 1640 medium supplemented with fetal calf serum (20%), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (10 mM), penicillin (250 U/ml), streptomycin (250 μg/ml), and L-glutamine (2 mM). On days 7 through 10, each culture was examined for characteristic cytopathic effects with syncytia formation and for reverse transcriptase activity in the supernatant fluid by techniques previously described (10). Neutralization was defined as the complete inhibition of syncytia formation and >90% reduction in reverse transcriptase activity compared with control cultures, which were similarly established except that the virus inoculum was preincubated with culture medium or seronegative control human serum. HIV serum neutralizing titers of 1:4 or greater were considered positive.

Of the 48 sera from seronegative controls, none showed neutralizing activity, whereas 157 of 175 sera from seropositive persons demonstrated virus neutralization at titers of 1:4 to 1:512 (Fig. 1A). The 10 healthy seropositive homosexual men who lacked serum neutralizing activity were all recent seroconvertors and had low titers (geometric mean titer, 1:18) of HIV-specific antibodies as determined by a fixed-cell indirect immunofluorescence assay (24). Serum neutralizing activity was not detected in these subjects for 4 to 8 months after the first signs of seroconversion (data not shown). Those sera from ARC and AIDS patients which lacked neutralizing activity also had low HIV antibody titers by immunofluorescence, and, overall, serum neutralizing and immunofluorescence titers were directly correlated (Fig. 1B). The reciprocals of the geometric mean neutralizing titers were 25.2 and 23.5 for healthy seropositive persons and ARC patients, respectively. In contrast, the reciprocal of the geometric mean titer for AIDS patients was 9.5, which is significantly lower ($P < 0.01$ by unpaired *t* test) than either of the previous two groups. In addition, nine of the ARC patients who subsequently developed AIDS or had >50% decline in T4 lymphocyte numbers also had significantly lower ($P < 0.01$) geometric mean neutralizing titers (8.0) than did the other ARC patients (30.3). These results suggest that low serum HIV-neutralizing titer is associated with

* Corresponding author.
† Present address: Infectious Diseases Division, Cedars-Sinai Medical Center, Los Angeles, CA 90048.

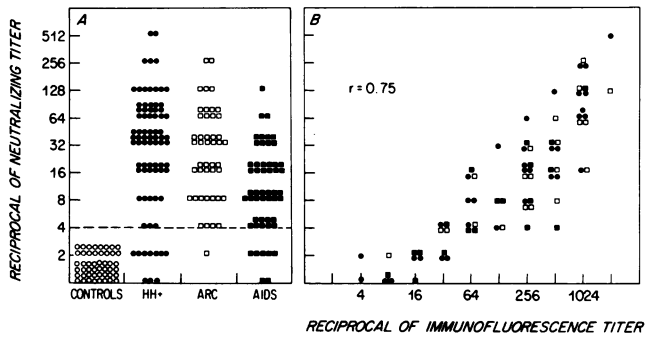

FIG. 1. (A) Reciprocals of serum HIV-neutralizing titers for seronegative controls, seropositive healthy homosexual men (HH+), and ARC and AIDS patients. (B) Correlation of HIV-neutralizing and immunofluorescence titers. Indirect immunofluorescence assay was performed using acetone-fixed, HIV-infected H9 cells as previously described (24).

advanced or progressive disease, as has been reported by other investigators (21). However, it is unclear whether low or absent serum neutralizing activity is causally involved in the disease progression or a mere reflection of the general deterioration in immune functions.

To determine whether the serum neutralizing factor is an antibody, immunoglobulin fractions were extracted from two sera that had previously demonstrated HIV-neutralizing activity, for use in neutralization assays. First, Affi-Gel–Protein A (BioRad Laboratories) columns were used to obtain total immunoglobulin fractions which showed nearly 100% of the original serum HIV-neutralizing activity. In addition, affinity chromatography employing disrupted HIV proteins attached to CH-Sepharose (Pharmacia Fine Chemicals) was performed to yield HIV-specific immunoglobulins, which also exhibited neutralizing activity. It is extremely unlikely that a nonimmunoglobulin factor would copurify with the immunoglobulins during the above procedures to account for the virus neutralizing activity. Therefore, we conclude that the HIV-neutralizing factor in serum is an antibody.

The importance of a specific type of target cell used in the HIV neutralization assay was studied. When other HIV-susceptible cells (Hut 78, CEM, U-937) were substituted for H9 cells in the assay, no changes in neutralizing titers were seen (the experiment was performed twice with four positive sera). The role of serum complement in HIV neutralization was also examined. Twelve seropositive sera were assayed for neutralizing activity, with and without prior heat inactivation (56°C, 60 min). No differences in neutralizing titers were observed. Furthermore, the addition of rabbit or guinea pig complement (Flow Laboratories) did not enhance HIV neutralization. Therefore, it appears that serum complement does not play a major role in the neutralization of HIV.

HIV genomic diversity is well described and is most prominent in the virus envelope (2, 28, 30). This heterogeneity may be a potential problem in vaccine development. Weiss et al. have previously reported that multiple HIV isolates were equally sensitive to a panel of human neutralizing sera (29). However, the degree of heterogeneity among those isolates was unknown. We therefore examined the susceptibility of four diverse isolates to neutralization by 15 human sera (13 HIV seropositive, 2 seronegative). Isolates HTLV-IIIB, HTLV-IIIRF, and HTLV-IIIMN were kindly provided by M. Popovic and R. Gallo (19, 30). They are known to differ from each other significantly, particularly in the *env* region (30). In fact, HTLV-IIIB and HTLV-IIIRF are different in 21.4% of the predicted amino acid sequences in the gp120 (28). Isolate HIV-AL was derived from the peripheral blood of a Haitian AIDS patient in our laboratory by methods already described (9). HIV-AL is known to be different from the other three isolates, based on restriction endonuclease analysis (unpublished data). Serum neutralization results with these viruses are summarized in Table 1. Despite the diversity, neutralization titers were the same or similar for 13 of the 15 sera tested. Moreover, serum 12 did not neutralize the "homologous" isolate (HIV-AL) with greater efficacy than the other isolates. Together, these findings suggest that HIV neutralizing antibodies are primarily directed against conserved epitopes and that broad cross-neutralization may be possible. Relative strain specificity in neutralization, however, was seen with two sera (no. 14 and 15).

Studies were also conducted to determine which viral antigens and epitopes are involved in antibody neutralization. First, we compared HIV antibody profiles on radioimmunoprecipitation assays (4) and Western blots (27) with HIV-neutralizing titers for 52 positive sera randomly selected from samples depicted in Fig. 1A. Serum neutralizing activity did not correlate with the presence, absence, or intensity of antibody reactivity to *gag* (p24 [25]) or *pol* (p51/p66 [3]) products. In contrast, since all 52 sera contained antibodies to at least one *env* product (gp41 or gp120) and 48 of 52 had neutralizing antibodies, a correlation between antienvelope antibodies and neutralization was suggested. In particular, 10 sera (3 shown in Fig. 2A) with reactivity to only envelope proteins (gp120 and gp41) and their precursor (gp160) (4) exhibited neutralizing capacity with titers of 1:4 to 1:64. These correlative findings were later supported by studies using a goat antienvelope serum. A preparation of gp41 and gp120, purified from disrupted virions on a lentil-lectin column as previously described (26) and illustrated in Fig. 2B, was used to immunize a goat. The resultant antiserum showed strong immunoreactivity with both envelope proteins and neutralized HIV at a titer of 1:32.

TABLE 1. Reciprocals of serum neutralizing titers against diverse HIV isolates[a]

Serum no.	Reciprocal of titer against HIV isolate:			
	HTLV-IIIB	HTLV-IIIRF	HTLV-IIIMN	HIV-AL
1	<2	<2	<2	<2
2	<2	<2	<2	<2
3	4	4	4	4
4	4	4	8	4
5	8	8	8	8
6	16	16	16	8
7	16	32	32	16
8	32	32	32	32
9	32	32	32	64
10	64	64	64	64
11	64	64	128	64
12(AL)	128	64	128	64
13	128	128	128	128
14	128	8	NT	8
15	32	256	NT	256

[a] All isolates were grown in H9 cells. The inoculum size was 50 50% tissue culture infective doses of cell-free virus for each isolate. When no neutralizing serum was added, the kinetics of reverse transcriptase activity for each of the four isolates at this inoculum were nearly identical. Not only were the endpoint neutralizing titers for sera no. 3 through 13 similar, but the kinetics of reverse transcriptase reduction were also comparable. NT, Not tested. Sera no. 1 and 2 were from seronegative controls.

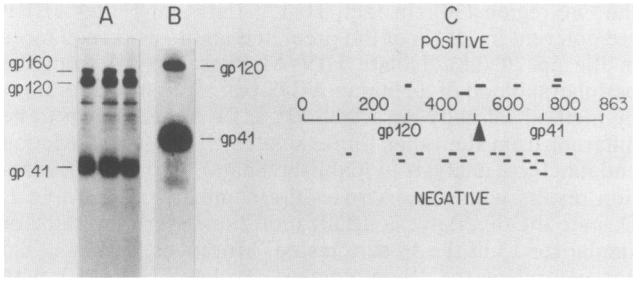

FIG. 2. (A) Three human HIV-neutralizing sera which react with only envelope proteins on radioimmunoprecipitation assay (4). The band below gp120 represents reactivity with a cellular product. (B) Lentil lectin-purified envelope proteins identified by a positive human serum on Western blotting (26). (C) Map of HIV envelope glycoproteins. The amino acid numbers are based on those of Ratner et al. (20), and the gp120-gp41 cleavage site is denoted by the triangle. The coordinates of those peptides which raised HIV-neutralizing sera are schematically represented on the top portion (POSITIVE). The coordinates of peptides which did not generate neutralizing sera are represented on the bottom portion (NEGATIVE).

These results show that HIV envelope proteins are major targets for neutralizing antibodies, a finding similar to those reported for other retroviruses (11). They are also consistent with recent studies demonstrating that goat, horse, and monkey antisera against purified gp120 (22) or rabbit and guinea pig antisera against recombinant gp130 (a slightly modified gp120) (14) can neutralize HIV.

The importance of both gp41 and gp120 in HIV neutralization was confirmed and extended by studies using a library of antibodies raised against synthetic envelope peptides. The peptides were synthesized on an Applied Biosystems model 430A synthesizer, using a modified version of the solid-phase method described by Merrifield (15) and utilizing the envelope amino acid sequence predicted from the nucleotide sequence analysis of Ratner et al. (20) on the HTLV-IIIB isolate. We deblocked and cleaved the protected peptides from the resin with anhydrous hydrogen fluoride containing 10% anisole, in a variation of the method of Sakakibara et al. (23). The peptides were then cleaved from the resin by partition chromatography and a Sephadex LH20 column using *n*-butanol–water (6:100) as eluent. The peptides were further purified by semipreparative high-pressure reversed-phase chromatography on an Altex Ultrasphere-ODS column by elution with an aqueous acetonitrile gradient containing 0.1% trifluoroacetic acid. The eluate was hydrolyzed with 6 N HCl for 18 h, and then sequence analysis was carried out on a Beckman amino acid analyzer to confirm the sequences of the peptides produced. Each of the peptides was coupled to keyhole limpet hemocyanin (Sigma Chemical Co.) by mixing 2 mg of peptide in 2 ml of sodium phosphate buffer (0.1 M, pH 8) with 5 mg of the hemocyanin in 2 ml sodium phosphate buffer. Glutaraldehyde solution (0.25%, 1 ml) was then added to the mixture in several portions over 1 h. The resulting mixture was stirred for 6 h and then dialyzed against phosphate-buffered saline overnight. Each of the keyhole limpet hemocyanin-coupled peptides was emulsified with Freund complete adjuvant at a 1:1 ratio. Subsequently, groups of three BALB/CJ mice (Jackson Laboratory) were immunized with each peptide by subcutaneous injection of 100 μg in 250 μl of the emulsion into each mouse. On days 14 and 35, each mouse received an appropriate booster injection of the same dose. Tail bleeds were taken on days 21 and 42, and sera were stored at −20°C until the time of assay.

A total of 85 overlapping peptides spanning *env* were synthesized. Peptide sizes were variable because of solubility and coupling considerations. The precise amino acid coordinates of the first 22 peptides and the immunoreactivity of their corresponding mouse antisera are summarized in Table 2. The complete characterization of all peptides and antisera will be published elsewhere (V. L. Sato, manuscript in preparation).

Two additional peptides (735-752 and 503-532, also according to Ratner et al. [20]) were selected and synthesized because of their hydrophilicity, predicted beta-turn secondary structure, and relative invariance (18). Peptide 735-752 was synthesized on a polystyrene resin with a C-terminal cysteine utilized for coupling to keyhole limpet hemocyanin (13), and peptide 503-532 was synthesized on a polyacrylamide-based resin (1a). Rabbit antisera raised against these two peptides were generated as previously described and were found to be reactive with the appropriate envelope glycoproteins (1a, 13).

Figure 2C and Table 2 summarize the neutralization results with the above antipeptide sera. Eighteen antisera, along with four normal mouse sera and three normal rabbit sera, were negative in the neutralization assay (Fig. 2C, bottom). In contrast, mouse antisera to peptides 298-314, 458-484, 616-632, and 728-751 were found to neutralize HIV at titers of 1:32, 1:4, 1:32, and 1:8, respectively (Fig. 2C, top). Rabbit antisera against peptides 503-532 and 735-752 also showed neutralizing activity, both at a titer of 1:32 (both prebleed serum neutralizing titers were ≤1:4). Taken together, the positive results point to four regions in the HIV envelope as important determinants for antibody neutralization. Two (amino acids 298-314 and 458-484/503-532) fall

TABLE 2. Immunoreactivity of antipeptide sera and their HIV neutralization titers[a]

Mouse antiserum to peptide:	Immunoreactivity to:		Neutralization titer
	Corresponding peptide	HIV gp160	
126-139	+	+	<1:4
272-284	+	−	<1:4
280-293	+	−	<1:4
298-314	+	+	1:32
336-351	+	+	<1:4
407-421	+	+	<1:4
426-450	+	−	<1:4
459-477	+	+	<1:4
458-484	+	+	1:4
483-499	+	−	<1:4
550-565	+	+	<1:4
575-584	−	−	<1:4
590-602	+	−	<1:4
616-632	+	+	1:32
627-639	−	−	<1:4
646-661	−	−	<1:4
667-680	+	+	<1:4
675-690	+	−	<1:4
686-699	−	−	<1:4
691-708	+	−	<1:4
728-751	+	+	1:8
768-785	+	−	<1:4

[a] Reactivity with the corresponding peptide was assayed by enzyme-linked immunosorbent assay (27); reactivity with the envelope precursor, gp160, was determined by radioimmunoprecipitation and polyacrylamide gel electrophoresis (4). The positive neutralization titers against isolate HTLV-IIIB were identical in two separate experiments.

primarily within the exterior glycoprotein, gp120. The other two (amino acids 728-752 and 616-632) are located in the transmembrane protein (4), gp41, suggesting that substantial portions of this molecule are external. All four sequences correspond to extremely hydrophilic regions of the envelope (best seen on the hydropathy plot of Muesing et al. [16]), and three (amino acids 458-484/503-532, 616-632, and 728-752) overlap significantly with highly conserved regions of the envelope glycoproteins identified by Starcich et al. (28).

In summary, we have reported the frequent detection of HIV-neutralizing antibodies in sera from infected individuals and noted a correlation between advanced disease and low neutralizing titers. We have also shown that multiple diverse isolates are equally sensitive to neutralization by most positive human sera and that the envelope glycoproteins are major targets for antibody neutralization of HIV. Furthermore, four regions important in neutralization have been defined in the virus envelope. Three of these four sites overlap significantly with conserved domains. These findings raise the possibility that the observed genomic diversity may not be a major deterrent to successful vaccine development.

We suggest that all domains which elicit neutralizing antibodies be selected for inclusion in a multivalent candidate vaccine. Furthermore, it may be useful to exclude nonneutralizing domains from such a vaccine preparation. Nonneutralizing epitopes may sterically hinder critical epitopes or may elicit "blocking antibodies" which interfere with the action of neutralizing antibodies. In addition, coating of virus by nonneutralizing antibodies could lead to enhancement of virus uptake by Fc receptor-bearing cells, such as monocyte/macrophages, as has been reported for dengue virus (6). This may be important, because the human monocyte/macrophage is capable of supporting HIV replication (5, 8, 17).

It is not clear which host responses, or which viral epitopes, will ultimately be useful in protection against HIV. Current studies in our laboratories are directed at defining the precise epitope specificities found in human neutralizing sera and at characterizing the importance of specific viral antigens in triggering different host immune responses. Further definition of these determinants should assist the development of a vaccine for AIDS.

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