HUMAN IMMUNODEFICIENCY VIRUS 1

Predominance of a Group-specific Neutralizing Epitope that Persists Despite Genetic Variation

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Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS (1, 2). Rapid progress in the isolation (3), cloning, and sequencing of the entire viral genome (4–6) has shown the remarkable propensity of HIV for genetic variation, particularly within the envelope gene (6). Since viral envelope proteins are often the target for neutralizing antibodies (7), this extensive variation may play an important role in the interaction between the virus and the host's immune system. For some viruses, such as influenza, rapid mutation is an important means of escape from neutralizing antibodies (8), which results in successive waves of influenza epidemics among previously infected populations. For visna virus, a sheep retrovirus, the mutation rate is believed to be so rapid as to allow antibody escape during the course of a single chronic infection (9). If similar mutants arise in humans infected with HIV, even during the course of multiple rounds of infection, it would be difficult to imagine a vaccine antigen that could keep pace with all the possible variants and prevent infection.

However, in spite of the observed rapid mutation rate, it is possible that the virus cannot mutate at certain sites, particularly those serving essential viral functions. For example, the CD4-binding site has been mapped to three relatively conserved regions of gp120 (10, 11). Divergent isolates bind soluble CD4 and are inactivated by it (12–14, and discussed in reference 15), suggesting conservation of the CD4-binding site. Presumably, if neutralizing antibodies were directed against this site or another site with an equally critical viral function, they would be active against numerous clinical isolates of HIV-1, and a vaccine capable of eliciting these antibodies might protect against infection by a broad spectrum of clinical pathogens.

To study this important question, we have developed a sensitive new plaquing assay for HIV-1 that divides the infection of a suitable monolayer into a series of individual viral infection events. Like plaquing assays for other viruses (16, 17), it is highly sensitive to neutralizing antibodies and can be readily adapted to compare divergent HIV-1 isolates under equivalent conditions. Using this new assay, we have found that nearly all HIV-1–infected patients make neutralizing antibodies that are group specific, as shown by the ability to neutralize two or three divergent HIV-1 isolates equally at each dilution of antibodies. Thus, HIV-1 isolates may be genetically diverse and still share a common group-specific neutralizing epitope, making them

immunologically equivalent with respect to neutralizing antibodies. Contrary to earlier interpretations based on genetic sequencing data, these results suggest that a successful vaccine is possible, provided that it can elicit antibodies to the group-specific neutralizing epitope before infection.

Materials and Methods

Cells. The adherent CD4⁺ HeLa human epithelial carcinoma cell line T4Ps5 (18) was generously provided by Drs. P. Maddon and R. Axel (Columbia University, New York). This line was constructed by transforming naturally adherant HeLa cells with the cloned gene for CD4 and was shown to support the growth of HIV-1 (18). Thus, it formed a monolayer that was suitable for detecting HIV-1 plaques. The cells were grown in the presence of 0.5 mg/ml Geneticin (G418 sulfate; Gibco Laboratories, Grand Island, NY) in complete medium containing DME supplemented with 4.5 g/liter glucose and 15% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Uninduced cells expressed surface CD4 on ~15% of cells (as measured by cytofluorometry), which increased to 30% after induction with epidermal growth factor (EGF)[1] (100 ng/ml) for 1 h. All assays were done with induced cells, except as noted.

Sera. Human sera were obtained from asymptomatic donors who were discovered to be seropositive by routine blood bank screening. They were confirmed positive by demonstrating reactivity to multiple bands on Western blot and were a gift of Dr. J. Wai-Kuo Shih of the NIH Department of Transfusion Medicine. Normal sera were obtained from laboratory workers, and three additional sera counted in this group were false positives by ELISA testing but were repeatedly Western blot negative over a period of 6 mo. Additional sera from patients with clinical AIDS were a gift of Dr. Robert Yarchoan of the NIH, and sera from patients with adult T cell leukemia, seropositive for HTLV-I, were a gift of Dr. Thomas Waldmann, NIH. Sera from patients with autoimmune diseases were a gift from Dr. Laurence Rubin, University of Toronto Medical School.

Virus. H9 cells infected with the IIIB (4) and RFII (6) isolates of HIV-1 as well as the NIHZ isolate of HIV-2 (19) were provided by Drs. Robert Gallo and Howard Streicher (National Cancer Institute) and by Drs. Hiroaki Mitsuya and Sam Broder (NCI), and A3.01 cells (20) infected with Zaire-1 (Z1) (21) were provided by Dr. Thomas Folks (NCI). Virus was passaged monthly by freshly infecting H9 cells, as confirmed by the appearance of viral p24 antigen in the culture supernatants (22). Infected H9 cells were irradiated (10,000 rad, Cs137 irradiator) before use in the assay.

Virus-specific DNA Probe. The probe was a slightly truncated 9.2-kb fragment of the cloned BH10 isolate of HIV-1 (4). It was ³²P labeled with a nick translation kit from New England Nuclear (Boston, MA), and purified on a Sephadex G50 spin column, giving a specific activity of ~2 × 10⁷ cpm/μg. As shown in Fig. 1, the probe could detect a dot containing the amount of viral RNA in 5,000 infected H9 cells, but there was no background signal from 10⁶ uninfected H9 cells. In subsequent experiments, with acutely infected H9 cells, dots containing as few as 1,000 infected cells were detectable.

Plaque-forming Assay. The assay can be divided into a virus growth step followed by a plaque detection step (Fig. 2). CD4⁺ HeLa cells were incubated for 1 h in the presence of 100 ng/ml EGF (Collaborative Research, Lexington, MA). They were then washed and infected by incubation with various dilutions of cell-free HIV-1 or with various numbers of infected H9 cells for 2 h at 37°. The cells were plated in 5-cm diameter tissue culture dishes (3060; Costar, Cambridge, MA) overnight in complete medium (without geneticin). The next day, the culture medium was replaced with an agarose overlay containing 4 ml of 0.8% Seaplaque agarose (Marine Colloids Inc., Rockland, ME) in complete medium. The cells in the culture dishes were diluted ~1:80 relative to confluence, and they grew to confluence in ~7 d.

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[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; PFU, plaque-forming units; rCD4, recombinant soluble CD4.

Zones of viral infection on the monolayer were detected by DNA-RNA hybridization, by a modification of the method of Villareal and Berg (23). When the infected monolayer cells reached confluence, the entire monolayer was transferred from plastic to nitrocellulose by removing the agarose and pressing down discs of nitrocellulose paper saturated with 0.05 M Tris-HCl, pH 7, and 0.15 M NaCl on the surface of the monolayer. When the discs were lifted off the plastic, the entire cell monolayer came off with them. The cells were lysed by placing the discs on a filter paper saturated with 6% formaldehyde in 6 × SSC and 50% formamide, for 10 min at 65°, followed by 6 × SSC and 0.1% SDS, and then 6 × SSC at room temperature. The discs were air dried and baked in a vacuum oven for 1 h at 80°C. After baking, the discs were washed in 6 × SSC and then prehybridized with a mix containing 6 × SSC, 10% dextran sulfate, 5% Denhardt's Solution, 0.1% SDS, 0.1% Poly adenosine (>100,000 mol wt), and 300 μg/ml boiled herring sperm DNA. After prehybridizing 4 h at 65°C, the discs were hybridized with a ^{32}P-labeled viral probe in fresh prehybridization mix for 18 h at 65°. After hybridization, the filters were washed twice at 55°C with 2 × SSC with 1% SDS, followed by 0.2 × SSC with 0.1% SDS at 50°C. The discs were then dried and placed in a photographic cassette with x-ray film.

Detection of Neutralizing Antibodies. A known titer of virus or virally infected H9 cells containing between 100 and 3,000 plaque forming units (PFU) of HIV-1 was incubated for 2 h at 37°C in the presence of various dilutions of serum from seropositive patients or immunized rabbits in a final volume of 50 μl. One-third or one-tenth of the surviving virus was used to infect CD4$^+$ HeLa cells during a further 2-h incubation, followed by the standard plaquing assay. The neutralizing titer was defined as the serum dilution giving a 50% reduction in plaque number.

Results

Plaque Formation. Previous attempts at detecting HIV-1 plaques were stymied by the lack of an adherent monolayer cell able to support the growth of the virus and by difficulty visualizing the cytopathic effects of the virus. We have now solved the monolayer problem by use of the genetically engineered CD4$^+$ HeLa cell, provided by Maddon and Axel (Columbia University, New York), which is naturally adherent and has acquired the ability to support the growth of HIV-1 (18). In addition, our HIV-specific detection system is sufficiently sensitive to detect the amount of virus in a single plaque. This was demonstrated by infecting monolayer cells with recombinant vaccinia virus vsc 25 (24) containing the *env* gene of HIV-1 and by detecting the lytic plaques with the ^{32}P-labeled HIV-specific probe (not shown).

We then used the hybridization method to detect infection of the CD4$^+$ HeLa monolayer by HIV-1. After 7-10 d in culture, we detected discrete, macroscopic viral plaques by hybridization, as shown in Fig. 3. Several HIV isolates, representing nearly the entire spectrum of HIV-1 as well as one HIV-2 isolate, were tested. In spite of differences in sequence (4-6, 19) and possibly in cytopathic effect, similar plaque
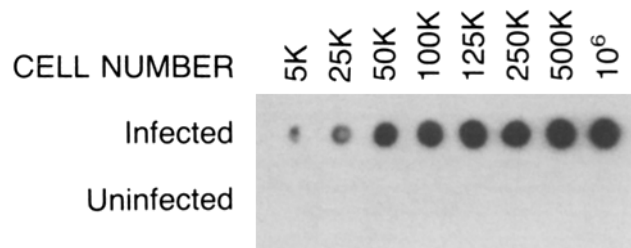

FIGURE 1. Detection of viral nucleic acid in infected H9 cells. Different numbers of infected or uninfected cells were blotted onto nitrocellulose paper and viral nucleic acid was detected by hybridization with a ^{32}P-labeled probe.

Virus + Antibody

2 h at 37°

Add CD4⁺ Hela Cells

4 h at 37°

Infected Foci
on CD4⁺ Monolayer

7–10 d

Culture Dish

Remove Medium

Transfer to
Nitrocellulose

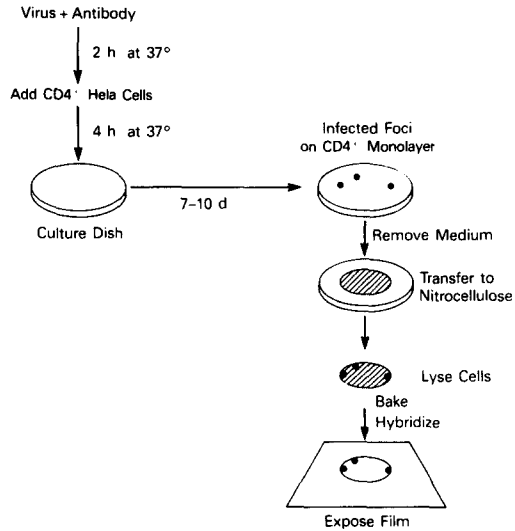Lyse Cells

Bake
Hybridize

Expose Film

FIGURE 2. Method for developing HIV-1 plaques. Virus is grown on the CD4⁺ monolayer for 7 d. Discrete foci of infection are detected by transferring the entire monolayer to nitrocellulose, followed by cell lysis, baking, and hybridization with a ^{32}P-labeled virus-specfic probe.

Third Codon Substitutions

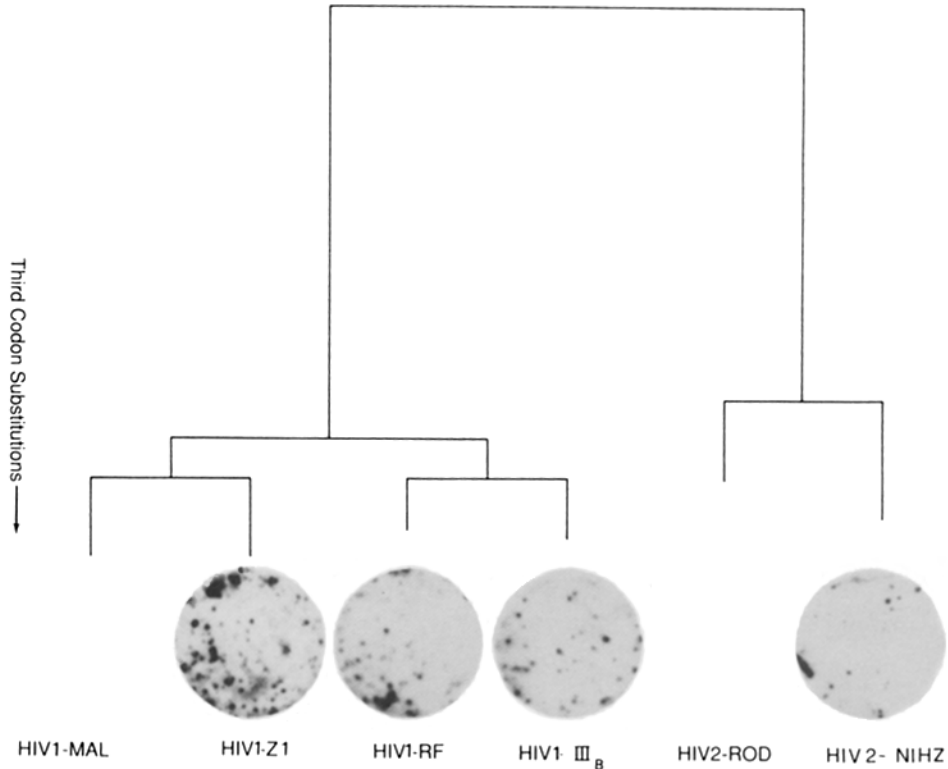HIV1-MAL   HIV1-Z1   HIV1-RF   HIV1· Ⅲ$_B$ HIV2-ROD   HIV 2- NIHZ

FIGURE 3. Divergent HIV isolates, arranged according to their deduced evolutionary tree (25), were detected as discrete plaques. The number of PFU observed after infecting the monolayer with virus from infected H9 cells were as follows: 63 PFU from 300 IIIB-infected cells, 121 PFU from 6,000 RFII-infected cells, 186 PFU from 1,000 Z1-infected cells, and 55 PFU from 1,500 HIV-2-NIHZ-infected cells. Vertical branch lengths indicate extent of genetic divergence, as measured by the number of third codon substitutions (adapted from reference 25). Strain Zaire-1 has been substituted for Zaire-3, which it resembles (21).

morphology was observed for each isolate. In Fig. 3, different isolates are arranged according to their deduced evolutionary tree, so that the vertical distance and the number of branches between isolates indicate evolutionary divergence as deduced from base substitutions in the envelope gene (25). If genetic variation among HIV-1 isolates is the result of antibody-mediated natural selection, then pairwise comparisons of variants for antibody resistance should correlate with the number of evolutionary branch points between them (discussed below). The plaques were macroscopic, 0.5–1.5 mm in size, and the number of PFU was readily counted by displaying the exposed film over an x-ray view box. The number of PFU was linearly proportional to the input of virus up to at least 160 plaques per culture, and no plaques were formed in the absence of virus or when a $CD4^-$ monolayer cell (CV-1 or normal HeLa cells) was substituted for the $CD4^+$ HeLa cell line. Thus, plaque formation is the result of viral growth on the monolayer and depends on the CD4 receptor of the monolayer cells. Microscopic observation of the monolayers revealed some giant cells, but, at the low multiplicity of infection used in this assay, there was no consistent difference between the infected and uninfected $CD4^+$ monolayer cells with regard to cytopathic effects or syncytium formation.

Comparison of the plaquing assay with the previously available antigen capture RIA (22) is shown in Fig. 4. There was an excellent correlation between PFU and antigen release into the culture medium over a 25-fold range of virus input. This enabled us to examine the effect of host cell activation on viral growth. It is well known that T cell activation by mitogens can enhance the recovery of HIV-1 (2), and a possible transcriptional mechanism involving the shared enhancer motif between the HIV-1 long terminal repeat and the NF-KB site of lymphocytes has been proposed (26, 27), so we tested whether activation of $CD4^+$ HeLa cells with EGF would have a similar effect on the efficiency of plaque formation. As shown in Fig. 4, activation of the monolayer cells gave increased numbers of PFU at each dose of virus, and the production of viral antigen increased proportionately. However, the slope of PFU vs. antigen production remained the same, indicating that average
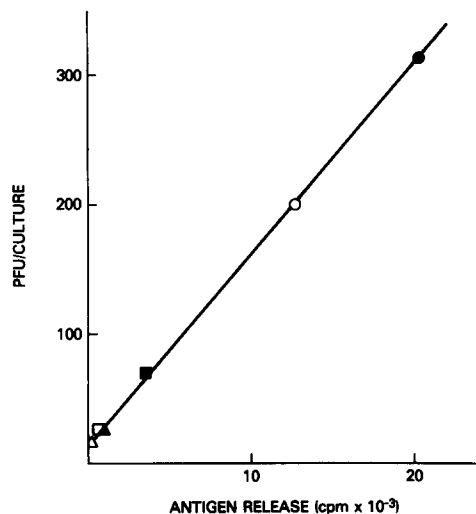

Figure 4. Correlation of PFU vs. antigen production per culture, as measured by antigen capture RIA (22). $CD4^+$ monolayer cells were infected with 2.5 × 10 ^{5} (circles), 5 × 10^4 (squares), or 10^4 (triangles) IIIB-infected H9 cells. PFU and viral antigen production were linearly proportional over the entire dose range, whether the cells were untreated (open symbols) or treated with EGF (closed symbols).

viral antigen production per plaque was unchanged by EGF activation. We conclude that EGF improves the plaquing efficiency, but it is still unclear whether this works primarily by stimulating the host cell to produce more CD4 receptors (see Materials and Methods) or by stimulating viral transcription.

Titration of Neutralizing Antibodies.   Since the number of plaques is linearly proportional to the input of cell-free virus, each plaque must result from infection by a single virus. If double infections occurred, then the number of double infections would increase with the second power of the input number of viruses. Inactivation of even a few viruses would cause a corresponding reduction in PFUs, making this assay highly sensitive to the effects of neutralizing antibodies.

For example, as shown in Fig. 5, serum from an asymptomatic seropositive pa-
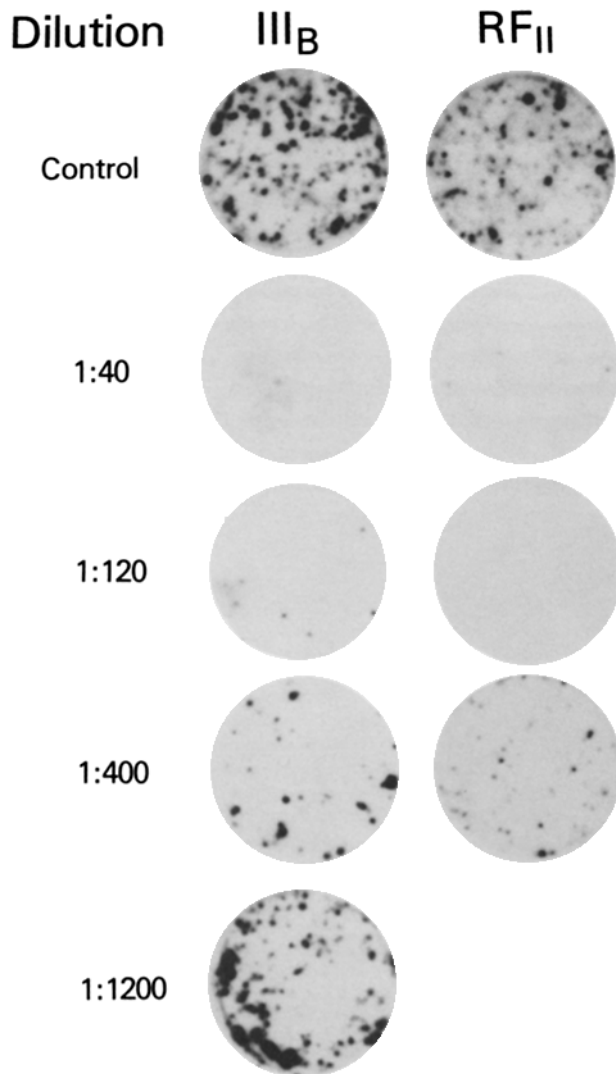

FIGURE 5. Antibody sensitivity of IIIB and RFII isolates. H9 cells infected with either isolate were incubated with various dilutions of a patient's serum or with an inactive control. The surviving plaques were measured in the standard assay. Counting the plaques directly from the exposed negatives gave the following numbers of PFU for IIIB, reading vertically: 140, 1, 9, 37, and 108 (some of which were lost in printing); and for RFII: 165, 7, 5, and 79. The 1:1,200 dilution was not tested on RFII.

tient was titered against two divergent HIV-1 isolates, IIIB and RFII. For the IIIB isolate, incubation with the 1:40 and 1:120 dilutions of serum gave nearly complete inhibition of viral plaques. The 1:400 dilution gave less neutralization, and there was only a slight antibody effect at 1:1,200. Essentially identical results were obtained when the serum was titered against RFII, including nearly total neutralization at the first two dilutions, with partial viral breakthrough at 1:400. The antibody titer against both isolates was estimated at 1:800, based on the dilution at which half of the PFU survived. Normal serum had no effect on either isolate at the 1:40 dilution. Thus, these two strains of HIV-1 are immunologically equivalent with regard to the neutralizing antibodies present in this serum, in spite of amino acid differences of 21% in the envelope proteins. The fact that the neutralizing titer is the same on both viruses also shows that the group-specific antibodies predominate over any type-specific neutralizing antibodies that may be present.

Numerical results for HIV neutralization with the same serum are shown in Fig. 6, but this time comparing the titration of the serum against IIIB and Z1. The ratio of surviving PFU divided by input PFU (V/V_o) is plotted against the antibody dilution used to treat the virus. Both isolates were neutralized 99.5% by the 1:50 dilution of antibodies, between 96 and 98% at 1:120, and between 86 and 89% at 1:400. Once again, we obtained a neutralizing titer of 1:800 for both viruses. Thus, in spite of highly divergent envelope sequences, both IIIB and Z1 are immunologically equivalent with regard to these neutralizing antibodies.

Because antisera neutralized IIIB and RFII equally, we tested whether the antibodies that neutralized both viruses were the same or different. To do this, another patient's serum with high titer group-specific neutralizing antibodies was adsorbed with recombinant viral envelope glycoproteins gp160 or gp41 or with core protein p24 of the IIIB type. After adsorption with these proteins, the residual neutralizing activity was measured on homologous IIIB virus or on variant RFII virus. As shown in Fig. 7, adsorption with IIIB envelope glycoprotein removed 90% of the neutralizing activity against the homologous IIIB virus and 80-90% of the neutralizing activity against the RFII variant. In contrast, adsorption with p24 or gp41 gave little or no reduction in neutralizing activity against either virus. Thus, nearly all of the neu-
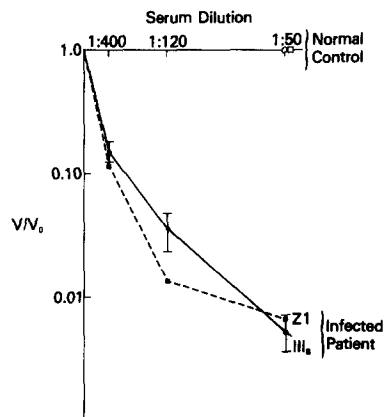

FIGURE 6. Antibody sensitivity of the IIIB and Z1 isolates. H9 cells infected with either isolate were incubated for 2 h with the same patient's serum as in Fig. 5 or with a normal control serum. The surviving fraction of virus is V/V_o, where V are the number of PFU surviving in the presence of antibody, and V_o are the number of PFU in the absence of antibody. V_o (corrected for dilution) for the untreated controls were: 602 for IIIB and 936 for Z1. V/V_o was determined in sextuplicate for IIIB, plotted as the arithmetic mean ± SD, and once for Z1. Normal serum had no effect on either virus.
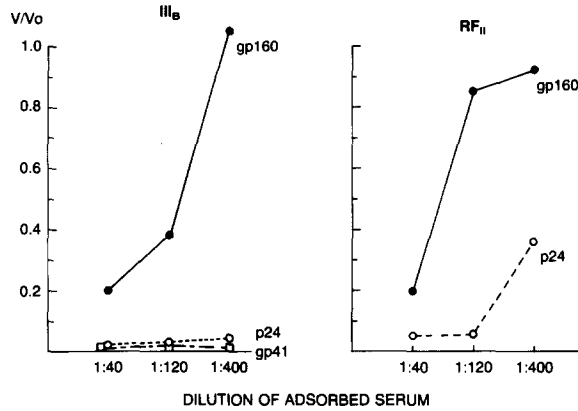
FIGURE 7. Adsorption of HIV-1 neutralizing antibodies by recombinant viral proteins. HIV-1 IIIB envelope glycoproteins gp160 and gp41, and p24 core antigen, were expressed in *Spodoptera frugiperda* cells infected with recombinant baculovirus (28). Patient serum was adsorbed with each cell-associated protein, and the residual neutralizing activity was tested against IIIB or RFII virus in the standard assay. The extent of antibody adsorption by gp160 was >32-fold and by p24 was eightfold as measured by an ELISA using recombinant viral proteins. V_0 was 110 for IIIB and 144 for RFII.

tralizing antibodies in this serum are directed against the envelope glycoprotein and are group specific, since nearly all of the antibodies that neutralize the RFII variant are adsorbable by IIIB envelope glycoprotein, and the major neutralizing epitope is shared by the envelopes of both viruses.

Clinical Results. Using this method, we have measured the neutralizing activity of 14 sera from asymptomatic seropositive patients on the IIIB isolate (Fig. 8) and 11 sera from patients with clinical AIDS. As controls, we also tested 11 seronegative
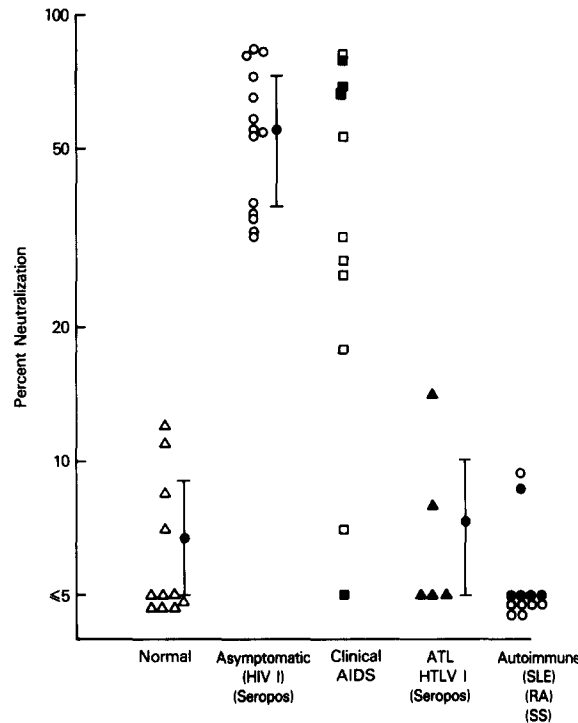

FIGURE 8. Clinical summary. Each patient's serum was assayed for neutralizing activity at 1:20 and 1:200 dilutions. Symbols are as follows: asymptomatic seropositives (*open circles*), AIDS patients with Kaposi's sarcoma (*closed squares*) or with opportunistic infections (*open squares*) autoimmune patients with systemic lupus (*closed circles*); or with rheumatoid arthritis or Sjogren's syndrome (*open circles*). The percent neutralization of IIIB by the 1:200 dilution of each serum is plotted logarithmically on the y-axis and is calculated by the formula: $100 \times (1-V/V_0)$, where V are the number of PFU surviving and V_0 are the number of PFU with no antibodies added. Arithmetic means ± SD are indicated.

normal sera, five sera from HTLV I–infected patients, and 12 sera from autoimmune patients. Each serum was tested at the 1:20 and 1:200 dilutions, and the percent neutralization at 1:200 is shown in Fig. 8. Sera from 11 normal controls gave <12% neutralization at either 1:20 or 1:200. In contrast, sera from half of the asymptomatic seropositive patients neutralized >50% at a dilution of 1:200. The median neutralizing titer of these patients' sera was 1:200, and some patients had titers as high as 1:2,000 and 1:6,000 in subsequent assays. These titers exceed by >1 log the highest titers detected by earlier assay methods (29–31), and the difference between the seropositive patients and normal controls was highly significant ($p < 0.0005$). Of eleven patients with clinical AIDS, three of four with Kaposi's sarcoma had levels of neutralizing antibodies similar to those found in asymptomatic patients, while seven with opportunistic infections had generally lower levels of neutralizing antibodies.

Sera from 12 autoimmune patients with systemic lupus, rheumatoid arthritis, or Sjögren's syndrome failed to neutralize HIV-1, indicating that this is not simply an autoimmune phenomenon mediated by antilymphocyte antibodies. The sera of five adult T cell leukemia patients with antibodies to HTLV-I failed to neutralize HIV-1 at either dilution, indicating that neutralization could distinguish between these two members of the human T cell retrovirus family. However, when sera from seven patients in the asymptomatic seropositive group were retested on a second HIV-1 isolate (RFII), they all neutralized IIIB and RFII equally (one of these sera is shown in Fig. 5), indicating that they all recognize a predominant group-specific neutralizing epitope for HIV-1.

Extent and Kinetics of Neutralization. The neutralization assay provides useful information both at antibody dilutions causing 50% inactivation, which define the titer, and at antibody excess, where the percent of virus surviving provides an upper limit for the frequency of spontaneously occurring antibody-resistant variants in each isolate. As shown in Figs. 5 and 6, both IIIB and Z1 contained <1 resistant plaque in 200, while RFII contained <1 in 100. In addition, the kinetics of inactivation of IIIB were linear over the first two logs of inactivation (Fig. 9), indicating that the last 5% of plaques to be inactivated were no less sensitive than the first 5%, since they were inactivated at the same rate. Thus, in spite of extensive molecular variation (6, 32), antibody-resistant variants of HIV-1 are actually quite rare, and the IIIB isolate is uniformly sensitive to neutralizing antibody.
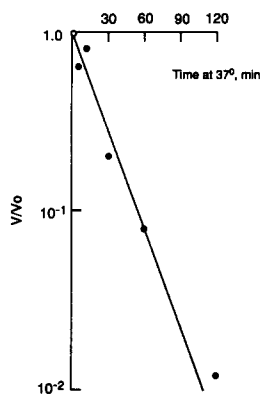

Figure 9. Inactivation kinetics of cell-associated virus. IIIB-infected H9 cells were incubated with patient antibodies at antibody excess (1:20 dilution) for various times, washed, and added to the CD4⁺ HeLa monolayer cells for plaquing. The fraction of viruses remaining infectious (V/V_0) was plotted on a log scale vs. time, showing linear inactivation kinetics for up to 99% of the starting inoculum. $V_0 = 2,500 \pm 10$ PFU (duplicates).
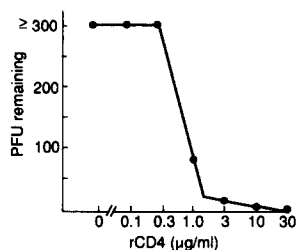
FIGURE 10. Soluble recombinant CD4 neutralizes HIV-1. Increasing doses of rCD4 (a gift of Dr. Daniel Capon, Genentech, Inc., South San Francisco, CA) were incubated with cell-associated virus for 2 h at 37°C followed by infecting the monolayer cells in the presence of the inhibitor for an additional 5 h. Recombinant CD4 inhibited PFU at concentrations of 1 μg/ml or higher.

Effect of soluble CD4.   Recent reports have demonstrated that soluble recombinant CD4 can neutralize HIV-1 in vitro (12–14), as shown by the inhibition of viral gene expression. With the plaquing assay we could determine whether the antiviral effect of CD4 prevented the initial infection or occurred during subsequent steps of the virus life cycle. As shown in Fig. 10, incubation of virus with soluble recombinant CD4 for 2 h before infection of the monolayer cells gave a marked reduction in PFU at CD4 concentrations of 1 μg/ml or greater. Thus, CD4 inhibits the number of infection events, perhaps by mimicry of the cellular receptor for HIV-1, or by competition with the receptor. These results also demonstrate that the plaquing assay provides a sensitive measure of viral inactivation, independent of the mechanism of inactivation.

Discussion

Using the plaque reduction assay, we have found significant new evidence that the human immune response to HIV-1 infection produces neutralizing antibodies against a broad spectrum of HIV-1 isolates, rather than a narrow band of HIV-1 serotypes closely related to the infecting strain. These are the predominant neutralizing antibodies elicited by infection and may play a major role in the host-virus interaction. Their frequent occurrence in nearly all infected patients implies that the group-specific neutralizing epitope is shared by multiple HIV-1 strains, in spite of the rapid mutation rate of HIV-1. If similar group-specific neutralizing antibodies could be elicited by vaccination, humoral immunity could play a role in preventing the spread of HIV-1.

Group specificity is not an artifact of our assay method, since neutralizing antibodies from a gp160-immune rabbit were found to be type specific for the homologous HIV-1 isolate (data not shown). Neither is it simply the aggregate result of multiple type-specific antibodies induced by infection with an unknown HIV-1 strain, since absorption studies with a recombinant HIV-1 envelope glycoprotein gp160 from one isolate indicate that the same antibodies neutralize both the IIIB and RFII isolates. Finally, virus neutralization in our assay is specific for HIV-1, as shown by the failure of sera from HTLV I–infected patients to inactivate HIV-1, despite their relatedness by Western blot (33).

The mechanism of viral neutralization by these antibodies is currently unclear, although they are most likely directed against conserved regions of gp160. Absorption experiments with recombinant viral proteins are in progress to map the location of these epitopes. Essential functions of gp160 include binding to the CD4 receptor (18, 34), penetration of cell membranes via the fusion peptide (35), or viral uncoating.

Thus, neutralizing antibodies that bind to critical regions of the viral envelope and interfere with these functions could block infection. For example, if they act on the CD4-binding site, they could mimic soluble CD4 and inactivate HIV-1 by competitive binding to the CD4-binding sites, which presumes near saturation levels of binding, or by triggering a conformational change in the virus that may require binding to only a few CD4 receptor sites per virion. The latter mechanism is found in poliovirus, where binding of a few neutralizing antibodies per virion inactivates the virus by triggering a conformational change detectable as a shift in the isoelectric point of the virus (36).

Why should a major neutralizing epitope of HIV be conserved in a virus that is known to mutate so rapidly? This is not so surprising when we consider other structural features and functions of gp120 that are conserved among all isolates. The gp120 glycoprotein is known to have five conserved regions interspersed among variable regions (37). Also conserved are the 20 cystines for disulfide bonds, important glycosylation sites (38), and the gp120-gp41 proteolytic processing site. In each variant, the gp120 function of CD4 binding is preserved, and they remain sensitive to inactivation by soluble CD4 (15). Also, gp120 retains its other physiologic functions in virus assembly, membrane fusion, and uncoating inside the cell. These essential functions place constraints on the virus that may limit the range of viable mutations in gp120 to some sites and not others. Since neutralizing antibodies could bind a site with an essential function, antibody escape mutations at this site may be lethal for the virus.

Another explanation for the failure of genetically divergent isolates to develop antibody resistance could be the tendency of HIV-1 to mutate in the absence of antibody-mediated selection. Mutation is a random process, limited only by the nonviability of mutations affecting essential viral functions. But these mutants only serve as the substrate for immunologic selection of antibody-resistant variants (39), which occurs when the virus tries to grow in the presence of neutralizing antibodies. Then, a slight growth disadvantage due to the mutation could be overcome by the selective advantage of antibody escape, leading to natural selection of antibody-resistant variants from all the available mutants. However, since HIV-1 rarely if ever reinfects previously immune individuals (40), and since it becomes an integrated provirus soon after infection (41), before the immune response has peaked (42), it is not surprising that numerous molecular variants of HIV-1 can be generated randomly without evolving toward antibody resistance. This hypothesis is supported by the evidence that IIIB and Z1 have progressed through a minimum of three evolutionary branch points since they diverged from a presumed common ancestor (Fig. 3) without developing antibody resistance. These branch points may have greater biologic significance than the overall number of amino acid substitutions, since antibody escape is usually due to point mutations (43), independent of the overall number of amino acid substitutions. If this hypothesis is correct, then the group-specific neutralizing epitope would be an ideal target for vaccine-induced antibodies, which would confront susceptible viruses of many types as soon as possible after infection, before they could hide as provirus or escape through mutation.

Further evidence of the inability of HIV-1 to escape from group specific neutralizing antibodies is the rarity of antibody-resistant variants among the three HIV-1 isolates tested. With the plaquing assay, we have analyzed hundreds of PFU at a time, in

order to detect rare variants. However, the nearly complete inactivation of PFU in the presence of excess neutralizing antibodies (demonstrated in Fig. 5 and 6) indicated that <0.5% of viruses in each isolate were potentially antibody resistant. Since this includes some viruses that survived due to the kinetics of neutralization (Fig. 9), the actual frequency of resistant variants may be much lower. This low frequency suggests that each isolate has not accumulated escape mutants at the group-specific neutralizing epitope, even after multiple rounds of viral replication in the host and unselected infections in culture. Perhaps mutations at this site produced defective virus, so that few or no variants were available for antibody selection. Alternatively, these mutants may be present in very low frequency, requiring rounds of infection under selective conditions in the presence of antibodies for enrichment to detectable levels.

Our results with patient sera suggest that group-specific neutralizing antibodies are produced by nearly all infected patients, suggesting that the epitope is widely shared among the HIV-1 isolates that infected our patients and is broadly immunogenic during infection. The general correlation between a patient's titer of neutralizing antibodies and his stage of disease, wheather asymptomatic seropositive, Kaposi's sarcoma, or AIDS with opportunistic infection, is consistent with some antiviral effect due to neutralizing antibodies, although it could also be the result of his clinical condition. The predictive value of high titered neutralizing antibodies is currently unknown, since most infected patients will eventually progress to clinical immunodeficiency in spite of having neutralizing antibodies. However, the prolonged duration of the asymptomatic infected state (44) suggests that the host's immune response may alter the course of infection. In addition, the rarity of reinfection (40) suggests that something about the host's immune response, quite possibly group-specific neutralizing antibodies, makes him resistant to second infections, even by divergent isolates. In that case, antibodies to the group-specific neutralizing epitope, if elicited by a vaccine antigen before infection, might help prevent the first infection. The broad spectrum anti-HIV-1 effect of antibodies to a conserved epitope implies interference with an essential and immutable viral function, which, if attacked early in the infection, might abort the infection. Thus, the ability to induce group-specific antibodies may serve as a biologically relevant marker in the search for a suitable vaccine antigen.

Summary

HIV-1 is known to show a high degree of genetic diversity, which may have major implications for disease pathogenesis and prevention. If every divergent isolate represented a distinct serotype, then effective vaccination might be impossible. However, using a sensitive new plaque-forming assay for HIV-1, we have found that most infected patients make neutralizing antibodies, predominantly to a group-specific epitope shared among three highly divergent isolates. This epitope persists among divergent isolates and rarely mutates, despite the rapid overall mutation rate of HIV-1, suggesting that it may participate in an essential viral function. These findings, plus the rarity of reinfections among these patients, suggest that HIV-1 may be more susceptible to a vaccine strategy based on a group-specific neutralizing epitope than was previously suspected.

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