JOURNAL OF VIROLOGY, Jan. 1995, p. 101–109
0022-538X/95/$04.00+0

Vol. 69, No. 1

Primary Isolates of Human Immunodeficiency Virus Type 1 Are Relatively Resistant to Neutralization by Monoclonal Antibodies to gp120, and Their Neutralization Is Not Predicted by Studies with Monomeric gp120

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Received 19 July 1994/Accepted 23 September 1994

A panel of anti-gp120 human monoclonal antibodies (HuMAbs), CD4-IgG, and sera from people infected with human immunodeficiency virus type 1 (HIV-1) was tested for neutralization of nine primary HIV-1 isolates, one molecularly cloned primary strain (JR-CSF), and two strains (IIIB and MN) adapted for growth in transformed T-cell lines. All the viruses were grown in mitogen-stimulated peripheral blood mononuclear cells and were tested for their ability to infect these cells in the presence and absence of the reagents mentioned above. In general, the primary isolates were relatively resistant to neutralization by the MAbs tested, compared with the T-cell line-adapted strains. However, one HuMAb, IgG1b12, was able to neutralize most of the primary isolates at concentrations of ≤1 μg/ml. Usually, the inability of a HuMAb to neutralize a primary isolate was not due merely to the absence of the antibody epitope from the virus; the majority of the HuMAbs bound with high affinity to monomeric gp120 molecules derived from various strains but neutralized the viruses inefficiently. We infer therefore that the mechanism of resistance of primary isolates to most neutralizing antibodies is complex, and we suggest that it involves an inaccessibility of antibody binding sites in the context of the native glycoprotein complex on the virion. Such a mechanism would parallel that which was previously postulated for soluble CD4 resistance. We conclude that studies of HIV-1 neutralization that rely on strains adapted to growth in transformed T-cell lines yield the misleading impression that HIV-1 is readily neutralized. The more relevant primary HIV-1 isolates are relatively resistant to neutralization, although these isolates can be potently neutralized by a subset of human polyclonal or monoclonal antibodies.

It has been known for almost a decade that human immunodeficiency virus type 1 (HIV-1) can be neutralized by antibodies directed at its envelope glycoproteins (29, 61, 74). The observations of virus neutralization in vitro by sera from naturally infected humans and by human or animal antisera to recombinant forms of the gp120 glycoprotein, or fragments thereof (5, 11, 22, 26, 30, 31, 36, 38, 59, 62, 64, 70), underlie much of the reasoning that humans might be successfully immunized against HIV-1 by subunit vaccines (6, 18, 27, 39), although the importance of cellular immunity in controlling HIV-1 infection should not be underestimated (33). However, until recently, neutralization assays have generally relied on the use of HIV-1 strains adapted to growth in transformed T-cell lines, as these viruses can be grown conveniently to high titers. It is now becoming clear that T-cell line-adapted strains are abnormally sensitive to neutralization by sera from humans naturally infected with HIV-1 or vaccinated with recombinant gp120 (13, 36, 37). This finding is consistent with a body of data obtained over the past few years demonstrating that primary HIV-1 isolates subjected to minimal passage only in peripheral blood mononuclear cells (PBMC) are relatively resistant to neutralization by soluble CD4 (sCD4)-based reagents directed at the CD4-binding site of gp120 (1, 8, 16, 41, 46, 51, 55, 57, 58, 67, 72). In contrast, T-cell line-adapted strains are very sensitive to sCD4 neutralization (16, 51). The difference in sCD4 neutralization sensitivity between primary isolates and variants selected to grow in transformed cell lines seems to reside in a subtle alteration in the structure of the oligomeric envelope glycoprotein complex on virions of the adapted variants (41, 51, 57). This alteration is manifested by an enhanced ability of the adapted strains to bind sCD4 (46), probably due to an increased on-rate (41, 57, 75, 76).

Because HIV vaccines must induce immune responses to counter primary strains of HIV-1 under in vivo conditions, it is important that we understand more about the properties of these strains (77). Since we believe that isolates cultured only in PBMC are more likely than T-cell line-adapted variants to reflect the properties of in vivo strains, we have started to analyze the mechanisms of neutralization of PBMC-grown primary isolates. Here we report that primary strains are relatively resistant to neutralization by a spectrum of monoclonal antibodies (MAbs) directed at different sites on gp120. The mechanism of resistance does not usually correlate with the absence of the antibody epitope from the monomeric form of gp120 of the test viruses. These findings with MAbs closely parallel those previously obtained with sCD4 (1, 8, 16, 41, 46, 51, 58, 72), and so we now suggest that the mechanism of resistance to antibodies shown by primary strains is broadly

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similar to the sCD4 resistance mechanism: it involves a decreased accessibility of the binding site on the native oligomeric glycoprotein complex. However, primary isolates are not intrinsically resistant in that they can be neutralized potently by at least one human MAb (HuMAb) to gp120 and by human serum antibodies to HIV-1.

MATERIALS AND METHODS

Viruses. The designations, locations, and disease statuses of the individuals whose virus strains were used in this study were as follows: AD-6 (New York, acute), N70-2 (New Orleans, asymptomatic), AC (San Diego, AIDS), LS (Los Angeles, AIDS), NYC-1 (New York, unknown), WM (Los Angeles, AIDS), AD-11 (New York, acute), and AD-13 (New York, acute). Some properties of the isolated strains, as well as partial envelope sequences, have been described elsewhere (33, 43, 52, 77). All the strains were of the non-syncytium-inducing phenotype except for LS, which was syncytium inducing, and WM, for which the phenotype has not been determined. The strains had been cultured only once or twice in mitogen-stimulated PBMC, and stocks used in the present experiments were grown in PBMC, essentially as outlined previously (16). The molecularly cloned HIV-1 JR-CSF and HIV-1 isolate JR-FL have been described elsewhere (55–57). Stocks of JR-CSF were prepared by infection of PBMC with supernatants initially obtained by DNA transfection (57). HIV-1 IIIB and HIV-1 MN are strains with extensive histories of passage in transformed T-cell lines (61). Stocks of these strains grown in H9 cells were passaged in mitogen-stimulated PBMC to prepare viruses that had been grown in the same cells as the primary viruses and to eliminate the influence of any host cell-dependent epigenetic factors on virus neutralization (66). The stock of PBMC-grown MN was a gift from Anthony Conley (Merck Research Labs, West Point, Pa.).

Antibodies. HuMAb 19b has been described elsewhere (52, 63, 69), as has HuMAb IgG1b12, which was derived from a Fab fragment isolated from a phage display library (3, 60). HuMAb 447-D (r1) is a recombinant form of HuMAb 447/52-D and was donated by Anthony Conley and Emilio Emini (Merck Research Labs). Properties of 447-D (r1) and of its parent MAb have been described previously (14, 23, 25, 42, 73). HuMAbs HT5, HT6, and HT7 were donated by Ciba-Geigy AG (Basel, Switzerland). CD4-IgG was a gift from Genentech Inc. (South San Francisco, Calif.) (10). HuMAbs HT5, HT6, HT7, and IgG1b12 recognize discontinuous epitopes that overlap the CD4-binding site on gp120 (CD4BS) (50, 60), which are similar to those described previously for other HuMAbs (28, 71). These MAbs block by 50% the binding of soluble CD4 to monomeric gp120 (IIIB) at concentrations comparable to those concentrations producing half-maximal binding to IIIB gp120 (Table 1 and data not shown). HuMAbs 19b and 447-D (r1) recognize determinants in the V3 region of gp120 (23, 25, 42, 69). Sera AD-18, AD-28, and AD-46 were obtained from individuals asymptomatically infected with HIV-1 for at least 6 years (9).

Virus neutralization. Virus neutralization was assessed by using phytohemagglutinin-stimulated PBMC as indicator cells, with determination of extracellular p24 antigen production as the end point, essentially as described previously (16). Briefly, PBMC were stimulated with phytohemagglutinin for 24 h before removal of the mitogen by washing. The lymphoblasts (10^6 in 1.5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum) were then exposed for 24 h to an inoculum of 50 50% tissue culture infective doses of HIV-1 that had been previously incubated for 30 min at 37°C with or without a test antibody. Several antibodies were tested simultaneously against each strain by using a common batch of PBMC. The antibodies were titrated in triplicate cultures in fivefold serial dilutions, usually starting at 25 μg/ml or a 1:8 dilution of HIV-1^+ serum. Excess virus and antibody were then removed by extensive washing, and the cultures were maintained for 5 to 7 days, depending on the growth kinetics of the strain. Virus production (p24 antigen) was measured during the logarithmic growth phase of the cultures by using a commercial kit (Abbott Labs, Abbott Park, Ill.). There was no interference of serum anti-p24 antibodies with the p24 assay after the washing procedure employed. The concentrations of each antibody or serum able to reduce p24 production by 50% and 90% (50% inhibitory dose [ID_{50}] and ID_{90} values) were calculated. ID_{50} and ID_{90} values usually varied up to threefold between experiments. However, experiments with 19b and isolate AD-6 produced more variable neutralization titers, and so a range of ID_{50} values derived from four experiments is presented for this antibody.

Binding of MAbs and human serum antibodies to monomeric gp120. PBMC-grown virus culture supernatants were treated with 1% Nonidet P-40 nonionic detergent to inactivate infectious virus and release gp120 from virions in soluble form. Culture supernatants (10 to 100 μl) were added to enzyme-linked immunosorbent assay (ELISA) wells (Immulon 2; Dynatech Inc.) coated with sheep antibody D7324 to the carboxy terminus of gp120, essentially as described previously (40, 46–49, 53). The volumes of the supernatants added were determined to produce approximately equivalent levels of bound gp120 for the different isolates, judged by binding of a saturating concentration (1 μg/ml) of CD4-IgG. When possible, the saturating optical density at 492 nm (OD_{492}) was in the range of 0.80 to 1.20. However, the gp120 concentrations in PBMC cultures of isolates AD-11 and AD-13 were low, and the OD_{492} values were correspondingly reduced (0.30 for AD-11 and 0.65 for AD-13); this reduced the precision of our data for these two isolates. After removal of unbound gp120, MAbs were titrated in TMTSS buffer and the amounts of bound MAb were determined with alkaline phosphatase-conjugated goat anti-human IgG (Accurate Chemicals Inc.) and the AMPAK ELISA Amplification system (Dako Diagnostics) (40, 46–49, 53). The MAb concentrations producing half-maximal binding were determined, to provide first-order approximations of K_d values for the binding of the MAbs to monomeric gp120 (40, 58). As saturation was rarely achieved with human sera (Fig. 1h), because of increasing assay backgrounds at dilutions of <1:1,000, the values presented in Table 1 reflect the serum dilution producing 50% of the background-corrected OD_{492} value observed at a 1:1,000 serum dilution.

We noted that the OD_{492} values found when saturating concentrations of 19b were reacted with gp120s from isolates WM, LS, N70-2, JR-FL, and JR-CSF were two- to threefold lower than expected from the extent of binding of other antibodies or CD4-IgG to these gp120s. This reduced binding was overcome by increasing by three- to fivefold the volume of culture supernatant used as the gp120 source in the assay. We believe that the binding of 19b was reduced because proteolytic cleavage of the V3 loop on a fraction of the total gp120 molecules occurred during the 5 to 7 days required to grow virus in PBMC cultures. Thrombin-mediated proteolysis of the V3 loop of purified MN gp120 destroys the 19b epitope, but it does not affect the binding of 447-D (r1) or other antibodies directed to the crown of the V3 loop (52).

TABLE 1. Binding of MAbs and HIV-1^+ sera to soluble gp120s from primary and T-cell line-adapted virus strains[a]

Reagent	Half-maximal binding concn (μg/ml or serum dilution) with gp120 from strain[b]:											
	AD-6	JR-CSF	N70-2	AC	LS	NYC-1	WM	AD-11	AD-13	JR-FL	IIIB	MN
MAbs												
CD4-IgG	0.220	0.060	0.065	0.060	0.085	0.080	0.090	0.170	0.180	0.060	0.100	0.110
HT6[c]	0.800	0.060	0.100	0.300	0.140	0.130	0.450	0.500	0.400	0.065	0.150	0.150
HT5[c]	0.420	0.040	0.030	0.085	0.065	0.060	0.090	0.110	>1.000	0.060	0.055	0.070
HT7[c]	0.190	0.025	0.025	0.025	0.050	0.025	0.100	0.110	0.150	0.025	0.025	0.150
IgG1b12[c]	0.270	0.210	1.000	0.320	0.100	0.060	0.045	0.045	—[d]	0.050	0.045	0.100
19b[e]	0.015	0.050	0.040	0.060	0.015	0.015	0.130	0.070	0.040	0.045	—	0.045
447-D (r1)[e]	—	0.025	—	0.200	—	0.040	—	>0.200	0.015	0.015	—	0.050
HIV-1^+ sera												
AD-28	1:8,800	1:8,000	1:5,300	1:5,000	1:8,000	1:6,400	1:7,400	1:4,300	1:8,300	1:6,400	1:4,200	1:7,700
AD-18	1:6,500	1:5,300	1:7,100	1:4,200	1:5,300	1:6,500	1:5,900	1:5,900	1:5,600	1:6,900	1:4,800	1:7,700
AD-46	1:7,100	1:5,300	1:4,200	1:5,300	1:5,900	1:6,200	1:8,300	1:3,100	1:6,700	1:8,300	1:4,200	1:4,300

[a] All strains were grown in PBMC.
[b] Data are MAb concentrations or serum dilutions giving half-maximal binding to the gp120s indicated.
[c] Binding site, CD4BS.
[d] —, no significant binding.
[e] Binding site, V3.

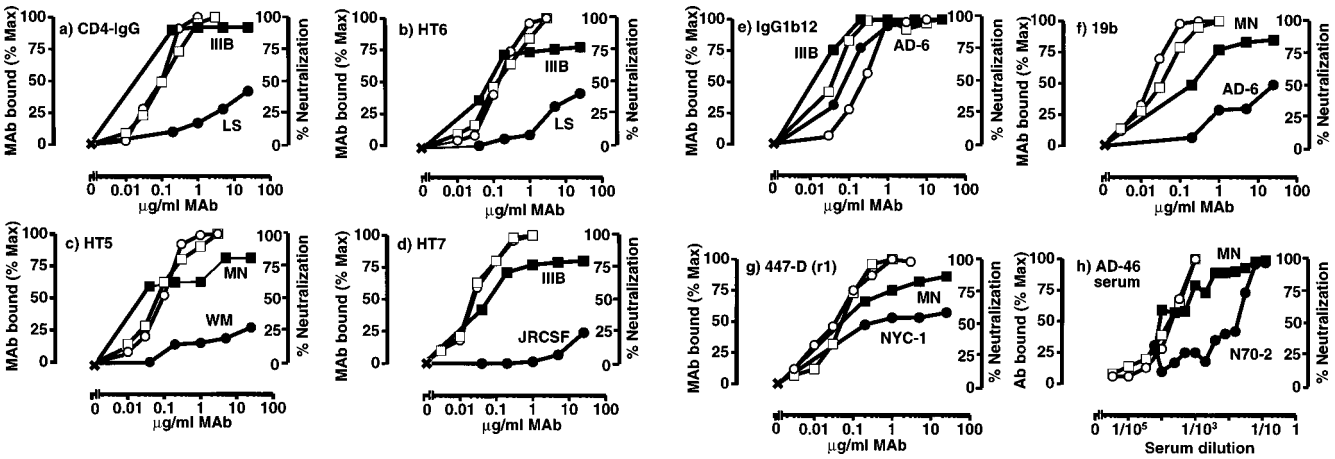

FIG. 1. Binding of MAbs to soluble gp120: relationship to HIV-1 neutralization. The extent of MAb binding to detergent-solubilized gp120 from PBMC-grown HIV-1 (○, □) is compared with virus neutralization data for the same MAb (●, ■). In each panel, the T-cell line-adapted strain IIIB or MN is represented by the squares, and the primary virus is represented by the circles. (a) CD4-IgG with IIIB and LS; (b) HuMAb HT6 with IIIB and LS; (c) HuMAb HT5 with MN and WM; (d) HuMAb HT7 with IIIB and JR-CSF; (e) HuMAb IgG1b12 with IIIB and AD-6; (f) HuMAb 19b with MN and AD-6; (g) HuMAb 447-D (r1) with MN and NYC-1; (h) AD-46 HIV-1^+ serum with MN and N70-2.

RESULTS

Neutralization of primary HIV-1 isolates by HuMAbs and HIV-1^+ sera. A panel comprising nine primary HIV-1 isolates cultured only in PBMC, one molecularly cloned primary strain (JR-CSF) grown in PBMC, and two prototypic, T-cell line-adapted strains (IIIB and MN) grown in PBMC was assembled. This test panel was used to evaluate the neutralization potencies of a set of six HuMAbs to various gp120 epitopes, the immunoglobulin-CD4 chimeric molecule CD4-IgG, and three samples of serum from individuals infected with HIV-1 for prolonged periods (at least 6 years) without clinical symptoms. Each reagent or serum sample was titrated in the presence of a fixed input inoculum of each virus strain (50 50% tissue culture infective doses). The highest concentration of each HuMAb tested and of CD4-IgG was 25 μg/ml, as this arguably represents the antibody concentration range that could be reasonably achieved in vivo by passive administration (67). Sera were tested at dilutions of 1:8 and greater, to avoid nonspecific effects that can occur at higher serum concentrations. Neutralization data (ID_{50} and ID_{90} values) for each MAb against each virus strain are presented in Fig. 2, and representative titration curves for a primary isolate and a T-cell line-adapted strain for most of the test reagents are depicted in Fig. 1.

In most cases, reduction of the infectivity of primary strains by HuMAbs was sporadic, and many different patterns of neutralization were observable from the titration curves (Fig. 1). These are discussed below. Of the three conventional HuMAbs against the CD4-binding site, only HT7 showed any significant activity, causing a 50% reduction in p24 production from two primary isolates (AC and WM) at 1 μg/ml (Fig. 2a) but no 90% infectivity reductions at ≤25 μg/ml (Fig. 2b). The V3 HuMAb 447-D (r1) was quite active against the molecularly cloned primary strain JR-CSF and the isolate JR-FL (ID_{50} and ID_{90} values of ≤0.2 μg/ml), and it also reduced the infectivities of isolates NYC-1 and AD-13 by 50% at concentrations of 1 to 5 μg/ml. However, except for JR-CSF and JR-FL, ID_{90} values of ≤25 μg/ml were not found for any primary isolate treated with 447-D (r1) (Fig. 2b). The other V3 HuMAb tested, 19b, reduced the infectivity of isolate AD-6 by 50% at concentrations that ranged from 5 to 25 μg/ml in four separate experiments, and it also caused a 50% infectivity reduction for JR-FL at 25 μg/ml (Fig. 2a). ID_{90} values of ≤25 μg/ml were not observed for 19b with any of the primary strains in this test panel (Fig. 2b).

Two reagents were active to some extent against most of the strains in the panel. CD4-IgG reduced the infectivity of 6 of 10 primary isolates by 50% at concentrations of 0.2 to 5 μg/ml, although only two primary strains (AC and JR-CSF) were 90% neutralized by CD4-IgG at ≤25 μg/ml. The most potent reagent tested was IgG1b12, a recombinant HuMAb to the CD4-binding site, which reduced by 50% the infectivities of all 10 primary strains at concentrations of ≤1 μg/ml and reduced the infectivities of 7 of these strains by 90% at concentrations of ≤5 μg/ml (Fig. 2). Sera from three HIV-1^+ individuals were active against primary strains, causing 50% infectivity reductions at dilutions of 1:8 to >1:64 for most of the strains in the panel (Fig. 2a). The performance of serum from individual AD-46 was particularly striking, for it reduced the infectivity of all 10 primary strains by 50% at dilutions of >1:64 and by 90% at dilutions of ≥1:32 (Fig. 2). A more extensive titration of AD-46 serum against one primary strain (N70-2) and MN showed that 50% neutralization of both strains occurred at serum dilutions of >1:1,000 (Fig. 1h). The degree of breadth and potency of primary virus neutralization shown by AD-46 serum is unusual for a human HIV-1^+ serum, in our experience; it may be significant that individual AD-46 is a long-term nonprogressor who has been infected with HIV-1 for 12 years without clinical symptoms (9). More detailed studies of serum AD-46 and other sera from rapid progressors and long-term nonprogressors will be described elsewhere (9).

The data presented above demonstrate that primary HIV-1 isolates grown only in PBMC can be potently neutralized by certain polyclonal and monoclonal antibodies but that most of the HuMAbs tested lack significant action against primary viruses. However, it is also clear from Fig. 1 and 2 that primary strains are usually relatively resistant to neutralization by MAbs and CD4-IgG compared with strains adapted to growth in transformed T-cell lines. This was demonstrated by using the prototypic isolates IIIB and MN, which have an extensive history of passage in cell lines. To avoid any influence of the cell type used for virus production on virus neutralization (66), stocks of IIIB and MN grown in PBMC were used for experiments involving PBMC as target cells. These strains were, in

a

ID_{50} values (μg/ml or serum dilution)

	Virus / Reagent	AD-6	JR-CSF	N70-2	AC	LS	NYC-1	WM	AD-11	AD-13	JR-FL	IIIB	MN
	CD4-IgG	5	<0.2	<0.2	<0.2	>25	25	1	>25	>25	5	<0.04	0.2
CD4BS	HT6	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	0.1	1
CD4BS	HT5	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	0.2	<0.04
CD4BS	HT7	>25	>25	>25	1	>25	>25	1	>25	>25	>25	0.2	1
CD4BS	IgG1b12	0.1	<0.04	<0.04	<0.04	1	1	<0.04	0.04	<0.04	<0.04	<0.04	<0.04
V3	19b	5-25	>25	>25	>25	>25	>25	>25	>25	>25	25	>25	1
V3	447-D (r1)	>25	0.2	>25	>25	>25	1	>25	>25	1	0.2	>25	<0.2
HIV-1+ sera	AD-28	1:64	>1:64	>1:64	>1:64	1 15	<1:8	1 32	<1:8	<1:8	<1:8	>1:64	>1:64
HIV-1+ sera	AD-18	1:32	1:16	1:16	>1:64	>1:64	1:16	>1:64	1:16	1:16	1:16	>1:64	1:32
HIV-1+ sera	AD-46	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	>1:64	1:10,000

b

ID_{90} values (μg/ml or serum dilution)

	Virus / Reagent	AD-6	JR-CSF	N70-2	AC	LS	NYC-1	WM	AD-11	AD-13	JR-FL	IIIB	MN
	CD4-IgG	25	1	25	5	>25	>25	25	>25	>25	>25	<0.04	1
CD4BS	HT6	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25
CD4BS	HT5	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25
CD4BS	HT7	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	0.2	>25
CD4BS	IgG1b12	1	0.2	5	5	25	>25	0.2	5	>25	0.2	0.2	5
V3	19b	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25
V3	447-D (r1)	>25	0.2	>25	>25	>25	>25	>25	>25	>25	0.2	>25	>25
HIV-1+ sera	AD-28	<1:8	<1:8	1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
HIV-1+ sera	AD-18	<1:8	<1:8	<1:8	1:32	1:32	<1:8	1:16	<1:8	<1:8	<1:8	1:8	1:8
HIV-1+ sera	AD-46	>1:64	1:32	1:16-1:64	>1:64	>1:64	>1:64	1:32	>1:64	>1:64	>1:64	1:16	1:64

FIG. 2. Neutralization of primary and T-cell line-adapted strains (PBMC grown) by MAbs and HIV-1^+ sera. ID_{50} (a) and ID_{90} (b) values are presented for the reagents and viruses indicated. Highlighted values are considered significant. A range of values is shown for HuMAb 19b and isolate AD-6 in panel a. IIIB and MN are T-cell line-adapted strains, and the others are primary strains, but all were grown in PBMC.

general, relatively sensitive to neutralization compared with the primary isolates (Fig. 1 and 2). Furthermore, the experiments with IIIB and MN also establish that most of the HuMAbs tested are effective at neutralizing T-cell line-adapted HIV-1 strains; the failure of many of them to neutralize primary strains is not, therefore, due to an inherent lack of activity.

Most primary strains that resist neutralization by HuMAbs possess the MAb epitope. The simplest explanation for the failure of an antibody to neutralize an HIV-1 strain is that the virus lacks the antibody epitope because of sequence divergence from the strain against which the antibody was raised. To assess whether this was, in fact, the case, we used an antigen-capture ELISA to test each MAb for its ability to bind gp120 released from each virus strain with nonionic detergent (Table 1). This procedure does not denature monomeric gp120 molecules, as demonstrated by the preservation of their high-affinity binding to CD4-IgG. This reagent bound half-maximally to the various gp120s at concentrations around 0.1 μg/ml, which corresponds to 0.7 nM (Table 1). Most MAbs bound strongly to most or all of the viral gp120s; indeed, HuMAbs to the CD4-binding site only rarely failed to react strongly with gp120 (Table 1).

The V3-specific HuMAb 447-D (r1) was the only antibody tested that did not react with several gp120s; 5 of the 12 strains tested in this panel lack the epitope for this antibody (Table 2). It was notable that the four primary isolate gp120s with the highest affinities for 447-D (r1) (JR-CSF, NYC-1, AD-13, and JR-FL) were from those strains most sensitive to neutralization by this antibody (Table 1 and Fig. 2). The other V3-specific HuMAb, 19b, was more broadly reactive than 447-D (r1) in terms of its ability to bind gp120, for only IIIB gp120 failed to bind 19b (Table 1). However, although 19b had affinities for several gp120s (e.g., NYC-1 and AD-13) that were comparable to those of 447-D (r1), this was not reflected in the results of the neutralization assays (Table 1 and Fig. 2).

Titration curves comparing the binding of CD4-IgG, the six HuMAbs, and HIV-1^+ serum antibodies to gp120s solubilized from a primary strain and a T-cell line-adapted strain grown in PBMC (IIIB or MN) are shown in Fig. 1. The corresponding

TABLE 2. Partial V3 sequences of viruses in test panel

Virus	Partial V3 sequence
AD-6	SITMGPGKVFYVT
JR-CSF	SIHIGPGRAFYTT
N70-2	SIHMGPGRAFYAT
AC	GIGIGPGRTVYTT
LS	Not done
NYC-1	SIPIGPGRAFYTT
WM	GIHIGPGKAFYTT
AD-11	SIPIGPGRAFYTT
AD-13	SIHIGPGRAFYAT
JR-FL	SIHIGPGRAFYTT
IIIB	RIRIQRGPGRAFVTI
MN	RIHIGPGRAFYTT

virus neutralization curves are also depicted for comparison. For each of the virus pairs shown, the test reagents bound with approximately equivalent affinity to the primary strain gp120 and the IIIB or MN gp120 (Fig. 1), although minor variations were observed (Fig. 1e; Table 1) and there were some rare examples of a gp120 lacking a MAb epitope (Table 1). In contrast to the gp120 binding data, the virus neutralization titration curves showed that the T-cell line-adapted, PBMC-grown strains were approximately 100-fold more sensitive than the primary strains to neutralization by CD4-IgG and the six HuMAbs (Fig. 1a through g), whereas both types of virus were strongly neutralized by serum from the long-term nonprogressor AD-46 (Fig. 1h and 2). Thus, the presence of the MAb epitope on primary virus monomeric gp120 need not predict virus neutralization, and there is no general correlation between the affinity of an antibody for the gp120 monomer and its ability to neutralize primary isolates of HIV-1.

DISCUSSION

Our studies demonstrate that primary isolates have an inherent resistance to neutralization by MAbs, in contrast to strains, such as IIIB and MN, that have been passaged extensively in T-cell lines, a conclusion which is exemplified by the data presented in Fig. 1. This observation is consistent with several reports that primary HIV-1 isolates are relatively resistant to neutralization by CD4-based reagents (1, 8, 16, 41, 46, 51, 55, 57, 58, 67, 72), by sera from HIV-1-infected people (36, 37), and by antisera to gp120 subunit vaccines (13, 37). Yet it is also clear that primary HIV-1 viruses can be neutralized. The development of neutralization escape mutants (17) and the presence of neutralized virus in plasma (68) document the ability of the humoral immune system to suppress HIV-1 infectivity in vivo, albeit with less than complete efficiency (78). In vitro, selected HIV-1^+ human sera and HuMAb IgG1b12 showed considerable breadth and potency against primary isolates in our PBMC-based neutralization assay (Fig. 1e and h and 2). Indeed, HuMAb 1gG1b12 caused 50 and 90% infectivity reductions in approximately the same concentration ranges for both primary strains and the T-cell line-adapted strains (Fig. 1e and 2). Thus, IgG1b12 and sera such as AD-46 provide the positive controls that validate the assay procedure we used.

Mechanistically, what may we infer from our data? Firstly, the virus is the major determinant of neutralization resistance. Both the IIIB isolate and the MN isolate, when grown in PBMC, are relatively sensitive to neutralization by a variety of agents compared with primary isolates grown in the same cells (Fig. 1 and 2). In our experience, differences in neutralization sensitivity between H9-grown and PBMC-grown stocks of IIIB and MN are minor when PBMC are used as target cells. We presume that the major differences in neutralization sensitivity between strains grown in PBMC and strains grown in T-cell lines that have been observed by others reflect, at least in part, the use of the MT-2 transformed T-cell line as the indicator of virus replication (66). Selection for retroviral variants can be very rapid (12), and passage of primary viruses into T-cell lines will select for variants that are better able to replicate in those cells. Either consequentially or coincidentally, the selected strains are sensitive to neutralization by sCD4 (1, 16, 41, 58, 72, 75, 76). We suggest that sensitivities to antibodies and to sCD4 are gained simultaneously (see below). There may also be undefined epigenetic factors that could influence neutralization efficiency (66).

We presented the data derived from the 120 combinations of reagents and virus strains tested in both gp120 binding assays and virus neutralization assays in tabulated form for logistical purposes (Table 1 and Fig. 2). However, many of the conclusions we draw are only truly apparent from the titration curves (Fig. 1 and data not shown). The 50% binding values shown in Table 1 provide a reasonable approximation of the K_d values for the MAb-gp120 interaction (40, 58), and so we compared the ID_{50} neutralization data (Fig. 2a) with these values to see whether there was any obvious relationship between gp120 binding and virus neutralization titers; in most cases, there was not. However, ID_{50} values are not the most reliable way to quantitate neutralization in PBMC-based assays, because the significance of a twofold reduction in HIV-1 infectivity, both in vitro and in vivo, is questionable and because of intra-assay variation. Thus, we also presented ID_{90} values (Fig. 2b), as a 10-fold reduction in infectivity is likely to be more meaningful. Yet ID_{90} value determinations are also problematical in the case of PBMC-based assays using nonclonal virus stocks that contain a swarm of infectious variants of HIV-1. With several combinations of MAb and virus, we observed that 50%, but not 90%, neutralization was achieved; indeed, it was not uncommon to observe a flattening out of the neutralization dose-response curve at >80%, but <90%, neutralization (Fig. 1c, d, f, and g and data not shown). We also saw examples of the extent of neutralization plateauing at around 50% (Fig. 1g and data not shown). Furthermore, >99% neutralization was rarely achieved for any virus-MAb combination, although it was possible to attain this degree of neutralization in some circumstances (Fig. 1e and h). The most probable explanation of the overall patterns of the data is that each virus stock contains a nonneutralizable fraction that varies in extent for different MAbs, although there are probably additional complexities to primary virus neutralization reactions that remain unsuspected and unresolved.

It is important to note that the performance of different antibodies in primary virus neutralization assays will depend to a considerable extent on the assay format used. For example, if antibody is maintained continuously in the PBMC cultures during the entire assay procedure (14, 15, 24), antibody concentrations lower than those seen in our assay, which required antibody to be removed from the cultures after 16 h, might be required for neutralization. Another commonly used procedure is the infectivity reduction assay, in which a fixed concentration of antibody (often up to 100 μg/ml) is incubated continuously with a variable virus inoculum during the life of a PBMC culture and the extent of reduction of virus infectivity is determined (14, 15, 52). The ability of such an assay to detect a quantifiable degree of infectivity reduction, especially at input MAb concentrations as high as 100 μg/ml, makes it an increasingly popular choice for primary virus assays. We have used a more traditional assay format based on antibody

titrations at a fixed virus inoculum, and we have chosen not to exceed an antibody concentration of 25 μg/ml to avoid generating unrealistic perceptions as to the neutralization potency of HuMAbs against primary viruses. The overall effect of the use of subtly or grossly differing assay formats is that it is very difficult, or impossible, to relate data obtained in one laboratory to those obtained in others when assessing the relative potencies of a group of MAbs. When we used a single assay format, HuMAb IgG1b12 was clearly the reagent with the greatest potency against primary virus strains. However, although several of the other MAbs lack the breadth and potency of IgG1b12, they do possess some activity against primary strains. For example, the V3 HuMAbs 447-D (r1) and 19b are able to significantly reduce the infectivity of certain primary viruses when the antibodies are present continuously at 100 μg/ml in an infectivity reduction assay (14, 52). These findings do not necessarily represent a discrepancy with our data, but they reflect the variations that are to be expected when different assay formats are followed and different virus strains are used (see above). Comparative data can only be obtained when reagents are compared within an assay, and we have used a rigorous format.

The inability of some antibodies, especially those directed at the variable domains, to neutralize certain primary strains may merely reflect the absence of the antibody binding site from the target strain. For example, the epitope for HuMAb 447-D (r1) is centered on the GPGR motif at the crown of the V3 loop (14, 23, 25). Isolates WM and AD-6 have a GPGK motif at the crown of the V3 loop (Table 2), and so the failure of 447-D (r1) to bind to or neutralize these strains is not surprising. 447-D (r1) bound most strongly to monomeric gp120 from primary strains JR-FL, JR-CSF, and AD-13 (Table 1), and these were the strains that were most sensitive to neutralization by 447-D (r1) (Fig. 2). Each of these isolates has the GPGR motif at the crown of the V3 loop (Table 2). However, other isolates with the same GPGR motif (N70-2, AC, NYC-1, AD-11, and IIIB) bound 447-D (r1) less avidly or not at all (Table 2) and were neutralized by this antibody at best only weakly (NYC-1) (Fig. 1g and 2). These observations presumably reflect the unpredictable effect of nearby or more distant residues on the epitope of 447-D (r1) and its very similar parent 447/52-D (23, 25, 42, 73), which may be especially important in the context of the oligomeric form of the envelope glycoproteins (7). Isolate LS has not been sequenced, and so we cannot speculate as to why 447-D (r1) fails to neutralize this strain.

The other V3-directed HuMAb, 19b, bound strongly to all the viral gp120s except that from IIIB. The binding site for 19b spans both sides of the V3 loop and involves amino acids -I----G--FY-T (e.g., for MN, rIhigpGraFYtT) (52). Amino acid substitutions at positions indicated with a dash do not strongly influence binding of 19b to monomeric gp120 (52). All of the sequenced virus strains in the test panel, except IIIB, contained the canonical 19b epitope (Table 2). Despite this fact, 19b showed only a limited ability to neutralize the primary strains in our panel at concentrations of ≤25 μg/ml. Primary virus neutralizing activity shown by 19b at 100 μg/ml in an infectivity reduction assay is described elsewhere (52). It was notable that while there was a reasonable correlation between gp120 binding affinity and primary virus neutralization potency for HuMAb 447-D (r1), no such correlation was observed with 19b (Table 1 and Fig. 2a). This may reflect extreme subtlety in the ways in which different V3 epitopes are presented on primary virus strains (7). For example, the segment of the V3 region that contains the binding site for 19b may be relatively inaccessible in the context of the oligomeric gp120/gp41 complexes on the surfaces of virions of HIV-infected cells.

Notwithstanding the observations with 447-D (r1), the simple absence of an antibody-binding site from monomeric gp120 is clearly not the major mechanism underlying the relative resistance of primary virus to most neutralizing antibodies. Conversely, the presence of a MAb epitope on monomeric gp120 from a primary virus does not predict that the virus will be neutralized. The affinities of certain antibodies for monomeric gp120 from a T-cell line-adapted strain such as IIIB or MN can be used to predict their virus neutralization potencies (4, 54). However, this relationship is not found for other antibodies (60), and it does not appear to extend generally to primary viruses, as discussed above for 19b. Although the binding of IgG1b12 to monomeric gp120 from AD-6 and IIIB appears to predict the relative neutralization potencies for these strains (Fig. 1e), this was not always observed (data not shown). Furthermore, at least one primary isolate that we are studying is neutralized by IgG1b12 despite the failure of the antibody to bind to monomeric gp120 from this isolate. This suggests that the binding site for IgG1b12 on the oligomeric spikes of the viral envelope may be quite complex. Further examples of the lack of any general correlation between MAb binding to the gp120 monomer and primary virus neutralization are provided by the antibodies to the complex epitopes around the CD4-binding site. Except for IgG1b12, HuMAbs directed at the CD4-binding site epitopes neutralized primary strains very poorly, if at all (Fig. 1b through d). For example, HuMAb HT7 bound very strongly to all 10 gp120s from primary strains, but it neutralized not one of these strains by 50% at concentrations of ≤25 μg/ml. Yet HT7 bound with comparable affinity to gp120 from IIIB and MN (Fig. 1d), and it reduced the infectivity of these strains by 50% at concentrations of 0.2 and 1 μg/ml, respectively (Fig. 2a). We have shown elsewhere that the ability of HIV-1^+ sera and HuMAbs to inhibit the binding of monomeric gp120 to sCD4 is not correlated with their ability to neutralize primary virus strains (43), and that finding is consistent with the present results.

The data derived from studies with the CD4-binding site-associated HuMAbs HT5, HT6, and HT7 are strikingly similar to the observations with CD4-IgG (compare Fig. 1a with Fig. 1b through d). The relative resistance of primary strains to neutralization by sCD4 is not attributable to an inability of monomeric gp120 from primary strains to bind sCD4 (1, 8, 46, 51), a result which was confirmed here by using CD4-IgG (Fig. 1a). However, measurements of the interactions of sCD4 with virions demonstrated that primary viruses had a reduced ability to bind sCD4 (46), due, it now appears, to a very slow on-rate for the reaction between sCD4 and these strains at 37°C (41, 57, 75, 76). The data obtained with sCD4 are supported by studies of the interaction of virus strains with CD4 on the cell surface: primary isolates require a much higher CD4 concentration for efficient infection of target cells than T-cell line-adapted strains do (21, 32).

It is logical to assume that HIV-1 did not evolve to resist neutralization by sCD4, and so we propose that HIV-1 in its unadapted form has an oligomeric envelope glycoprotein configuration in which access to the epitopes around the CD4-binding site is restricted. We note that a similar conclusion concerning the V3 loop of primary strains has been reached recently; this loop is relatively inaccessible on the oligomeric form of the envelope glycoproteins of these strains, and it provides a poor target for neutralizing antibodies (7). An envelope configuration of this nature could confer a selective advantage to HIV-1 in an environment rich in neutralizing antibodies: human serum (43–45). Thus, resistance to sCD4 may be merely coincidental to this natural mechanism of resistance to antibodies directed at roughly the same site (45),

a site that must be conserved in order for HIV-1 to be viable. We suggest, therefore, that the relative resistance of primary strains to neutralization by antibodies, despite the presence of the antibody binding site on the gp120 monomer, may be due to a reduction in the rate of binding of the neutralizing ligand to the virus particle. Passaged into T-cell lines, HIV-1 variants that become unnaturally sensitive to neutralization by antibodies and CD4-based reagents are selected for (1, 16, 41, 57, 72, 75). We propose that the selection pressure operating is for variants with a more accessible CD4-binding site and thence a higher rate of binding to $CD4^+$ cells and virus-cell fusion. The acquisition of neutralization sensitivity may be simultaneous and coincidental to this. The mechanism underlying these adaptative events is unknown, pending more knowledge of the structure and function of the virus envelope. It may or may not be relevant that the envelope glycoprotein oligomer of T-cell line-adapted strains appears to be unstable, compared with that of primary strains, leading to increased shedding of gp120 from the former strains at 37°C (41, 46, 57, 75, 76).

Do the results of in vitro neutralization experiments using primary strains, whatever the assay format, have any relevance to the interactions of HIV-1 and antibodies under in vivo conditions? We believe that they do. Our conclusion is based partly on studies with sCD4 and CD4-IgG: the weakness of these reagents in neutralizing primary strains in vitro is consistent with the failure of these reagents to induce clinical benefits in human trials (reviewed in reference 51). Furthermore, Baldinotti et al. have shown that there is no relationship between the potent neutralizing activity of cat sera against high-passage isolates of feline immunodeficiency virus and the ability of the sera to protect cats from infection by unadapted isolates that are relatively resistant to neutralization (2). Thus, the acquisition of the neutralization-sensitive phenotype after prolonged passage in vitro may be a general property of lentiviruses.

It is important for vaccine development to understand how primary strains can be neutralized, for it is clear that if the right reagents are used they can be neutralized, albeit often less easily than T-cell line-adapted strains. Thus, serum from individual AD-6 can neutralize the autologous primary isolate with an ID_{90} of >1:640 in a PBMC-based assay (33, 43), and serum samples from long-term nonprogressors such as AD-46 can be extremely potent (Fig. 1h). A more detailed characterization of the neutralizing activity of sera from rapid progressors and long-term nonprogressors will be described elsewhere (9), for it is beyond the scope of the present study. The specificity of the neutralizing antibodies in AD-46 serum is not yet known. Perhaps some are similar to the recombinant HuMAb IgG1b12; others may recognize as-yet-unidentified neutralization sites on gp120 and/or gp41. One possibility that merits exploration is that some critical neutralizing antibodies are against sites that are better represented on gp120/gp41 oligomeric complexes than on monomeric gp120 (19). Such antibodies would, perhaps, not be induced by subunit vaccines based on recombinant, monomeric gp120. Preliminary results indicate that the Fab version of HuMAb IgG1b12 binds unusually well to native oligomeric HxBc2 gp120 compared with other, less effective Fabs that have affinities for monomeric gp120 similar to that of IgG1b12 (60). We cannot otherwise determine from studies with wild-type and mutant monomeric HxBc2 gp120 molecules why IgG1b12 has such outstanding neutralization potency (60).

Although our results do suggest that neutralization of physiologically relevant HIV-1 strains is more difficult than was hitherto appreciated, an HIV-1 vaccine based wholly or in part on the induction of humoral immunity is clearly not impossible. But its creation may depend on our ability to identify and further characterize those types of antibodies that are able to neutralize primary strains and then to find the best ways to induce these antibodies. Our belief is that this will require a greater understanding of the structure and antigenicity of the native, oligomeric envelope glycoprotein complex of HIV-1 and its interaction with the surfaces of target cells and neutralizing antibodies (19, 20, 32, 34, 35, 48, 65, 76).

ACKNOWLEDGMENTS

We thank Jamie Franklin and Wendy Chen for assistance with graphics and Shu-Wing Poon for technical assistance. We appreciate the gifts of HuMAb 447-D (r1) from Anthony Conley and Emilio Emini (Merck Research Labs, Inc.); of HuMAbs HT5, HT6, and HT7 from Heinz-Kurt Hochkeppel (Ciba-Geigy AG); and of CD4-IgG from Genentech, Inc.

This study was supported by The Aaron Diamond Foundation and by NIH awards AI24030, AI25541, AI36082, AI35168 (Antigenic Variation of HIV-1), AI45218 (Correlates of HIV Immune Protection; PI S. Wolinsky), and AI27742 (Center for AIDS Research). D.R.B. and C.F.B. were supported by NIH award AI33292.

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