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Increased Envelope Spike Density and Stability Are Not Required for the Neutralization Resistance of Primary Human Immunodeficiency Viruses

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Previous observations that the gp120 envelope glycoprotein contents of some primary, clade B human immunodeficiency virus type 1 (HIV-1) isolates were higher than those of laboratory-passaged HIV-1 isolates suggested the hypothesis that increased envelope glycoprotein spike density or stability contributes to the relative neutralization resistance of the primary viruses. To test this, the structural, replicative, and neutralization properties of a panel of recombinant viruses with HIV-1 envelope glycoproteins from divergent clades were examined in an *env* complementation assay. In this system, although the spike density and stability of envelope glycoproteins from primary HIV-1 isolates were not greater than those from a laboratory-adapted isolate, relative resistance to neutralizing antibodies and soluble CD4 was observed for the viruses with primary envelope glycoproteins. Thus, neither high envelope glycoprotein spike density nor stability is necessary for the relative neutralization resistance of primary HIV-1 viruses.

The infection of the host cell by human immunodeficiency virus type 1 (HIV-1) is initiated by the virus binding to the CD4 receptor on target T lymphocytes or monocytes/macrophages (10, 24, 27). The residues in the viral exterior envelope glycoprotein, gp120, that contribute to CD4 binding form a discontinuous structure (39) that is well conserved among different HIV-1 isolates (34, 36). Other conserved, discontinuous structures in the gp120 glycoproteins contribute to the noncovalent association with the gp41 transmembrane glycoprotein (21), which is anchored in the viral membrane.

In addition to conserved envelope glycoprotein structures, five regions of variability exist in the HIV-1 gp120 glycoproteins (29, 42). Variation in *env* has been used to classify HIV-1 into subtypes with distinct geographic distributions (11, 36, 41). The M (major) group of HIV-1 contains isolates classified into five clades (A to E), but additional clades (F to I), with fewer members so far, have recently been identified (23, 25, 26a). The O (outlier) group consists of a few extremely divergent HIV-1 isolates from Cameroon (20, 47).

In addition to the high degree of sequence diversity among geographically distant HIV-1 isolates, studies of the HIV-1 envelope glycoproteins are further complicated by important functional differences between primary virus isolates and virus isolates that have been extensively passaged on transformed T-cell lines (laboratory-adapted viruses). Primary isolates are defined as those that have never been passaged on transformed T-cell lines. Two key differences between primary and laboratory-adapted virus, namely, neutralization sensitivity and target cell tropism, are specified by the envelope glycoproteins (5–6, 15, 37, 43, 48). One characteristic of primary virus isolates, addressed herein, is that they are highly unresponsive to neutralization by antibodies and by soluble CD4 (sCD4) compared with laboratory-adapted viruses (9, 19, 32). For example, a 200- to 2,700-fold-higher concentration of sCD4 may be required to achieve neutralization of primary isolates compared with laboratory isolates (9).

At least three mechanisms have been proposed to explain the resistance of primary HIV-1 isolates to neutralization by sCD4, and these may also apply to neutralizing antibodies. First, when binding of purified virus to sCD4 was measured, it was found that primary virus isolates exhibited a 10- to 30-fold-lower affinity to the soluble receptor than laboratory-adapted virus (35). This difference was not apparent when binding experiments were performed using monomeric gp120 from the different viruses (2, 4, 35, 46), suggesting that the multimeric envelope complex on a primary virus may have a less accessible CD4 binding site than that on a laboratory-adapted virus. Since a large fraction of neutralizing antibodies generated in HIV-1-infected individuals is directed against epitopes closely related to the CD4 binding site (33), this lack of accessibility may also be relevant to the relative insensitivity of primary isolates to antibody neutralization. Second, primary viruses do not generally infect transformed T-cell lines, but they do efficiently infect primary cells such as human peripheral mononuclear cells (PBMCs) and purified macrophages (5a, 14). It is therefore plausible that other cell surface molecules present on primary cells may assist CD4 in the early binding of primary viruses, rendering antiviral approaches that block gp120-CD4 interaction less effective (4, 26). Third, it has been reported that primary viruses exhibit a two- to threefold-higher level of gp120 per virion than was seen on laboratory-adapted isolates (30, 38). It is unknown whether the higher gp120/p24 ratio observed for primary isolates reflects a more efficient incorporation of envelope glycoproteins into virions or an increased

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stability of the noncovalent gp120-gp41 interaction. It has been proposed that higher envelope spike density could contribute to neutralization resistance, since a larger number of functional envelope complexes would require inactivation to effect virus neutralization.

Here we test the hypothesis that envelope glycoprotein spike density contributes to differences in sensitivity to neutralization between primary and laboratory-adapted isolates. Since functional studies of both primary and laboratory-adapted HIV-1 envelopes performed to date have almost exclusively used subtype B viruses (the predominant genotype in the United States and Europe), we included viruses with envelope glycoproteins derived from viral subtypes A, C, D, and E to obtain a broader perspective.

MATERIALS AND METHODS

Proviral DNA constructs and envelope expressor plasmids. HXBH10Δ envCAT is a derivative of HXBH10 with a deletion in the envelope gene (ΔBglII), a bacterial chloroamphenicol acetyltransferase (CAT) gene in place of the *nef* gene and a functional *vpu* gene derived from HIV-1 BH10 (44). HXBH10-gag$^-$ is a variant of HXBH10 that is unable to express p55^{gag} because of a premature termination codon in place of codon 8 of the *gag* gene and an additional frameshift mutation in the CA coding region (12). The HXBH10-gag$^-$ plasmid was used as a control in these studies. To create expressor plasmids for the envelope glycoproteins from primary HIV-1 isolates of various clades, *env* sequences were obtained from PCR-amplified fragments derived from viruses propagated in PBMCs (17, 18, 28) or, in the case of the clade D *env* expressor (pSVIIIenv-eli), from a molecular proviral clone (1). For the former sequences, a *Kpn*I-*Kpn*I fragment spanning the 5′ end of *env* to the *nef* gene was cloned into the pSVIIIenv(Kpn) plasmid, which was derived from the pSVIIIenv plasmid (21) by reintroduction of the 3′ *Kpn*I site. For the clade A *env* (92RW020.5) and clade E *env* (TN243), one of the *Kpn*I sites was introduced by PCR mutagenesis, while the clade B (92BR020.4) and clade C (92BR025.9) fragments contained naturally occurring *Kpn*I sites at the desired locations. The pSVIIIenv-eli plasmid was created by introducing a *Bam*HI site into the 3′ portion of the *env* gene by PCR and replacing the *Kpn*I-*Bam*HI fragment of pSVIIIenv with the amplified fragment. The pSVIIIenv plasmid expresses the HXBc2 envelope glycoproteins from a clade B, laboratory-adapted virus. The envelope glycoprotein expression plasmids for the HXBc2 and *env* genes from the viruses belonging to clades A to E will be referred to hereafter as HXBc2, Rw20-5, Br20-4, Br25-9, Eli, and TN243, respectively (17, 18, 28). The pSVIIIenvΔKS plasmid, which has a deletion in *env*, was included as a negative control and is referred to hereafter as ΔKS.

Cell culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium, the human T-cell lines Molt 4 clone 8 and SupT1 were maintained in RPMI 1640 medium, and human PBMCs were prepared by Ficoll separation and maintained in RPMI 1640 medium, 10% fetal calf serum (PAA Laboratories Inc., Newport Beach, Calif.), 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 mg of streptomycin per ml was supplemented to all cultures. Human PBMCs were stimulated with 1 μg of phytohemagglutinin (Murex Diagnostics Ltd., Dartfield, United Kingdom) per ml for 48 to 72 hours, and 24 h prior to infection, 5% interleukin 2 (T-stim; Becton Dickinson Labware, Bedford, Mass.) was added.

sCD4 and MAbs. sCD4 was a gift from Raymond Sweet at Smith, Kline and Beecham (King of Prussia, Pa.). The CD4 binding site-directed monoclonal antibody (MAb) F105 was obtained from M. Posner (40).

Analysis of viral protein content. HeLa cells (7×10^5/plate) were transfected by the calcium phosphate precipitation technique (8) with pHXBH10ΔenvCAT and the different envelope expressor plasmids to generate recombinant virus particles. To control for envelope glycoproteins not associated with virions, the HXBH10-gag$^-$ construct was used (12). Envelope glycoproteins are expressed by this plasmid, but because of a frameshift mutation in *gag* no particles are formed. Two days after transfection, the cells were labeled with 50 μCi (each) of [^{35}S]cysteine and [^{35}S]methionine per ml for 12 h, harvested, and centrifuged, and the virus-containing supernatants were passed through 0.45-μm-pore-size filters. The cells were lysed in radioimmunoprecipitation (RIPA) buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and clarified by centrifugation. Virions were pelleted through a 20% sucrose (in phosphate-buffered saline [PBS]) cushion for 90 min at 4°C and 27,000 rpm with a Beckman SW28 rotor and lysed in 100 μl of RIPA buffer. An aliquot of each supernatant, retained above the sucrose after ultracentrifugation, was taken out (inactivated with 1/4 the volume of 5× RIPA buffer) to determine the amount of soluble gp120 present in the medium. HIV-1-encoded proteins were immunoprecipitated from cell lysates, supernatants, and viral lysates with a mixture of sera from HIV-1-infected individuals and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry. A mixture of sera from individuals infected with clade B HIV-1 or with HIV-1 viruses from clades A, B, C, D, and E (a gift from John Moore, Aaron Diamond AIDS Research Center) was used for immunoprecipitations.

Single-round infectivity assays. Recombinant virus was generated by cotransfecting pHXBH10ΔenvCAT and the different envelope expressor plasmids into HeLa cells as described above. The medium was replaced 16 h prior to harvest to obtain a synchronized virus population, and viral supernatants were harvested and normalized to reverse transcriptase (RT) activity. PBMCs (1.5×10^6), Molt 4 clone 8 cells, or SupT1 cells (10^6) were infected with the same amount of each recombinant virus (2,500 cpm [RT units]) and cultures were incubated for 60 to 68 h. The target cells were harvested, washed once in PBS, lysed in 0.25 M Tris-HCl (pH 7.8), and assayed for CAT activity as previously described (21).

Neutralization assays. Recombinant virus was generated by cotransfecting pHXBH10ΔenvCAT and the different envelope expressor plasmids into HeLa cells as described above. Neutralization experiments were performed by incubating 0.5 ml of viral supernatant (normalized to 5,000 cpm/ml) with increasing concentrations of antibodies or sCD4 for 2 h at 37°C prior to the addition of human PBMCs (1.5×10^6 target cells added to 0.5 ml of viral supernatant in 24-well plates). Culture medium (2 ml) containing 5% interleukin 2 was added 1 day after the infection, and cultures were harvested and processed as described above. Relative neutralization was determined by quantitating the percentage conversion in the CAT assay relative to that of the untreated control.

Neutralization of virus subjected to ultracentrifugation. To confirm that the virions retained their neutralization characteristics when they had been subjected to ultracentrifugation, viral supernatants were placed on a sucrose cushion and pelleted for 90 min at 27,000 rpm with a Beckman SW28 rotor as described above. The virus pellets were resuspended in culture medium and preincubated with different concentrations of the MAbs for 2 h followed by the addition of target PBMCs. Samples were processed as described for the other neutralization assays.

Binding of MAbs to monomeric envelope glycoproteins. To determine if the envelope glycoproteins from HIV-1 viruses from different clades retained the epitopes for CD4 binding site MAbs, the envelope expressor plasmids were transfected into HeLa cells supplying *tat* in *trans*. Two days after transfection, the cultures were labeled with 75 μCi of [^{35}S]cysteine per ml for 12 h, and the cells and supernatants were separated by centrifugation. Each supernatant was divided in four parts, and soluble gp120 was immunoprecipitated with a mixture of sera from HIV-1-infected individuals to normalize for expression, and the F105, 21h, and 15e MAbs (22, 40, 45). MAb binding to the different envelopes was quantitated by SDS-PAGE and densitometry.

RESULTS

Analysis of virion-associated envelope glycoproteins. Recombinant HIV-1 virions containing the laboratory-adapted virus envelope glycoprotein (HXBc2) or the envelope glycoproteins derived from primary HIV-1 isolates were produced transiently following transfection of HeLa cells. The results of the analysis of virally encoded proteins in the transfected HeLa cells are shown in Fig. 1. The degree of processing of the gp160 precursor glycoproteins varied (Fig. 1A). The HXBc2 envelope glycoprotein precursor was most efficiently processed followed by that of the Eli and TN243 isolates. Next in the rank order of processing were the Br20-4, Rw20-5, and Br25-9 precursor glycoproteins. Only very low levels of Rw20-5 gp120 and virtually no Br25-9 gp120 glycoproteins were detected. The HXBH10-gag$^-$ construct expressed the HXBc2 envelope glycoproteins but not p55^{gag} precursor, while the p55^{gag} protein but no envelope glycoproteins were detected in the ΔKS control, as expected.

The amount of free gp120 present in the medium directly mirrored the level of processing of gp160 in the cells (Fig. 1B). High levels of HXBc2 gp120 were detected, with smaller amounts of gp70 and gp50 proteins observed. The latter products result from cleavage in the V3 loop (7) and were not seen for the other envelope glycoproteins. High levels of supernatant gp120 were also observed for the Eli, TN243, and Br20-4 envelope glycoproteins, but less Rw20-5 gp120 and almost no Br25-9 gp120 were detected in the culture medium.

Immunoprecipitation of virion proteins revealed that the incorporation of the gp120 envelope glycoprotein into virus particles varied (Fig. 1C). The amount of gp120 glycoprotein precipitated from the pellet derived from the HXBH10-gag$^-$ transfection was very low, indicating that the vast majority of the envelope glycoproteins detected were specifically virion associated. When the ratios of gp120/p24 proteins in the viri-

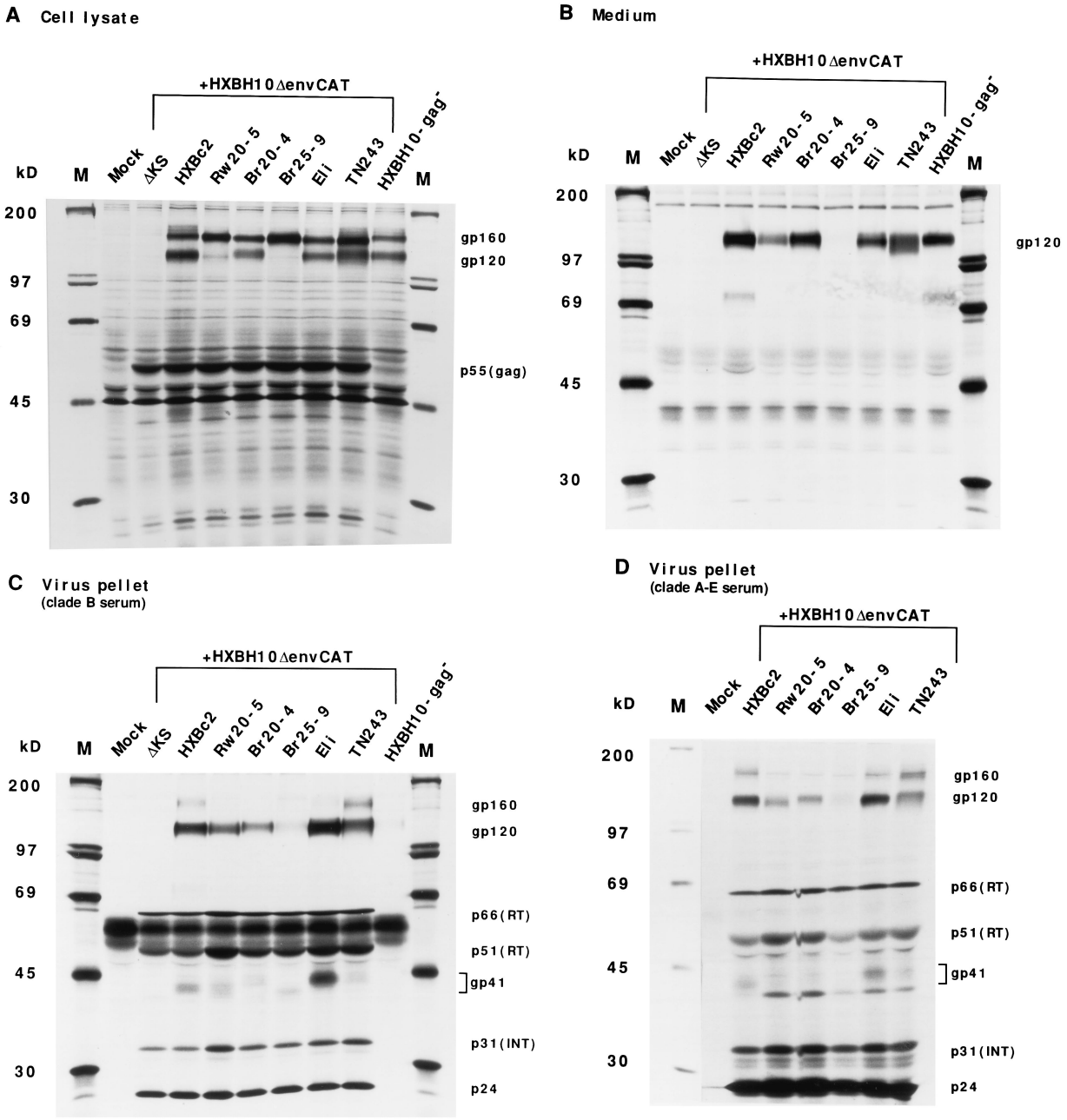

FIG. 1. Analysis of viral proteins. HIV-1-encoded proteins were immunoprecipitated with clade B patient sera from transfected HeLa cell lysates (A), supernatants (B), and virion lysates (C) or with a pool of patient sera from patients infected with virus of clades A through E from virion lysates (D) and resolved on 8.5% reducing SDS-polyacrylamide gels. From each transfection, 1/40 of the total cell lysates, 1/30 of the total volume of supernatant, and all of the pelleted virus lysate was used for immunoprecipitations. The outer lanes (M lanes) show molecular mass markers (in kilodaltons). The following three controls were included in panels A to C): mock transfection, ΔKS, and HXBH10-gag$^-$. In panel D, the control was mock transfection. As indicated over the lanes, cells were transfected with the pHXBH10ΔenvCAT plasmid and the respective envelope expressor plasmids.

ons were determined, the following rank order was observed: Eli > HXBc2 > TN243 > Rw20-5 = Br20-4 > Br25-9 (Table 1). At least in this system, the virion gp120 content associated with primary virus envelope glycoproteins was not necessarily higher than that of the laboratory-passaged HXBc2 envelope glycoproteins.

The gp160 precursor glycoproteins of the HXBc2 and the TN243 envelope glycoproteins were detectable in the virion preparations (Fig. 1C). The reason why this was seen with only some envelope glycoproteins and not others is not clear, but the result does indicate that gp160 molecules are not absolutely excluded from becoming incorporated into virus particles (13).

The gp41 transmembrane glycoprotein was detected in the virion immunoprecipitates (Fig. 1C). This allowed a calculation of the gp120/gp41 ratio, a measurement reflecting the stability of the noncovalent gp120-gp41 interaction. The data in Table 1 indicate that the gp120/gp41 ratios were similar for the different envelope glycoproteins.

To confirm that the serum mixture used for the immuno-

TABLE 1. Properties of HIV-1 envelope glycoproteins

Envelope glycoprotein	Clade	Passage history	gp120/p24 ratio[a]		gp120/gp41 ratio[b]	PBMC entry[c]
			Clade B sera	Clade A to E sera		
Eli	D	Primary	1.45	1.05	2.6	90.0
HXBc2	B	Laboratory-adapted	0.97	0.97	2.8	16.9
TN243	E	Primary	0.85	0.52	3.6	15.3
Rw20-5	A	Primary	0.55	0.40	2.9	8.2
Br20-4	B	Primary	0.53	0.43	3.8	22.7
Br25-9	C	Primary	0.22	0.28	2.9	1.0

[a] These values were derived by densitometric analysis of the SDS-polyacrylamide gels shown in Fig. 1C and D. The values obtained for the precipitation with the sera of clades A to E were normalized to the value obtained for the HXBc2 envelope glycoproteins with the clade B sera.
[b] These values were derived by densitometric analysis of the SDS-polyacrylamide gel shown in Fig. 1C.
[c] These values represent the percentage conversion of chloramphenicol to acetylated forms in the experiment using human PBMC target cells shown in Fig. 2. The background activity in the assay observed for the ΔKS control plasmid was 0.2%. In different experiments, the activity associated with the Br25-9 varied from 1 to 7% conversion.

precipitations (obtained from patients infected with clade B viruses) did not selectively recognize the two clade B envelopes (HXBc2 and Br20-4), the experiment was repeated using a pool consisting of equal parts of sera from patients infected with viruses of clades A through E (31) (Fig. 1D). The relative gp120 content on virions quantitated from this immunoprecipitation was similar to that obtained with the clade B sera, as shown in Table 1.

Function of envelope glycoproteins. To investigate the function of the envelope glycoproteins, recombinant viruses were harvested at 60 h following the transfection of HeLa cells, normalized according to RT activity (2,500 cpm), and used to infect target cells. The CAT activity in the target cells produced by the pHXBH10ΔenvCAT provirus was assayed 64 h after infection. Target cells were either SupT1 lymphocytes, Molt 4 clone 8 lymphocytes, or phytohemagglutinin-stimulated human PBMCs.

In human PBMC targets, all of the envelope glycoproteins were able to support a level of virus entry detectable above that of the ΔKS negative controls (Fig. 2). The activities observed in PBMC target cells may be affected in part by the level of virion-associated envelope glycoproteins, since the Eli glycoproteins supported the highest activity, whereas the Br25-9 glycoproteins exhibited an activity only slightly above the background level of the assay (Table 1). The activity of the primary virus envelope glycoproteins, relative to that of the HXBc2 envelope glycoproteins, was generally lower in Molt 4 clone 8 or SupT1 target cells. The Eli glycoproteins were an exception, supporting efficient virus entry into both cell lines. The Br20-4 envelope glycoproteins supported efficient virus entry into Molt 4 clone 8 cells, consistent with previous reports that some primary HIV-1 isolates replicate in this cell line (16, 43).

Recognition of monomeric gp120 by CD4 binding site antibodies. To investigate the relative sensitivity to neutralization of viruses containing the different envelope glycoproteins, MAbs reactive with HIV-1 epitopes that are conserved across the clades are required. The neutralization epitopes near the CD4 binding site of gp120 are reasonably well-conserved among HIV-1 isolates (34) so MAbs F105, 21h, and 15e, which recognize these epitopes (22, 40, 45) were examined for the ability to precipitate the envelope glycoproteins used in this study. Figure 3 shows that only the 21h antibody recognized all of the envelope glycoproteins tested. The F105, 21h, and 15e antibodies all recognized the clade B (HXBc2 and Br20-4) and clade D (Eli) gp120 glycoproteins, whereas the clade A (Rw20-5) and clade E (TN243) gp120 glycoproteins were rec-

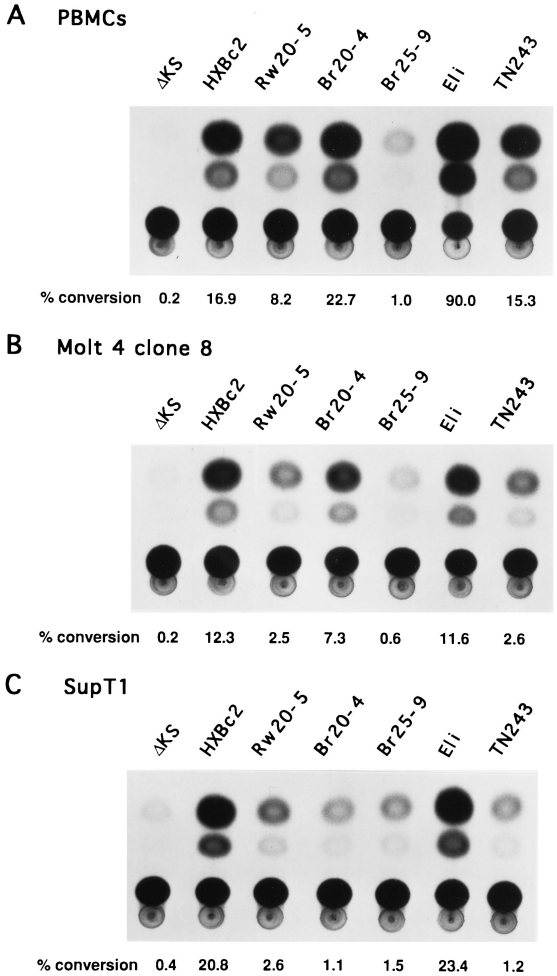

FIG. 2. Abilities of different envelope glycoproteins to mediate virus entry in a single-round infectivity assay. Recombinant virions were produced by transient transfection of HeLa cells and normalized for RT activity. Either human PBMCs, Molt 4 clone 8 cells, or SupT1 cells were infected, and the CAT activity was assayed in target cell lysates 3 days following infection. Percentage conversion of [^{14}C]chloramphenicol to acetylated forms is indicated for each sample.

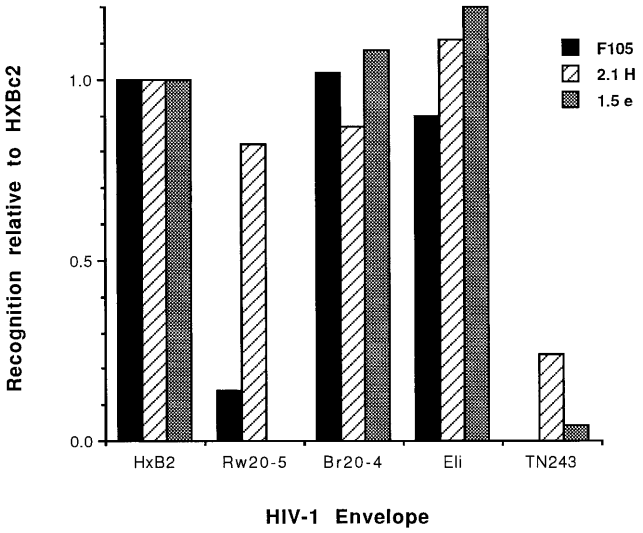

FIG. 3. Recognition of monomeric envelope glycoproteins by MAbs. HeLa cells were transfected with the different envelope expressor plasmids, and monomeric gp120 was immunoprecipitated from the culture medium either with an excess of a mixture of sera from HIV-1-infected individuals or with the F105, 21h, or 15e MAb. SDS-polyacrylamide gels were scanned by densitometry, and the recognition of the different envelope glycoproteins was quantitated relative to that observed for the HXBc2 envelope glycoprotein, after normalization for the amount of gp120 precipitated by the mixture of sera.

ognized by only a subset of these antibodies. Since the amount of Br25-9 (clade C) gp120 glycoprotein present in the supernatant was low, the recognition of this envelope glycoprotein by the MAbs was not tested.

Virus neutralization assays. Recombinant viruses containing the different envelope glycoproteins were tested for their sensitivity to neutralization by sCD4 and the MAbs (Fig. 4). All of the antibodies were tested against all viruses, even when the epitope was shown not to be present (see above). The latter cases served as useful controls for possible nonspecific inhibitory effects of antibody preparations and are depicted as broken lines in Fig. 4. Compared with viruses with the HXBc2 envelope glycoproteins, all of the viruses with primary envelope glycoproteins were resistant to neutralization by sCD4 and by the MAbs.

Neutralization of virus subjected to ultracentrifugation. To confirm that the neutralization results obtained with unpelleted virions could be correlated with the virion protein content results (obtained from immunoprecipitations of pelleted virions), control neutralization assays were set up with virus supernatants that had been subjected to ultracentrifugation and subsequently resuspended into the original volume. Pelleted virions complemented with the HXBc2 envelope exhibited nearly identical neutralization sensitivities to both F105 and 15e antibodies as unpelleted virus, while pelleted viruses with two primary envelope glycoproteins, Br20-4 and Eli, were relatively resistant to neutralization by the F105 (Br20-4) and 15e antibodies (Br20-4 and Eli) (Fig. 5). These results are consistent with those obtained with the unpelleted viruses complemented with these envelope glycoproteins.

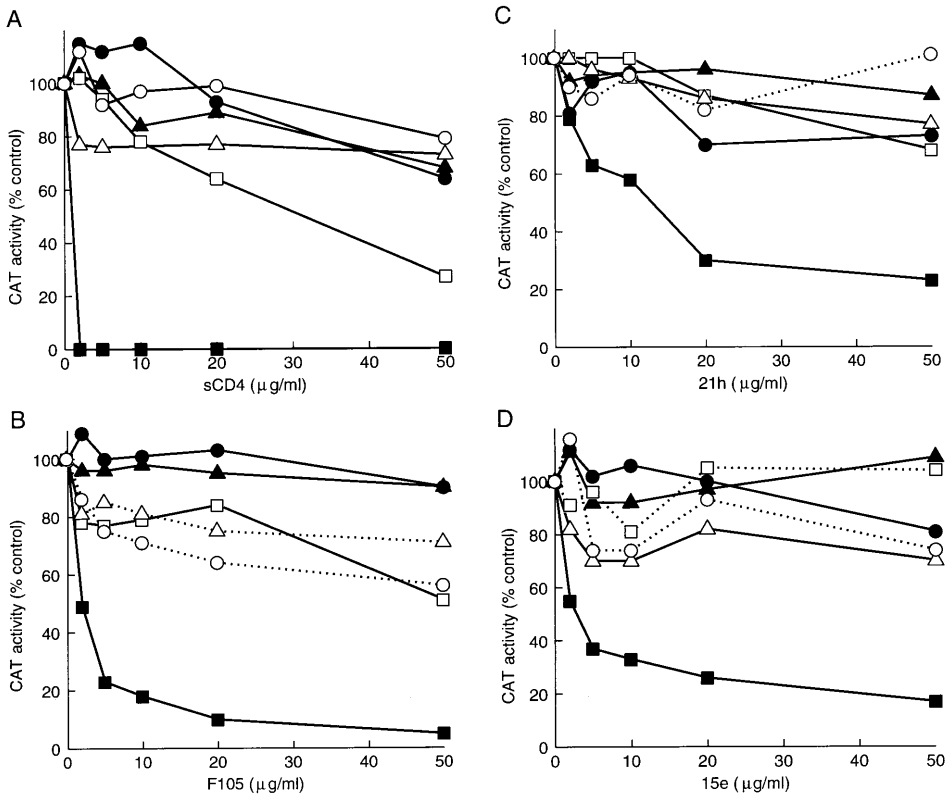

FIG. 4. Neutralization of recombinant viruses by sCD4 and MAbs. Recombinant viruses, carrying either HXBc2 or primary envelopes, were preincubated with the indicated concentrations of sCD4 (A), F105 (B), 21h (C), or 15e (D) for 2 h at 37°C. Human PBMCs were used as target cells, and the experiments were repeated two or three times to control for variability in donor PBMCs (graphs show representative experiments). Broken lines are used in cases where the envelope glycoproteins were not recognized by the MAb. Symbols: ■, HXBc2; □, Rw20-5 (clade A); ●, Br20-4 (clade B); ○, Br25-9 (clade C); ▲, Eli (clade D); △, TN243 (clade E).

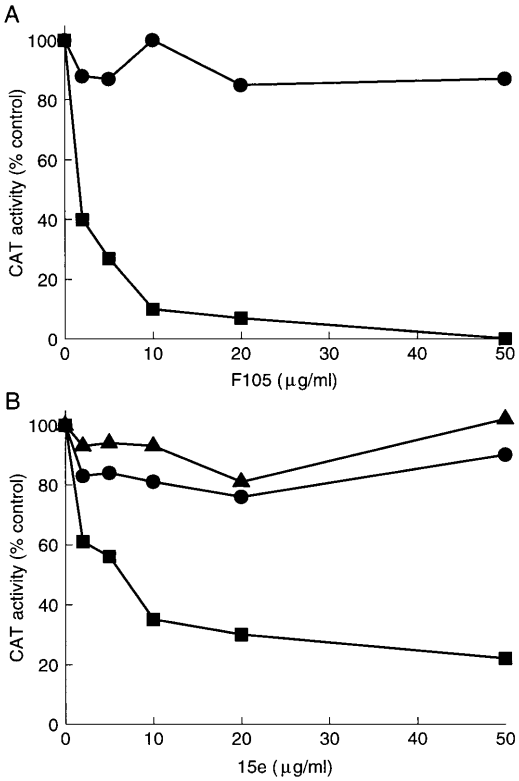

FIG. 5. Neutralization of virions following ultracentrifugation. Viral supernatants were placed on sucrose cushions and pelleted followed by resuspension into the original volume. Neutralization sensitivity was assayed by using the F105 (A) and 15e (B) MAbs, with human PBMCs as target cells. Symbols: ●, Br20-4; ■, HXBc2; ▲, Eli.

DISCUSSION

These studies document that insensitivity to neutralization is a property conferred on HIV-1 by envelope glycoproteins derived from primary viruses of diverse clades. In addition to other factors such as the accessibility of neutralization epitopes on the assembled oligomer of primary viruses (3, 32, 43), it has been suggested that the higher observed envelope glycoprotein density of primary HIV-1 isolates might contribute to this resistance. Our results indicate that envelope glycoproteins derived from primary HIV-1 isolates, even when present on virions at levels comparable to or lower than those of laboratory-adapted envelope glycoproteins, still render the virus relatively resistant to neutralization by sCD4 and MAbs. In addition, since the gp120/gp41 ratios on virions containing envelope glycoproteins from laboratory-passaged and primary viruses were similar, it is unlikely that major differences in the intrinsic stability of gp120-gp41 association exist between these groups of viruses. While primary virus envelope glycoproteins synthesized in contexts more natural than those used herein (e.g., in relevant host cell types or in the presence of homologous Gag proteins) might exhibit higher virion gp120 content, our results indicate that neither envelope glycoprotein incorporation nor stability plays a necessary role in specifying neutralization resistance. Differences in the density or stability of the envelope glycoproteins might be important for other properties of HIV-1 isolates such as those related to target cell tropism. Further understanding of the factors underlying primary HIV-1 resistance to neutralizing antibodies may aid in directing vaccine efforts appropriately.

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