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Neutralizing Antibodies against the V3 Loop of Human Immunodeficiency Virus Type 1 gp120 Block the CD4-Dependent and -Independent Binding of Virus to Cells

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The CD4 molecule is an essential receptor for human immunodeficiency virus type 1 (HIV-1) through high-affinity interactions with the viral external envelope glycoprotein gp120. Previously, neutralizing monoclonal antibodies (MAbs) specific to the third hypervariable domain of gp120 (the V3 loop) have been thought to block HIV infection without affecting the binding of HIV particles to CD4-expressing human cells. However, here we demonstrate that this conclusion was not correct and was due to the use of soluble gp120 instead of HIV particles. Indeed, neutralizing anti-V3 loop MAbs inhibited completely the binding and entry of HIV particles into CD4$^+$ human cells. In contrast, the binding of virus was only partially inhibited by neutralizing anti-CD4 MAbs against the gp120 binding site in CD4, which, like the anti-V3 loop MAbs, completely inhibited HIV entry and infection. Nonneutralizing control MAbs against either the V3 loop or the N or C terminus of gp120 had no significant effect on HIV binding and entry. HIV-1 particles were also found to bind human and murine cells expressing or not expressing the human CD4 molecule. Interestingly, the binding of HIV to CD4$^+$ murine cells was inhibited by both anti-V3 and anti-CD4 MAbs, whereas the binding to human and murine CD4$^-$ cells was affected only by anti-V3 loop MAbs. The effect of anti-V3 loop neutralizing MAbs on the HIV binding to cells appears not to be the direct consequence of gp120 shedding from HIV particles or of a decreased affinity of CD4 or gp120 for binding to its surface counterpart. Taken together, our results suggest the existence of CD4-dependent and -independent binding events involved in the attachment of HIV particles to cells; in both of these events, the V3 loop plays a critical role. As murine cells lack the specific cofactor CXCR4 for HIV-1 entry, other cell surface molecules besides CD4 might be implicated in stable binding of HIV particles to cells.

The CD4 molecule, expressed on the surfaces of T cells and macrophages, serves as a receptor of the human immunodeficiency virus (HIV) by interacting with the external envelope glycoprotein gp120 of HIV type 1 (HIV-1). The interaction between gp120 and CD4 is of high affinity, which under appropriate conditions can lead to virus and cell membrane fusion, thus allowing viral entry and initiation of the viral replicative cycle (12, 24; for a review, see reference 3).

In CD4$^-$ human cells, HIV-1 viral particles attach but productive infection either does not occur or happens with low efficiency. For such cells galactosyl ceramides have been described as potential receptors for viral particles (19). On the other hand, the expression of human CD4 in heterologous cells does not always render them permissive to HIV entry. The latter observation indicates that viral entry requires other species-specific cell surface molecules besides CD4. Recently the chemokine receptors CXCR4 and CCR5 have been described to be specific cofactors for the entry of T-cell-tropic and macrophage-tropic HIV-1 isolates, respectively (10, 14–18). The natural ligand of the CCR5 receptor, RANTES, has been shown to block HIV entry without affecting virus attachment to cells (43). Similarly, SDF-1 and MIP1α/β, which are the natural ligands of CXCR4 and CCR5, respectively, appear to interfere with a postbinding fusion step that leads to blockade of HIV infection (16, 40). Other cell surface components reported to be implicated directly or indirectly in the HIV attachment and/or entry process are tryptase TL_2 (39), CD26 (5, 42), heparan sulfates (44, 47), and adhesion molecules (22, 50).

gp120, which is highly N glycosylated, has a complex secondary structure in which five conserved regions (C1 to C5) and five hypervariable regions (V1 to V5) have been defined (51, 55). This complex structure is stabilized by several disulfide bonds occurring between conserved cysteine residues. A variety of antibodies have been used to map the gp120 epitopes involved in CD4 binding. Cumulative results indicate that epitopes at the C3 and C4 regions are important for the binding to CD4 (41), although other regions may contribute to the integrity of the CD4 binding site (3). It should be emphasized that the results obtained with soluble forms of gp120 should be considered cautiously, since gp120 presented by HIV particles probably has a much more complex structure due to its oligomeric nature and the fact that it is associated noncovalently with the transmembrane glycoprotein gp41 (38).

Consistent with the relevance of HIV binding to CD4 for viral infectivity, antibodies directed against the D1 domain of CD4, which impair binding of viral particles, completely abolish HIV infection, and thus they are considered neutralizing antibodies (3). In HIV-infected individuals, neutralizing immune responses reveal the existence of two major targets in gp120: the CD4 binding domain and the third hypervariable region, referred to as the V3 loop (37). Accordingly, antibodies directed against the conformational CD4 binding site in gp120

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prevent binding of virions to CD4 and abolish viral infection, whereas antibodies against the V3 loop prevent viral infection despite the fact that they do not inhibit the binding of soluble gp120 to CD4 (31). Although the mechanism by which these anti-V3 loop antibodies neutralize infection has not been completely elucidated, the lack of effect on soluble gp120 binding to CD4 suggested that they inhibit post-CD4-binding events, assuming that the V3 loop interacts with cell surface molecules other than CD4 (reviewed in reference 36). McDougal et al. (32) have recently studied the mechanisms of HIV-1 neutralization by antibody-positive serum from infected individuals and have suggested that the effect of neutralizing antibodies on HIV-1 infectivity may occur before or independently of viral attachment to $CD4^+$ cells.

The aim of this work was to characterize the effect of neutralizing monoclonal antibodies (MAbs) specific for either CD4 or the V3 loop on the process of HIV-1 binding to and entry into cells. The results demonstrate that anti-CD4 antibodies, which block binding of soluble gp120 to $CD4^+$ cells and prevent viral infection, do not completely block virus attachment to these cells. Similar results were obtained with neutralizing MAbs against the CD4 binding domain in gp120. Thus, in addition to the CD4 molecule, which is the functional site for viral entry, there should be CD4-independent binding sites for HIV-1 interaction with cells. Anti-V3 loop antibodies completely blocked virus binding to $CD4^+$ and $CD4^-$ cells, thus indicating that they affect both CD4-dependent and CD4-independent binding sites. Our results indicate that the V3 loop is essential for interaction of the HIV particle gp120 with CD4 and other cell surface components, suggesting the existence of V3 loop-dependent events necessary for optimal binding, and thus entry, of HIV-1 particles.

MATERIALS AND METHODS

Cells. CEM cells (clone 13) selected for the high expression of CD4 were obtained from L. Montagnier, Institut Pasteur, Paris, France. Chronically HIV-1-infected H9/IIIB cells were obtained from R. C. Gallo, National Institutes of Health, Bethesda, Md. The A2.01 CEM cell line, derived from CEM cells but lacking CD4 expression, was provided by R. P. Sekaly (Laboratoire d'Immunologie, IRCM, Montréal, Canada). C8166 cells (from G. Farrar) were generously provided by the Medical Research Council AIDS Directed Program Reagent Project, United Kingdom. U937 and Jurkat cells were routinely used in our laboratory. All these cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. The murine hybridoma T-cell line $58\alpha^-\beta^-$ and its clone T54S1 stably expressing human CD4 were kindly provided by U. Blank (Institut Pasteur) (2). These cells were cultured in Dulbecco's modified Eagle's medium (ICN) supplemented with 10% FCS and 2 mM glutamine.

Antibodies. A MAb specific to human CD4 and reacting with the gp120 binding site (clone CB-T4-2) was kindly provided by E. Bosmans (Eurogenetics, Tessenderlo, Belgium). Other MAbs specific to human CD4 and reacting with the gp120 binding site, MAbs OKT4A and Leu-3A, were purchased from Ortho Diagnostics Systems and Becton Dickinson, respectively. MAb N11-20 (also called 110-H) against the V3 loop of gp120 (amino acids 317 to 325 [GPGRAFVTI]), MAb 110-C against an epitope in gp120 corresponding to amino acids 282 to 284, MAb 110-D against an epitope situated at residues 381 to 394 and MAb 110-A reacting with a peptide comprising amino acids 314 to 318 (IQRGP) of gp120 and used for immunoblotting were provided by J. C. Mazie, Hybridolab, Institut Pasteur. Other anti-gp120 antibodies, MAb 110-4 recognizing an epitope located between amino acids 303 and 323 in the V3 loop and MAb 110-1 recognizing an epitope located between residues 489 and 511 of gp120 (31, 52), were obtained from Genetics Systems (Seattle, Wash.). MAb ADP390 against the CD4 binding domain in gp120 (from J. Cordell and C. Dean) was provided by the Medical Research Council AIDS Reagent Project (35); this MAb blocks the binding of gp120 to CD4 when used at high concentrations (11). MAb IgGb12, also directed against the CD4 binding site in gp120 but showing a higher neutralizing capacity, and its Fab fragment (1, 4) were kindly provided by Q. J. Sattentau (Centre d'Immunologie de Marseille-Luminy, Marseille, France). MAb V3-21 against the INCTRPN sequence at residues 298 to 304, containing the N-terminal end of the V3 loop, was obtained from J. Laman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (27). When necessary, purified preparations of these antibodies (purified by the Ultralink immobilized protein G method [Pierce]) were obtained for use in the assays.

Viral preparations. A highly infectious preparation of HIV-1 was generated by several consecutive passages of the original HIV-1 Lai isolate on CEM cells in culture. For the preparation of an HIV-1 stock, CEM cells were infected with one synchronous dose of HIV-1 Lai, and the culture supernatants (after centrifugation at $5,000 \times g$ for 10 min) were recovered at 3 to 4 days postinfection, when cells were still viable, and stored at −135°C (30). Prior to use, freshly thawed aliquots were filtered through 0.22-μm-pore-size filters in order to eliminate aggregates and/or cell debris.

HIV binding, entry, and infection. HIV entry into permissive cells was monitored by measuring the intracellular concentration of p24 after digestion of extracellular bound virus by trypsin treatment. For this purpose, after incubation with HIV, cells were washed to remove unbound virus before treatment (or not) with trypsin. Cells were then extracted, and the concentration of p24 was measured. Thus, in the absence of trypsin treatment, the concentration of p24 represents both the virus which is still bound on the cell surface and the virus which has already entered into cells, and this is referred to as virus associated with cells. Accordingly, viral association (binding and entry) with CEM cells was studied by incubating 6×10^6 cells in the presence of HIV-1 Lai (25 ng of p24) for 1 h at 37°C in FCS-supplemented RPMI 1640 medium (final volume, 1 ml). When indicated, viral preparations were incubated prior to the binding in the presence of anti-gp120 antibodies, while cells were incubated in the presence of the anti-CD4 MAbs. After incubation of cells with HIV, cells were washed once with phosphate-buffered saline containing 2 mM EDTA and twice with supplemented RPMI. Then, 5×10^6 cells were lysed with 100 μl of E buffer (20 mM Tris HCl [pH 7.6], 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml, 0.5% Triton X-100, and 7 mM 2-mercaptoethanol); the lysates were centrifuged ($350 \times g$ for 5 min, at 4°C) in order to eliminate nuclei, and supernatants were assayed for the content of HIV-1 major core protein p24 with an HIV-1 p24 core profile enzyme-linked immunosorbent assay (ELISA) kit (DuPont). Thus, the amount of p24 in such cell extracts can be defined as the total virus associated with cells, since it represents both virus bound to cells and virus that has entered into cells. In experiments designed to estimate viral entry, after the first wash with phosphate-buffered saline–EDTA, cells were treated with 2.5 mg of trypsin per ml for 5 min at room temperature to eliminate extracellular bound virus. After three washes with culture medium, cell extracts were prepared and the concentration of intracellular p24 was measured (25). The strategy used for determination of HIV-1 Lai binding allows specific detection of viral particles, avoiding interference with soluble gp120. Furthermore, background levels associated with the binding of soluble p24 are undetectable, as could be illustrated by the complete blockade of HIV binding (monitored by the p24 concentration) observed in the presence of neutralizing anti-V3 loop MAbs (Fig. 1).

When infection studies were performed in parallel with the association and entry assays, cells were incubated as described above and an aliquot of washed cells (10^6) was cultured for 1 week (by passing twice at days 3 and 5) in order to monitor HIV production by measuring the concentration of p24 in culture supernatants.

^{125}I-labeled gp120 and ^{125}I-labeled sCD4 binding. Recombinant soluble gp120-IIIB (or Lai) produced by the baculovirus expression system and soluble CD4 (sCD4) (both from Neosystem Laboratories) were iodinated by using the Bolton-Hunter reagent (DuPont NEN) as described before (54). For the gp120 binding to $CD4^+$ cells, CEM cells (10^6) were preincubated (37°C, 15 min) in the absence or presence of anti-CD4 neutralizing antibodies before further incubation (37°C, 30 min) with ^{125}I-labeled gp120 (1 nM) which had also been preincubated (37°C, 15 min) with or without anti-gp120 MAbs. The binding of sCD4 to membrane-expressed gp120 was performed with chronically infected H9/IIIB cells, which express the HIV-1 envelope gp120-gp41 complex on the surface (23). To investigate the latter binding, H9/IIIB cells (10^6) were incubated (37°C, 30 min) with ^{125}I-labeled sCD4 (1 nM) which had been preincubated (37°C, 15 min) or not with anti-CD4 MAb. All binding experiments were carried out in unsupplemented RPMI 1640 medium in a total reaction volume of 200 μl. Cells were washed three times with ice-cold RPMI 1640 before lysis in buffer E as described above; such nucleus-free lysates were diluted with 1 volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue) and analyzed by electrophoresis according to the method of Laemmli (26). Following SDS-PAGE analysis, gels were fixed and dried and radioactivity associated with labeled gp120 or sCD4 bands was quantified in a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Analysis of gp120 shedding from HIV particles. Aliquots of an HIV-1 Lai stock, containing 50 ng of p24, were incubated in 1 ml of supplemented RPMI 1640 medium in the absence or in the presence of different anti-gp120 antibodies or sCD4 (Neosystem Laboratories) for 1 h at 37°C. The different suspensions were then ultracentrifuged ($100,000 \times g$ for 1 h at 4°C), and viral pellets and supernatants were assayed for their gp120 content by ELISA with MAb 110-4. The quantitation of gp120 was also carried out by immunoblotting as described previously (54).

Analysis of MAb binding to cells and HIV particles. MAb binding to the surfaces of chronically infected H9/IIIB cells or uninfected H9 cells was determined by fluorescence-activated cell sorter analysis as described previously (54).

TABLE 1. Inhibition of HIV-1 Lai infection by different MAbs[a]

Reagent	Concn (μg/ml)	p24 (ng/ml)[b]	% Inhibition of HIV production
None (control)		724 ± 10	0
AZT[c]		17 ± 4	97
CB-T4	0.1	675 ± 94	7
	1	<0.01	>99
	10	<0.01	>99
110-4	0.1	751 ± 3	0
	1	266 ± 148	64
	5	0.19 ± 0.01	>99
N11-20	0.1	685 ± 55	6
	1	161 ± 89	78
	5	<0.01	>99

[a] Cells incubated with virus and the different reagents were washed and then cultured (300,000 cells/ml) in supplemented RPMI 1640 medium as described in Materials and Methods. At day 7 postinfection, the concentration of p24 in the culture supernatant was determined.

[b] Results are means ± standard deviations from a representative experiment performed in triplicate.

[c] AZT was added at a concentration of 5 μM.

The amount of MAb bound to viral particles was also monitored in order to demonstrate the ability of neutralizing MAbs to bind to gp120 expressed on HIV particles. Briefly, extracts of viral pellets containing gp120-MAb immunocomplexes were captured on sCD4 (100 ng/well)-coated plates, and bound MAb was revealed by using horseradish peroxidase-coupled goat antimouse antibody and *o*-phenylenediamine as a substrate.

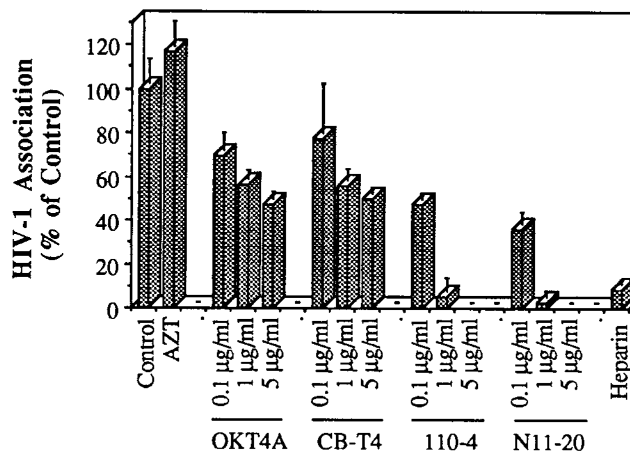

FIG. 1. Effect of anti-V3 loop and anti-CD4 MAbs on HIV-1 association (attachment plus entry) with CEM cells. AZT (5 μM), heparin (5 μg/ml), and different MAbs were assayed for their abilities to inhibit the association (attachment and entry) of HIV-1 to CEM cells. For some samples, cells were preincubated (15 min, 37°C) in culture medium with or without anti-CD4 MAbs OKT4A and CB-T4 at the indicated concentrations before the addition of virus. For other samples, the viral stock (HIV-1 Lai) was preincubated (15 min, 37°C) in culture medium with or without the anti-V3 loop MAbs (110-4 and N11-20) or heparin at the indicated concentrations. Incubation of cells with HIV-1 was performed for 1 h at 37°C. After processing of the samples as indicated in Materials and Methods, p24 core protein levels in cell lysates were determined. The control association was 450 ± 60 pg of p24/10^7 cells. Values are means ± standard errors of the means from a representative experiment performed in triplicate. Under these experimental conditions, the control MAb 71/10 (at 5 μg/ml), specific for the interferon-induced protein kinase PKR (29), had no effect.

RESULTS

Inhibition of the association of HIV-1 particles by anti-CD4 and anti-V3 loop MAbs. We first investigated the effect of different neutralizing MAbs against human CD4 and against the V3 loop of HIV-1 gp120 on virus infection. To confirm the neutralizing activity of these antibodies, CEM cells were infected in the presence of different concentrations of anti-CD4 and anti-V3 MAbs. Zidovudine (AZT) was used as a control to demonstrate the severe impairment of viral replication by this inhibitor of reverse transcriptase (Table 1). At day 7 postchallenge, infection was completely prevented in cells treated with the anti-CD4 MAb CB-T4 at concentrations equal to or higher than 1 μg/ml. With the two anti-V3 loop MAbs at a concentration of 1 μg/ml, infection was markedly impaired, more strongly in the case of MAb N11-20 than in the case of MAb 110-4. For these antibodies a complete block of virus infection occurred at a concentration of 5 μg/ml (Table 1).

We then investigated the effect of AZT and the different MAbs on the association of HIV-1 Lai with CEM cells. These experiments were carried out at 37°C rather than 4°C, in order to mimic the physiological conditions of virus infection. For this purpose cells were incubated with HIV-1 particles in the presence or absence of the different reagents; after 1 h of incubation and three washes, cells were lysed and the content of p24 in the postnuclear lysate was measured. This was defined as HIV-1 association with CEM cells, which represented the sum of virus that had entered into cells and virus still attached on the cell surface. AZT did not affect HIV association, whereas two different antibodies against the CD4 molecule, OKT4A and CB-T4, partially inhibited association in a dose-dependent manner (Fig. 1). At 0.1, 1, and 10 μg of MAb CB-T4 per ml, inhibition of viral association with CEM cells with respect to control values was 23, 42, and 50%, respectively (Fig. 1). Interestingly, the two antibodies against the V3 loop tested, 110-4 and N11-20, were more efficient inhibitors of HIV-1 association with CEM cells; both MAbs inhibited the process of binding plus entry by more than 95% at concentrations of 1 μg/ml or higher (Fig. 1). These results demonstrated that although 1 μg of anti-CD4 MAb CB-T4 per ml inhibited virus infection completely (Table 1), under the same conditions it inhibited virus attachment to cells by less than 50% (Fig. 1). These observations indicate the existence on the cell surface of CD4-independent binding sites, which are V3 loop related and are not sufficient for entry of infectious virus. In addition to the neutralizing anti-V3 loop MAbs, heparin at 5 μg/ml inhibited HIV-1 Lai association with CEM cells by more than 90% (Fig. 1). Heparin is a polyanion which inhibits HIV entry (25), probably by interacting with the V3 loop of gp120 (21).

A similar set of experiments was performed with other $CD4^+$ cell lines, such as Jurkat and U937 cells and phytohemagglutinin-activated peripheral blood mononuclear cells. The neutralizing anti-V3 loop MAb 110-4 at 5 μg/ml inhibited viral association with the different human cell types by more than 80% (not shown). Thus, these results indicate that irrespective of the cellular context, the binding of HIV particles to $CD4^+$ cells is dependent on the integrity of the V3 loop.

Effect of antibodies on HIV binding and entry to CEM cells. To distinguish whether the antibodies were inhibiting attachment or entry, experiments were carried out in parallel to assess viral association with and entry into CEM cells. Viral entry was monitored by measuring the intracellular p24 in trypsin-treated cells. AZT, which had no effect on the global association of HIV with CEM cells, did not affect entry, in accord with its efficiency as inhibitor of retrotranscription of viral RNA. Consistent with the fact that neutralizing MAbs against the V3 loop blocked viral association with cells (Fig. 1

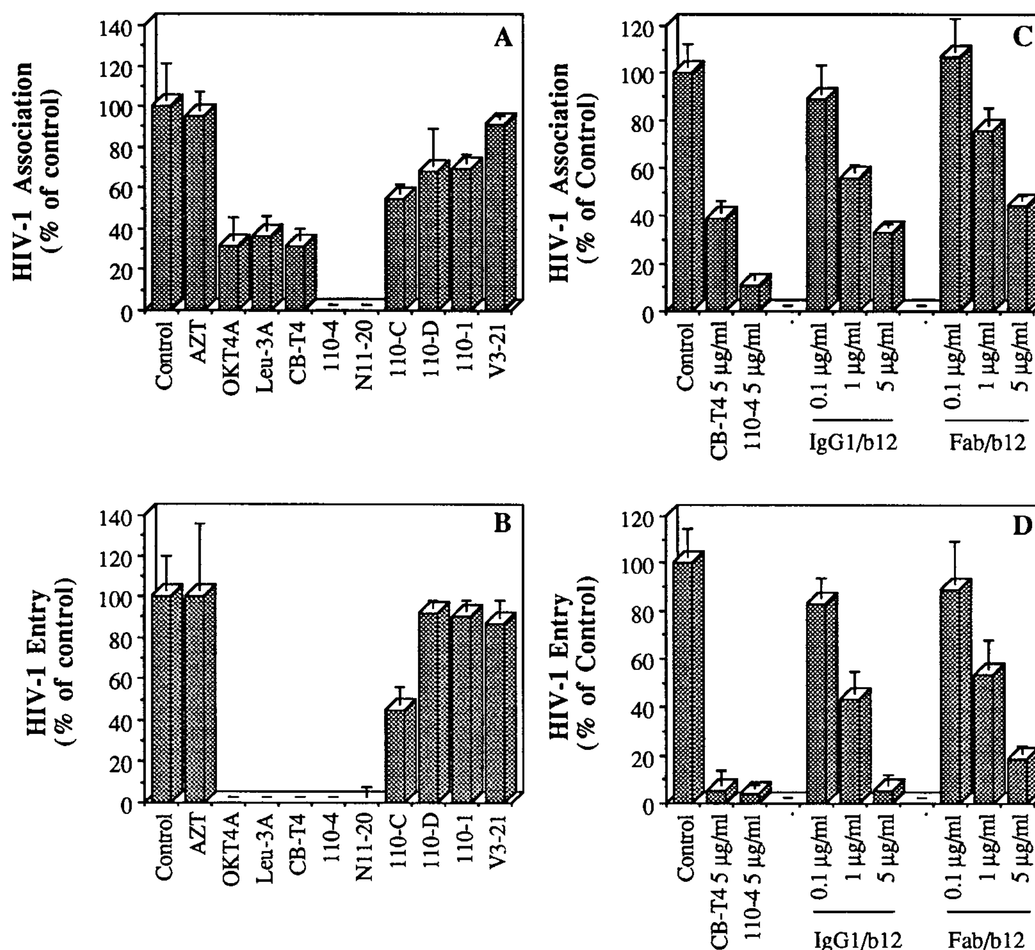

FIG. 2. Effect of anti-gp120 and anti-CD4 MAbs on HIV-1 association with and entry into CEM cells. AZT (5 μM) and different MAbs were assayed for their abilities to reverse HIV-1 association with and entry into CEM cells. (A and C) Effect on total association of HIV particles, with the procedure described in the legend to Fig. 1. (B and D) Effect on viral entry. In this case, after 1 h of incubation, cells were washed and treated with trypsin to remove viral particles attached to the cell surface (see Materials and Methods). The concentration of the different MAbs was 5 μg/ml or as indicated. Viral content was assessed by the quantity of p24 in cell lysates. Control association or entry values (in picograms of p24/10^7 cells) were 360 ± 71 for panel A, 180 ± 35 for panel B, 412 ± 50 for panel C, and 240 ± 27 for panel D. The experiments for panels A and B and for panels C and D were performed at different times, and for this reason the control values are slightly different. Values are means ± standard errors of the means from a representative experiment performed in triplicate.

and 2A), no p24 protein was detected inside the cells when the experiments were performed in the presence of 5 μg of MAb 110-4 or N11-20 per ml (Fig. 2B). Interestingly, although three different neutralizing MAbs against CD4 only partially inhibited viral attachment, they blocked entry completely, since it was not possible to detect intracellular p24 when cells were incubated with these antibodies (Fig. 1 and 2). These results suggest that the CD4-independent HIV binding sites on the cell surface are not sufficient to lead to detectable virus entry.

By considering the values of p24 obtained for viral association and entry (Fig. 1 and 2), we can conclude that neutralizing anti-CD4 MAbs reduce by only about 50% the amount of HIV which becomes associated with cells. Therefore, the other 50% associated with cells represents the amount of CD4-independent binding of HIV to cells, which is not functional for viral entry. A similar conclusion was obtained by using the neutralizing MAb IgG1b12 and its Fab fragment (33), which are directed against the CD4 binding site in gp120 (1, 4). Both of these preparations of MAb b12 inhibited the association of HIV with CEM cells in a dose-dependent manner. With these antibodies at 5 μg/ml, the inhibition was to an extent similar to that observed with neutralizing anti-CD4 MAbs (Fig. 2C). Similarly, HIV entry was inhibited by both IgG1b12 and Fab b12 in a dose-dependent manner, and at 5 μg/ml the viral entry was inhibited by more than 90 and 75%, respectively (Fig. 2D). The slightly lower efficacy of Fab b12 in inhibiting HIV entry was also correlated with the lower efficacy of this preparation in inhibiting HIV infection, thus indicating that the Fab fragment manifests lower activity than IgG1b12. It should be noted that like neutralizing anti-CD4 MAbs but unlike neutralizing anti-V3 loop MAbs, both IgG1b12 and Fab b12 did not affect the CD4-independent binding of HIV (Fig. 2). Therefore, antibodies directed against the CD4 binding domain in gp120 do not interfere with the CD4-independent binding of HIV particles to the cell surface. Another antibody directed against the CD4 binding domain in gp120, MAb ADP390, manifested only a slight effect on HIV binding and entry when used at 5 μg/ml (not shown). This was probably due to the lower affinity of MAb ADP390 for binding virus-expressed gp120 (11, 35).

In HIV entry experiments, we also tested a set of nonneutralizing MAbs directed against different regions of gp120 (see Materials and Methods): MAb 110-1 against the C-terminal

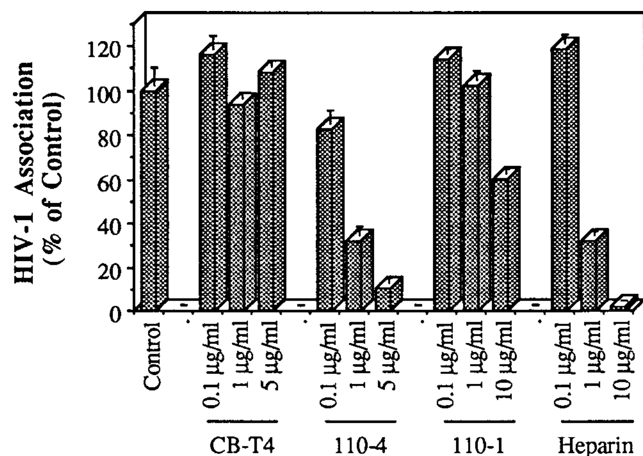

FIG. 3. Characterization of CD4-independent binding to A2.01 cells. Human A2.01 cells, which do not express CD4, were tested for their ability to bind HIV particles in the presence of the indicated concentrations of anti-CD4 MAb CB-T4, anti-V3 loop MAb 110-4, anti-gp120 MAb 110-1, and heparin. Cells were processed as described in Materials and Methods, and p24 in cell lysates was assayed. The control association value was 312 ± 27 pg of p24/10^7 cells. Values are means ± standard errors of the means from a representative experiment performed in triplicate.

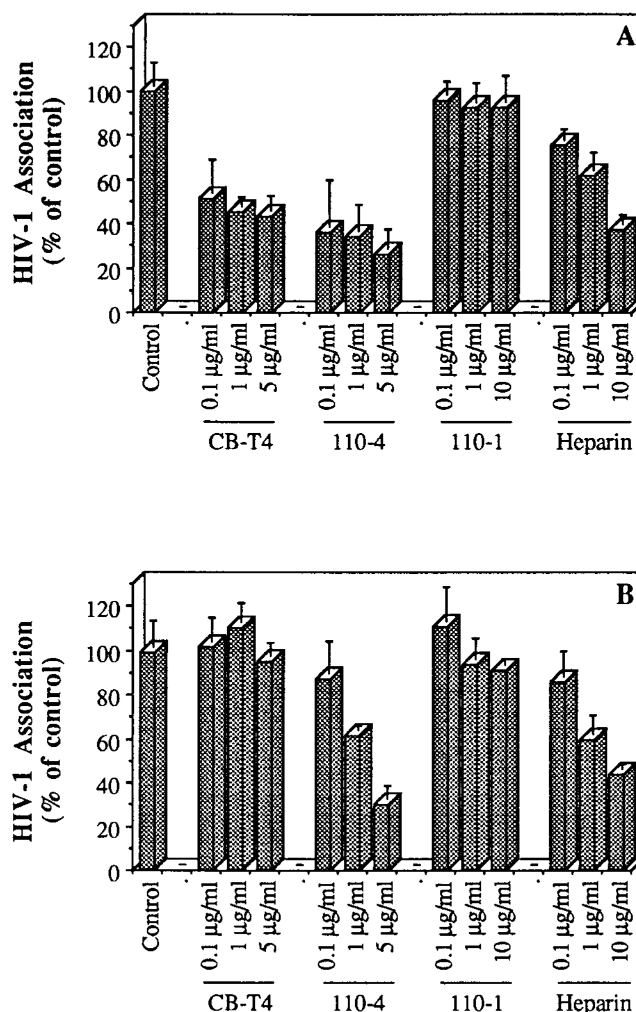

FIG. 4. Effect of neutralizing anti-V3 loop and anti-CD4 MAbs on HIV-1 Lai binding to murine cells. Murine T54S1 cells expressing human CD4 (A) and the parental control $58\alpha^-\beta^-$ cells lacking human CD4 (B) were tested for their abilities to bind HIV particles in the presence of the indicated concentrations of anti-CD4 MAb CB-T4, anti-V3 loop MAb 110-4, anti-gp120 MAb 110-1, and heparin. Cells were processed as described in Materials and Methods, and p24 in cell lysates was assayed. Control association values were 152 ± 14 and 148 ± 26 pg of p24 in 10^7 $CD4^+$ and $CD4^-$ cells for panels A and B, respectively. Values are means ± standard errors of the means from a representative experiment performed in triplicate.

region of gp120, MAb V3-21 recognizing the N-terminal end of the V3 loop, MAb 110-D against residues 381 to 394 of gp120, and MAb 110-C against the N-terminal region of gp120. When these antibodies were used at 5 µg/ml, except for MAb 110-C, none of the antibodies resulted in a dramatic effect on HIV entry into and association with cells as was observed with MAbs Leu-3A, CB-T4, OKT4A, 110-4, and N11-20 (Fig. 2). MAb 110-C mediated only a slight decrease in HIV-1 attachment and entry.

All the antibodies used in these experiments were assayed for their capacities to inhibit HIV infection. Consequently, the concentration of neutralizing antibodies was selected according to their capacities to inhibit significantly HIV infection (Table 1). For example, at 1 µg/ml the neutralizing anti-CD4 and anti-V3 loop antibodies resulted in more than 50% inhibition of HIV infection, and therefore we used them at 1 and 5 µg/ml. The concentrations of the other antibodies, which were nonneutralizing, were selected according to their titers against soluble gp120 obtained by ELISA. Interestingly, with the different nonneutralizing MAbs used in our assays (MAbs 110-C, 110-D, 110-1, and V3-21) at 1 µg/ml, titers similar to those obtained with neutralizing anti-V3 loop antibodies at 1 µg/ml were observed. For these reasons, the antibodies were used at 5 µg/ml.

Effect of antibodies on binding of HIV-1 to human $CD4^-$ cells. In order to confirm the existence of CD4-independent HIV binding sites on the cell surface, we studied the viral association with a CEM-derived cell line which lacks CD4 expression, the A2.01 cell line. As shown in Fig. 3, HIV-1 Lai particles were found to bind A2.01 cells. Interestingly, the absolute value of association to $CD4^-$ CEM cells was somewhat comparable to that observed with $CD4^+$ CEM cells. As expected, the CD4-independent association of HIV with A2.01 cells was not affected by the anti-CD4 MAb CB-T4, whereas the neutralizing MAb 110-4 against the V3 loop and heparin interfered with viral association in a dose-dependent manner, inhibiting more than 90% at 5 and 10 µg/ml, respectively. In contrast, the nonneutralizing anti-gp120 MAb 110-1 (52) showed only a partial inhibitory effect only at the highest concentration tested (10 µg/ml). These results confirm the existence of CD4-independent binding sites for HIV-1 Lai on the surfaces of human cells and indicate their direct relationship to the V3 loop of the virus-expressed gp120 (Fig. 3). Consistent with the hypothesis that the CD4-independent binding is not sufficient for HIV entry, no viral production was detectable in the $CD4^-$ A2.01 cells (not shown).

Effect of antibodies on binding of HIV-1 to murine cells expressing or not expressing human CD4. In order to further characterize the binding of HIV-1 particles to cell surface-expressed human CD4, the effect of neutralizing antibodies was also investigated with murine cells expressing (T54S1) or not expressing ($58\alpha^-\beta^-$) the human CD4 molecule (Fig. 4). Binding of HIV to murine cells stably expressing human CD4 was inhibited by MAb CB-T4 (60%) and also by MAb 110-4 (75%) and heparin (65%). In the case of the parental murine cell line, HIV binding was significantly inhibited only by MAb

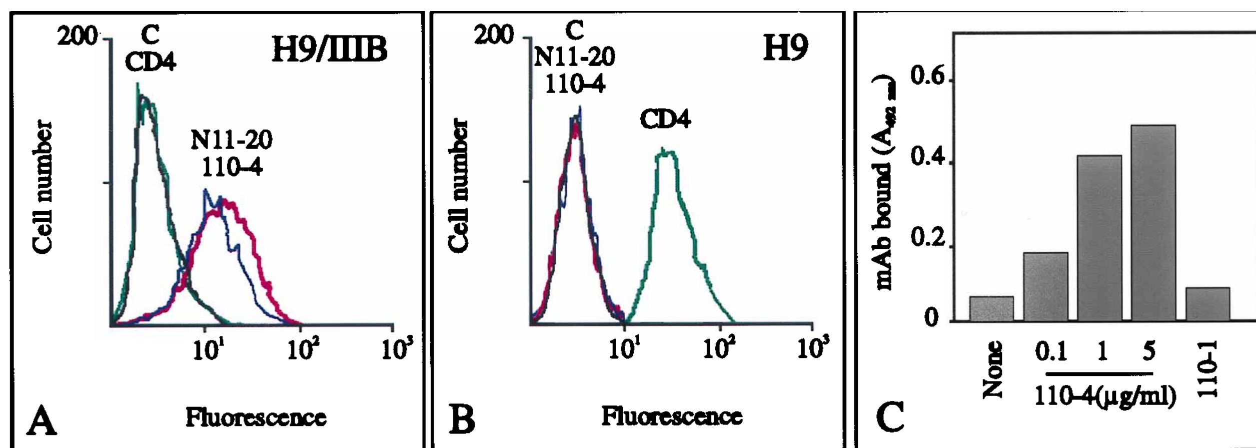

FIG. 5. Specific binding of neutralizing anti-V3 loop antibodies to cell surface and virus-expressed gp120. (A and B) Chronically infected H9/IIIB (A) and uninfected H9 (B) cells were assayed for their abilities to bind neutralizing anti-V3 loop MAbs 110-4 and N11-20 or the anti-CD4 MAb CB-T4 (each at 1 μg/ml). After two washes, cells were incubated with a fluorescein isothiocyanate-coupled goat antimouse antibody, washed twice, and analyzed as described before (54) with a FACScan flow cytometer (Beckton Dickinson, Mountain View, Calif.). The lack of CD4 expression in chronically infected H9/IIIB cells is due to severe down regulation of CD4 in such infected cells (23). (C) HIV-1 viral particles were incubated with the neutralizing anti-V3 loop MAb 110-4 (at the indicated concentrations) or with the nonneutralizing MAb 110-1 against gp120 (at 5 μg/ml) for 1 h at 37°C. Viral suspensions were then ultracentrifuged, and the MAb content in the viral pellet was determined by ELISA (see Materials and Methods). Results from a representative experiment are shown.

110-4 (75%) and heparin (60%), whereas MAb CB-T4 had no effect, consistent with the lack of human CD4 with which the CB-T4 MAb would interact. In both cell types, the nonneutralizing anti-gp120 MAb 110-1 had no effect on HIV association, even at 10 μg/ml. It should be noted that in this heterologous model, the maximum inhibitory effect of the anti-V3 loop MAb 110-4 did not exceed 75%, whereas in human CEM cells, the effect was consistently at least 90% (Fig. 4). This is probably due to additional interactions independent of the V3 loop, which might occur in certain types of cells. Despite the ability to interact with the cell surface, HIV-1 particles are not able to enter murine cells, even those transfected with human CD4, due to the fact that they lack the specific cofactor, CXCR4, necessary for HIV-1 entry (18).

Mechanism of HIV neutralization by anti-V3 loop antibodies: anti-V3 loop MAbs specifically interact with virion gp120 but not with cell surface components. In an attempt to understand the mechanism by which neutralizing anti-V3 loop antibodies block the binding of HIV to cells, we first investigated the affinity of these antibodies to react specifically with cell surface-expressed gp120. For this purpose, we used chronically infected H9 cells, which continuously express the gp120-gp41 complex on their surface. Fluorescence-activated cell sorter analysis with uninfected H9 cells and chronically infected H9/IIIB cells showed that neutralizing anti-V3 loop MAbs manifest no apparent reactivity with cell surface components other than gp120; i.e., they react with chronically infected H9/IIIB cells but not with uninfected H9 control cells (Fig. 5A and B). It should be noted that the expression of CD4 was not detectable in the chronically infected H9/IIIB cells (Fig. 5A), due to the previously reported down regulation of CD4 expression in such HIV-producing cells (23). Second, we studied the binding of these antibodies to viral particles in an assay in which HIV-1 Lai was incubated with the antibodies prior to pelleting by centrifugation. The results demonstrated that the neutralizing anti-V3 loop MAb 110-4 was recovered with the viral pellet, whereas the nonneutralizing mAb 110-1, specific to gp120, was not (Fig. 5C), thus providing evidence that the neutralizing anti-V3 loop MAb indeed binds HIV-1 particles. Consequently, the neutralization of HIV by the anti-V3 loop antibodies should occur prior to the virus binding step. These observations are consistent with the hypothesis that the neutralizing effect of anti-V3 loop antibodies is a direct consequence of their ability to bind HIV-1 particles.

sCD4, but not anti-V3 loop MAbs, causes a significant degree of gp120 shedding from HIV-1 particles. To investigate whether the effects of neutralizing anti-V3 MAbs on binding and entry of HIV were due to a possible release of gp120 from virions (shedding effect), HIV-1 particles were incubated (1 h, 37°C) with different concentrations of MAb 110-4, and the amount of gp120 shedding was estimated by monitoring gp120 content in viral pellet and viral supernatant fractions. In the absence of MAbs, gp120 was found to be partially released from viral particles; however, such a spontaneous release was estimated to be less than 15% of the total gp120 in the HIV-1 preparation used. sCD4 was used as a positive control since it is known to cause the shedding of gp120 (46). Consistent with this, more than 50% of gp120 shedding occurred in the presence of 10 μg of sCD4 per ml (Table 2). On the other hand, the anti-V3 loop MAb 110-4 led to a dose-dependent, but only a

TABLE 2. Release of gp120 induced by anti-gp120 MAbs and sCD4[a]

Reagent	Concn (μg/ml)	% of gp120[b] (mean ± SD) in:	
		Viral supernatant	Viral pellet
None[c]		13 ± 2	92 ± 2
sCD4	10	55 ± 4	41 ± 10
110-4	0.1	10 ± 4	91 ± 7
	1	20 ± 6	88 ± 12
	5	22 ± 7	84 ± 12
110-1	5	15 ± 2	92 ± 10

[a] Aliquots of HIV-1 Lai stock were treated as indicated for 1 h at 37°C. After centrifugation, viral supernatants and pellets were assayed for their gp120 content as described in Materials and Methods.
[b] A value of 100% is defined as the total content of gp120 in the virus stock and was calculated from a viral stock lysate as described previously (54).
[c] Spontaneous shedding.

slight degree of, gp120 shedding from HIV-1 Lai particles, even at 5 μg/ml, which causes more than 99% inhibition of HIV infection (Tables 1 and 2). The control antibody MAb 110-1 did not lead to shedding of gp120 above the levels found for spontaneous shedding (Table 2). Consequently, these results suggest that the slight shedding caused by MAb 110-4 cannot be considered as a major mechanism to account for the neutralization effect of this anti-V3 loop antibody.

Effect of antibodies on binding of soluble gp120 to cells or on binding of sCD4 to membrane-expressed gp120. Binding of ^{125}I-labeled recombinant soluble gp120 IIIB to CEM cells was measured in the presence or absence of the different MAbs against CD4 and gp120. Binding was completely blocked by the anti-CD4 MAb, whereas three different anti-gp120 MAbs, two neutralizing (110-4 and N11-20) and one nonneutralizing (110-D), did not diminish but instead increased the binding of gp120 to cells (Fig. 6A). Conformational changes induced by these MAbs may result in an overall increase of the affinity of the interaction between soluble gp120 and CD4, as has been previously described by others (45). These results confirm the differences between soluble and virus-expressed gp120 and provide evidence that the anti-V3 MAbs used here behave like the MAbs reported previously; i.e., they do not inhibit the binding of soluble gp120 to CD4.

Analogously, the effects of different antibodies against gp120 on the binding of sCD4 to the gp120-gp41 complex expressed on the surfaces of chronically infected H9/IIIB cells were tested, in order to determine the integrity of CD4 binding domain of gp120 in the presence of neutralizing anti-V3 loop antibodies. None of the anti-gp120 MAbs was able to modify significantly the binding of sCD4 to the HIV-1 envelope glycoprotein complex, whereas MAb CB-T4 completely prevented its binding (Fig. 6B), thus suggesting that the CD4 binding site is not modified by conformational changes induced by anti-V3 loop MAbs binding to gp120.

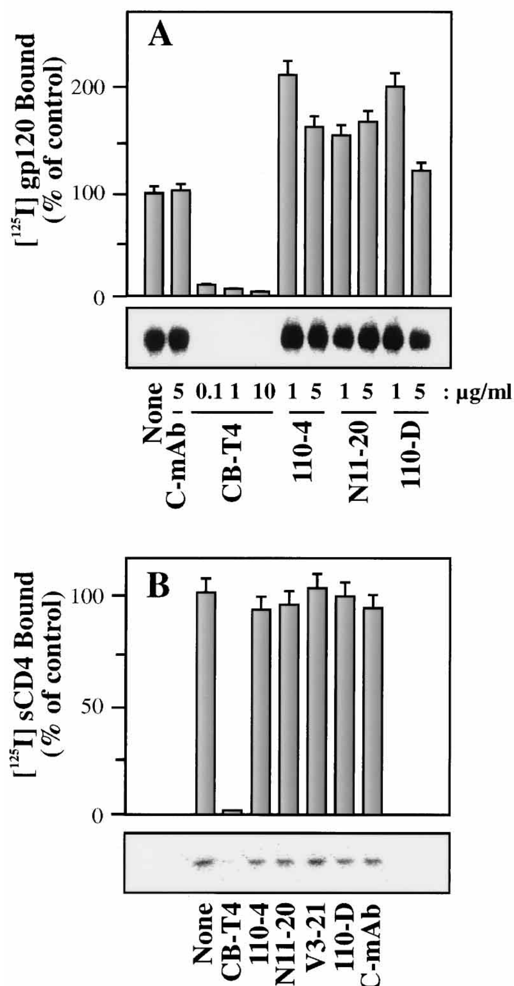

FIG. 6. Binding of ^{125}I-labeled soluble gp120 to CEM cells and of ^{125}I-labeled sCD4 to membrane-expressed gp120. (A) The binding of 1 nM ^{125}I-labeled gp120 to $CD4^+$ CEM cells was assayed by SDS-PAGE as described in Materials and Methods. Prior to the addition of ^{125}I-labeled gp120, cells were incubated (15 min, 37°C) with the indicated concentrations of MAb CB-T4. ^{125}I-labeled gp120 was also incubated in unsupplemented RPMI 1640 medium alone (lane None) or containing the indicated concentrations of different anti-gp120 MAbs. Results of a typical experiment are presented, with a section of the autoradiogram showing the labeled 110-kDa band corresponding to cell-bound ^{125}I-labeled gp120. The histograms give a quantification of the associated radioactivity (means ± standard errors of the means from triplicate samples). (B) The binding of 1 nM ^{125}I-labeled sCD4 to the gp120-gp41 complex expressed on the cell surfaces of chronically infected H9/IIIB cells was assayed by SDS-PAGE as described in Materials and Methods. In this case, prior to binding cells were incubated (15 min, 37°C) with 5 μg of the indicated anti-gp120 MAbs per ml, and sCD4 was incubated with 5 μg of MAb CB-T4 per ml. Results of a typical experiment are presented, with a section of the autoradiogram showing the 50-kDa labeled band corresponding to cell-bound ^{125}I-labeled sCD4. The histograms give a quantification of the associated radioactivity (means ± standard errors of the means from triplicate samples). Lanes C-mAb, irrelevant antibody (at 5 μg/ml) specific for the protein kinase PKR (29) used as a control.

DISCUSSION

The results presented here suggest the existence of two distinct binding events, CD4 dependent and CD4 independent, responsible for the gp120-mediated HIV-1 attachment to the cell surface. Neutralizing anti-V3 MAbs block both interactions, whereas neutralizing anti-CD4 MAbs block only the CD4-dependent interaction. Nevertheless, the lack of viral replication in the presence of anti-CD4 MAbs emphasizes the essential role of CD4 in the HIV entry process. The portion of CD4-independent HIV attached to cells does not progress towards detectable viral entry and infection. It should be noted that in different experimental models, neutralizing anti-V3 loop MAbs fail to inhibit the binding of sCD4 to cell surface-expressed gp120 or the binding of soluble gp120 to cell surface-expressed CD4. Therefore, the binding of soluble gp120 or sCD4 to their targets, expressed as components of the cell membrane, is qualitatively different from the interaction established between the cell surface CD4 and gp120 molecules exposed on the surface of viral particles. It is well known that there are conformational differences between soluble gp120 and the gp120 complexed to the HIV-1 envelope transmembrane glycoprotein gp41 on the surface of HIV-1 particles. For example, some of the domains which are exposed in the soluble molecule are hidden in the viral particle and vice versa (reviewed in reference 48). Probably the high conformational flexibility of gp120 in solution (36) is compromised on the surface of the viral particle, where gp120 molecules are tightly packed together in an oligomeric form. Our results demonstrate that under these conditions, neutralizing MAbs against the crown of the V3 loop bind HIV particles and completely prevent their association with the cell surface, thus accounting for the neutralizing capacity of these antibodies. This should be the consequence of the inhibition of the gp120 interaction with CD4 rather than inhibition of a post-CD4-binding event, which was the mechanism previously accepted. Interestingly, by using a different experimental approach, Sattentau and coworkers have also concluded that neutralizing anti-V3 MAbs block the

binding of HIV-1 particles to $CD4^+$ cells (49). In contrast to neutralizing antibodies, nonneutralizing MAbs specific for gp120 might display reduced affinity for the virus-expressed gp120, and consequently, such MAbs do not affect virus infection because of their failure to bind virus particles.

Recently, it has been reported that a variety of MAbs directed against different regions of gp120, including the V3 loop, induce gp120 dissociation from viral particles and thus could contribute to the neutralizing capacity of some anti-V3 loop antibodies (46). However, it should be noted that this effect was observed at concentrations much higher than those which interfere with HIV binding and infection. Accordingly, under our experimental conditions, different anti-V3 loop MAbs which at 5 μg/ml completely inhibited HIV binding and infection did not trigger significant shedding of gp120 molecules from HIV particles (Tables 1 and 2). In other recent work, McDougal et al. (32) studied the mechanism of neutralization of HIV-1 by antibody-positive sera from HIV-infected individuals. They came to the conclusion that the neutralizing capacity is not due to agglutination of virus causing a blockade of binding, nor is it the consequence of dissociation of gp120 from virions (32). Based on their findings, they suggested that anti-gp120 antibodies may induce neutralization by a mechanism that can occur even prior to and independently of HIV-1 binding, perturbing some metastable property of the envelope that is required for viral entry. This possible perturbation of gp120 induced by anti-V3 loop MAbs would not lead to disruption of the CD4 binding site, because we have demonstrated here that the gp120-gp41 complex is still able to bind soluble CD4 in the presence of the anti-V3 MAbs 110-4 and N11-20 (Fig. 6). It also has been reported that neutralizing anti-V3 loop antibodies do not inhibit the binding of a MAb directed against the CD4 binding site in the multimeric gp120 expressed on the viral membrane (34). Although these results should be evaluated cautiously because of the use of soluble molecules, they are consistent with the suggestion that the CD4 binding site in membrane-expressed gp120 is not modified by anti-V3 loop antibodies.

The observations that anti-V3 loop antibodies completely inhibit the interaction of virus-associated gp120 with $CD4^+$ cells, without an apparent effect on the CD4 binding domain in gp120, suggest a direct or an indirect role of the V3 loop in virus binding. Thus, blockade of the V3 loop may compromise its interaction with cell surface components that stabilize the binding of virus particles on $CD4^+$ cells, or alternatively, it may modify other regions of gp120 necessary for binding to such components. In favor of these hypotheses, here we have shown that reagents blocking the V3 loop of gp120, such as neutralizing anti-V3 loop MAbs and heparin, abolish HIV-1 binding to cell surface CD4. Moreover, we have recently reported that the V3 loop-mimicking pseudopeptide 5(KPR)-TASP, which binds to a cell surface-expressed protein(s) (6, 7), blocks HIV-1 binding to $CD4^+$ cells and virus infection. Logical candidates for cell surface components involved in HIV-1 attachment are chemokine receptors, i.e., CXCR4 for lymphotropic HIV-1 isolates (17). However, by using soluble gp120, it has been demonstrated that CXCR4 becomes recruited only after binding of gp120 to $CD4^+$ cells (28), suggesting that CXCR4 is not critical in early steps in HIV binding. Consistent with this, SDF-1, which is the natural ligand of CXCR4, inhibits HIV infection (40) without affecting the binding of HIV-1 particles to $CD4^+$ cells (8, 49). In the case of infection with monotropic HIV-1 isolates, which use the chemokine receptor CCR5 as a cofactor of CD4 (53, 56), RANTES, the natural ligand of CCR5, also blocks HIV infection without having any effect on virus binding (43). These observations and our results indicate that potential cell surface components responsible for stabilizing the attachment of HIV particles on $CD4^+$ cells are distinct from CD4 and its cofactors CXCR4 and CCR5.

We have shown here that HIV-1 particles bind human and murine cells lacking human CD4 expression (Fig. 3 and 4). This CD4-independent binding must represent virions bound or adhered to the cell surface, since no detectable HIV-1 entry occurs in $CD4^-$ human cells and in murine cells. Moreover, this binding appears to be specific, since heparin and neutralizing MAbs against the V3 loop inhibit by more than 85% HIV-1 binding to both human CD4-negative and -positive cells, whereas other anti-gp120 MAbs have a very small effect (Fig. 2, 3, and 4). Similar results were also obtained by using murine cells, although a complete inhibition of virus association by anti-V3 loop MAbs or heparin could not be observed. These results suggest that the hypothetical complementary cell surface component(s) is probably not species specific. Consistent with this, expression of human CD4 and of CXCR4 or CCR5 in heterologous cells is sufficient for efficient entry of lymphotropic or monotropic HIV-1 isolates (10, 14–18).

The identities of cell surface components responsible for the interaction with the V3 loop and their potential role in the mechanism of HIV particle binding to $CD4^+$ cells remain to be determined. Previously, different regions of the gp120 molecule, including the V3 loop, have been shown to be involved in the interaction of HIV-1 with galactosyl ceramides (20, 57). In addition, cell surface heparan sulfate proteoglycans have been reported to interact with the V3 loop of HIV-1 particles (47). Niwa et al. (39) have found that tryptase TL_2 binds HIV-1 gp120 and is able to cleave the V3 loop in vitro. Another cell surface peptidase, CD26, has also been implicated in the HIV-1 entry (5, 42), and a synthetic V3 loop peptide was reported to bind CD26 (9). Finally, we have recently identified three cell surface V3 loop binding proteins of 95, 40, and 30 kDa which are implicated in the mechanism of HIV-1 binding to $CD4^+$ cells (7, 8). Considering that the binding of HIV particles to cells is a complex event, the involvement of several cell surface components besides CD4 cannot be ruled out. Whatever is the case, CD4 plays the key role that results in functional binding which leads to viral entry and infection. An intriguing observation from our data is the high level of HIV particle binding in the presence of anti-CD4 neutralizing antibodies (Fig. 1 and 2). Such CD4-independent binding can also be observed with CD4-negative cells (Fig. 3), as has been reported previously (13). An important question which remains to be answered is the physiological significance of the CD4-independent HIV binding to cells. We have recently shown that HIV-1 Lai particles are able to inhibit adenosine deaminase binding to CD26 independently of CD4 expression, an event which may result in defective adenosine deaminase-CD26 costimulatory signaling in T-cell activation (54). Therefore, a better understanding of the role of CD4-independent HIV binding to cells could provide further insights to explain HIV pathogenesis.

The results presented here provide the basis to dissect out two qualitatively different types of interaction between HIV-1 viral particles and $CD4^+$ cells, both of which are completely prevented by anti-V3 loop neutralizing antibodies. The integrity of the V3 loop, therefore, is essential for HIV interactions with the cell surface. Neutralizing anti-V3 loop antibodies, through their capacity to bind virus particles, inhibit the binding of HIV virions to permissive cells. Thus, further understanding of the neutralizing mechanism of V3 loop-blocking reagents is essential for the development of anti-HIV therapy, in both pharmacological and vaccine-based approaches.

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J.B. and A.V. contributed equally to this work.

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