Inhibition of Virus Attachment to CD4$^+$ Target Cells Is a Major Mechanism of T Cell Line–adapted HIV-1 Neutralization

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Summary

Antibody-mediated neutralization of human immunodeficiency virus type–1 (HIV-1) is thought to function by at least two distinct mechanisms: inhibition of virus–receptor binding, and interference with events after binding, such as virus–cell membrane fusion. Here we show, by the use of a novel virus–cell binding assay, that soluble CD4 and monoclonal antibodies to all confirmed glycoprotein (gp)120 neutralizing epitopes, including the CD4 binding site and the V2 and V3 loops, inhibit the adsorption of two T cell line–adapted HIV-1 viruses to CD4$^+$ cells. A correlation between the inhibition of virus binding and virus neutralization was observed for soluble CD4 and all anti-gp120 antibodies, indicating that this is a major mechanism of HIV neutralization. By contrast, antibodies specific for regions of gp120 other than the CD4 binding site showed little or no inhibition of either soluble gp120 binding to CD4$^+$ cells or soluble CD4 binding to HIV-infected cells, implying that this effect is specific to the virion–cell interaction. However, inhibition of HIV-1 attachment to cells is not a universal mechanism of neutralization, since an anti-gp41 antibody did not inhibit virus–cell binding at neutralizing concentrations, implying activity after virus–cell binding.

Neutralization of enveloped viruses by antibody alone, in the absence of other factors such as complement and antibody-dependent cellular cytotoxicity, is mediated by various mechanisms, including viral aggregation, the inhibition of virus binding to its cellular receptor, and interference with later events such as virus–cell membrane fusion (for review see references 1 and 2). Of these diverse mechanisms, the inhibition of virus binding to its target cell is conceptually simple, in that a virus that cannot bind cannot infect, but is considered to occur only rarely (1, 2). Inhibition of virus–cell binding has nevertheless been implicated as a mechanism of antibody-mediated neutralization for several enveloped viruses: Newcastle disease virus (3), rhinovirus (4), mouse mammary tumor virus (5), visna virus (6), and HIV-1 (7–11). Most of these studies were carried out either with polyclonal antisera, or a single mAb, and the precise relationship between neutralization and inhibition of virus–cell binding was generally not well established. Thus, the relative importance of this mechanism of virus neutralization remains unclear.

Neutralizing activity in the serum of HIV-1–infected individuals is directed predominantly to the surface envelope glycoprotein (gp)120,[1] although neutralization can also be mediated by a transmembrane glycoprotein (gp41)–specific fraction of antibodies (for review see references 12–15). The anti-gp120 neutralizing response has been mapped by the use of mAbs of rodent, chimpanzee, and human origin, allowing the identification of a number of neutralization epitope clusters on gp120 and gp41 (12, 13). The majority of the neutralizing activity against T cell line–adapted (TCLA) viruses in human antisera is reactive with two regions of gp120; the CD4 binding pocket and associated structures (known as the CD4 binding site or CD4bs), and the V3 loop (16). Other confirmed gp120-specific neutralizing activity is directed to the hypervariable loops V1/V2, or to complex, discontinuous epitopes clustered around the base of the variable loops (17–20). By contrast with most TCLA viruses, primary isolates are generally difficult to neutralize; higher concentrations of antibody are required and fewer neutralizing epitopes are available (12, 13, 15).

[1]*Abbreviations used in this paper:* bs, binding site; GM, growth medium; gp, glycoprotein; i, induced; s, soluble; TCLA, T cell line adapted; WB, wash buffer.

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Little is understood of the mechanisms by which antibodies neutralize HIV-1. Antibodies to the V3 loop of gp120 have long been assumed to inhibit HIV infection at a stage after virus–cell binding, since these antibodies inhibit soluble (s)gp120-CD4 binding weakly or not at all (21–23). However, direct evidence to support postbinding activity is limited (24–26). A cluster of gp120-specific mAbs, including some which recognize the V3 loop and related structures, has been demonstrated to induce gp120 dissociation from gp41 on TCLA HIV-1, suggesting that this may contribute to viral inactivation (27). Recently it has been shown that neutralizing anti-gp120 mAbs to regions other than the CD4bs, including some specific for the V3 loop, inhibit the interaction of sgp120 with the HIV-1 coreceptor CCR5 (28, 29). These studies imply that HIV-1 neutralization may be mediated primarily by inhibition of the interactions between gp120 and the CD4 coreceptor complex.

The binding to CD4 of recombinant, monomeric sgp120, derived from TCLA viruses, is blocked by anti-CD4bs antibodies, implying that their mechanism of neutralization may be based, at least in part, on competition for virus–receptor binding (8, 30, 31). However, the interaction between sgp120 and sCD4 or sgp120 and $CD4^+$ cells is unlikely to adequately represent the true virus–cell binding interaction, since the conformation and quaternary structure of sgp120 and gp41-associated, oligomeric gp120 are different (32–34), as is the valency of potential interaction sites with cellular proteins. Moreover, the binding affinity for CD4 of oligomeric, gp41-associated gp120 may be substantially lower than that of monomeric sgp120 from the same viral background (35–37). Direct measurement of virion binding to $CD4^+$ cells is obviously the assay of choice for the analysis of HIV-1–cell binding and the inhibition of this process by cellular and viral ligands. Few studies have reported the inhibition of HIV virion binding to $CD4^+$ cells by antibodies. McDougal et al. demonstrated that neutralizing human antisera prevented viral adsorption to $CD4^+$ cells (7), Posner et al. observed that a neutralizing anti-CD4bs mAb inhibited HIV-1–cell binding in a dose-dependent manner (10), and Bahraoui et al. found partial inhibition of HIV–1 cell binding by two anti-V3 loop–specific mAbs (22). However, one drawback with the analyses of HIV-1–cell binding carried out in these studies is that sgp120 present in the virus preparation or released from virions during the assay would also bind to the $CD4^+$ cells, and would be detected with the anti-gp120–reactive antibodies used. Assuming that sgp120 has a higher affinity than virion gp120, this would then bias the assay readout in favor of the sgp120–CD4 interaction and prevent a precise analysis of virion–cell interactions. Moreover, the prebinding of neutralizing mAbs or sCD4 to virion gp120 is likely to influence later detection of virus by gp120-reactive antibodies.

To clarify the influence of neutralizing mAbs on HIV-1–cell binding, we analyzed the ability of a panel of neutralizing mAbs specific for different gp120 neutralizing epitopes to inhibit HIV-1–cell binding, using a novel assay in which virion–cell binding is measured directly without contamination of the detection signal by sgp120. We found that all gp120-specific mAbs tested inhibited virus–cell binding, and that this inhibition correlated strongly with virus neutralization. The only neutralizing mAb unable to inhibit HIV-1–cell binding was the gp41-specific mAb 2F5. We therefore conclude that the inhibition of virus–cell binding is a major mechanism of HIV-1 neutralization, and that mAb 2F5 neutralizes at a stage after virus–cell binding.

Materials and Methods

Cells and Viruses. The HLA-$DR^-/CD4^+$ line A3.01 and its $CD4^-$ derivative A2.01 were obtained from T. Folks (Center for Disease Control, Atlanta, GA) and were grown in RPMI 1640 supplemented with 10% FCS (growth medium, GM). The HLA-$DR^+/CD4^+$ PM1 cell line derived from HUT78 by P. Lusso et al. (38) was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD. The HIV-1 molecular clone Hx10 and the TCLA isolate MN were obtained from A. Fisher (Royal Postgraduate Medical School Hammersmith Hospital, London, UK; reference 39) and H. Holmes and the Medical Research Council (MRC) AIDS Reagent Project, respectively.

Antibodies and Recombinant Proteins. The anti-CD4 mouse mAbs Q4120, Q425, and L120 (from the MRC AIDS Reagent Project) were previously mapped to the first, third, and fourth domains of CD4, respectively (40). Q4120 competes with sgp120 for CD4 binding and inhibits HIV infection and syncytium formation, Q425 inhibits HIV infection and syncytium formation but not sgp120–cell binding, and L120 does not influence sgp120–cell binding and only weakly interferes with infection and syncytium formation (40). Anti-sCD4 sheep antiserum was prepared by D.M. Konys and M. Page (Biogen, Cambridge, MA) and was obtained from the MRC AIDS Reagent Project. Anti–HLA-DR mAb L243 was obtained from the American Type Culture Collection (Rockville, MD), DA6 from M. Moore, and BL2 and biotinylated B8.12.2 were from Immunotech SA (Marseille, France). Rodent anti-gp120-V3 mAbs were from the following sources: 110.5 (41) from Genetic Systems (Seattle, WA) and ICR41 (42) from J. Cordell (Institute for Cancer Research, Sutton, Surrey, UK). The human anti–V3 loop mAb 447-52D (43, 44) was purchased from Cellular Products Inc. (Buffalo, NY). The chimpanzee anti-gp120-V2 mAb C108G was prepared as previously described (45). The human mAbs specific for the CD4bs, 21h, the CD4-induced epitope (CD4i) 48d (17, 46), and the V3 loop, 19b (47) were from J. Robinson (University of Connecticut, Storrs, CT). The anti-CD4bs monospecific antibody IgG1b12 (48), and its Fab, b12 (49–51), and the anti–V3 loop monospecific antibody Loop 2, and its Fab, were prepared as previously described (52, 53). The human anti-gp120 mAb 2G12 (18, 19) and the anti-gp41 mAb 2F5 (54) were prepared by H. Katinger, and were obtained from the MRC AIDS Reagent project. Anti–HIV-1 gp120 antibody D7324, raised against a peptide synthesized from a highly conserved sequence in the gp120 COOH terminus, was from Aalto BioReagents Ltd. (Dublin, Ireland). Recombinant sCD4 was from L. Burkly (Biogen; reference 55). HIV-1 MN (Baculovirus-produced) was provided by J. Raina and IIIB (Chinese hamster ovary cell–produced)–derived purified recombinant sgp120s were obtained from the MRC AIDS Reagent Project.

Virus Binding and Neutralization Assays. 2×10^7 PM1 cells in-

fected overnight with Hx10 or MN at a multiplicity of infection of ~0.1 were cultured in GM, which was renewed every 24 h to prevent the accumulation of heat-inactivated virus particles (56) and to allow culture of the cells at high density. The production of viral p24 protein was followed by p24 ELISA (57), and the supernatant containing the peak production of p24 was harvested. The virus supernatant was clarified by centrifugation at 3,000 *g*, filtrated through a 0.45 μm filter, and then concentrated by ~10-fold using a 300-kD cutoff Macrosep centrifugal concentrator (Filtron Technology Corp., Northborough, MA). The concentrated virus was aliquotted and stored at −80°C until use. Mock-infected supernatants from uninfected PM1 cells were prepared and treated in the same way. The virus–cell binding assay was carried out by incubation of 50 or 100 μl of concentrated virus (for Hx10 or MN, respectively), or dilutions thereof, at 37°C with 3×10^5 A3.01 or A2.01 cells for 30 min unless otherwise stated, in a total volume of 70 or 120 μl for Hx10 and MN, respectively, unless otherwise stated. Next, the cells were washed twice in PBS/1% FCS/0.02% sodium azide (wash buffer, WB), and then resuspended in 50 μl of either a mixture of three non–cross-competing anti–HLA-DR mAbs, L243, DA6, and BL2 (for MN and Hx10 virus), or the biotinylated HLA-DR mAb BL8.12.2 (for Hx10). The mixture of the three anti–HLA-DR mAbs was used with some virus stocks to increase detection sensitivity, as the signal was too weak when the single biotinylated mAb was used. After 1 h at 4°C, the cells were washed twice in WB, and then fixed overnight in 0.2% formaldehyde in WB. No vortexing was carried out during the washing steps to avoid removing virus bound weakly to the cell surface. After washing three times in WB, the cells were incubated for 1 h at 4°C in WB containing either a 1:100 dilution of anti–mouse IgG-phycoerythrin or a 1:20 dilution of streptavidin-phycoerythrin (Immunotech SA). Bound virus was detected by flow cytometry using a FACScan® with Lysis II software. Cells gated on side scatter and forward angle light scatter were analyzed, and each datum point represents the acquisition of 10,000 gated events. Inhibition by CD4 mAbs was carried out by preincubating the A3.01 cells for 1 h at 4°C with different concentrations of mAbs before addition of virus. Virus neutralization was measured using 5 μl of the same virus sample used for the binding test, and was carried out as follows. Serial dilutions made in GM at 4°C (in duplicate) of virus pretreated or not with anti-Env mAbs was incubated with 3×10^5 A3.01 or C8166 cells for 2 h at 37°C before adding fresh medium. The cells were cultured in 200 μl GM for 10 d, during which time 100 μl of medium was exchanged for fresh GM every 2 d, and the culture medium monitored for p24 in the supernatant by p24 ELISA (57). The viral titer (50% tissue culture infectious dose) was determined as the final dilution (from duplicate wells by the method of Karber, reference 58) of virus containing supernatant yielding a positive p24 signal at peak cell-free p24 production, in which the cutoff for p24 positivity was taken as a signal greater than twice that of the negative control signal. The binding and neutralization tests were not carried out under identical conditions, in that the antibody-treated virus was incubated for 2 h longer with the cells in the neutralization assay than in the cell binding assay, to allow entry of nonneutralized virus. Results were analyzed for correlation as follows. The 10-logs of the relative virus binding and $TCID_{50}$ values were calculated. Simple regression of the second order of a linear relationship of $\log TCID_{50}$ as a function of the log(relative virus binding) was performed as included in the Statview program (Abacus Concepts, Inc., Berkeley, CA). The *P* values were calculated with F and *t* tests.

sgp120 Cell Binding Assay. 5×10^5 cells were incubated for 30 min at 37°C with agitation with different concentrations of sgp120, pretreated or not with mAb, in a total volume of 50 μl in a 96-well plate. The cells were then washed twice with WB, resuspended in 50 μl of 2 μg/ml D7324, and incubated with agitation for 1 h at 4°C. Anti–goat-FITC (Sigma Chemical Co., St. Louis, MO) was added to the cell pellet at a 1:20 dilution in 50 μl WB, and then the cells were agitated at 4°C for 1 h and washed twice in WB. Labeled cells were analyzed by flow cytometry as described above. For mAb inhibition studies, sgp120 at 10 μg/ml was preincubated with serial dilutions of anti-gp120 mAbs for 2 h at 37°C before addition to the target cells as described above.

sCD4-infected Cell Binding Assay. The assay was carried out essentially as previously described (37). In brief, H9 cells were infected with either Hx10 or MN virus at a multiplicity of infection of ~0.1, and cultured for 8–10 d, respectively, before use. At this time, the cells were surface $CD4^-/gp120^+$ as demonstrated by mAb staining and flow cytometric analysis. The infected cells were incubated for 2 h at 37°C in the presence or absence of anti-HIV mAb at varying dilutions in RPMI/5% FCS, and then biotinylated sCD4 was added to achieve a final concentration of 2 μg/ml, and incubated for 1 h at 4°C. After washing, the cells were fixed in 0.2% formaldehyde in WB overnight before washing and labeling with streptavidin-phycoerythrin (Immunotech SA) at a 1:20 dilution for 1 h at 4°C. After final washes, fluorescence was analyzed by flow cytometry.

Results

Detection of Virus–Cell Binding by Anti–HLA-DR Antibodies. The detection of HIV–cell binding by the use of anti–HIV-specific reagents (anti-gp120 mAbs or anti–HIV-specific antisera) has two major disadvantages. First, these reagents will detect cell-associated sgp120 which has been shed from the virus before or during the assay, and second, if the virus has been pretreated with HIV-specific antibodies, these may interfere with binding of the detection antibody. To eliminate these potential problems, we took advantage of the fact that during budding, HIV incorporates large numbers of HLA-DR molecules into its membrane. The binding of $HLA\text{-}DR^+$ virus particles to $CD4^+/HLA\text{-}DR^-$ cells allows detection of bound virions with anti–HLA-DR mAbs, followed by indirect immunofluorescent staining and flow cytometric analysis. Binding was carried out with $CD4^+/HLA\text{-}DR^-$ A3.01 cells, and the sister cell line, A2.01 ($CD4^-/HLA\text{-}DR^-$), was used as a control for specificity. Moreover, we included mock-infected control preparations, since cell culture supernatants contain large numbers of membrane vesicles that carry molecules of cellular origin (59, 60). In preliminary experiments, we incubated concentrated Hx10 or MN virus or mock virus with A3.01 or A2.01 cells for different times at 37 and 4°C. Since the binding at 4°C was weak or undetectable and considered nonphysiological (results not shown), and we were concerned that incubation periods of >30 min at 37°C would lead to significant levels of virus–cell membrane fusion which might affect detection of cell-bound, virion-associated HLA-DR, we selected 30 min at 37°C for later experiments. The binding of 50 μl of an undiluted Hx10 preparation (Fig. 1 *A*) or 100 μl of MN (Fig. 1 *B*) to A3.01 and A2.01 cells are represented as flow cytometry

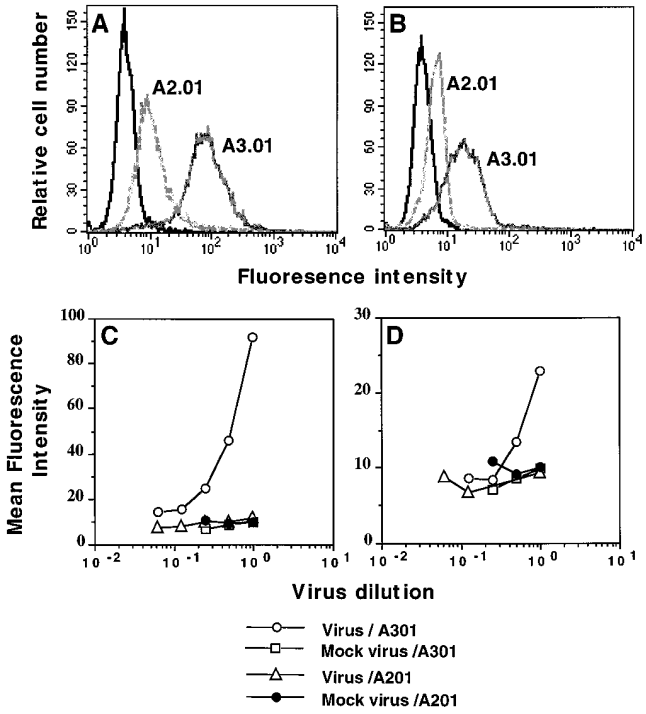

Figure 1. Binding of HLA-DR$^+$ HIV-1 to HLA-DR$^-$/CD4$^-$ and CD4$^+$ cells. Concentrated HIV-1 or mock virus was incubated either undiluted or at the dilutions shown for 30 min at 37°C with either A3.01 or A2.01 cells, then cell-bound virus was detected by staining with anti–HLA-DR mAbs followed by indirect immunofluorescent staining and analysis by flow cytometry. Each point represents the mean fluorescence intensity of 10,000 accumulated events transformed into percentage of inhibition. *A* and *B* show flow cytometry profiles for Hx10 and MN, respectively; the left peak corresponds to the background staining with anti–HLA-DR mAb alone on A3.01 cells in the absence of virus. *C* and *D* show the concentration dependence of Hx10- and MN-cell binding respectively as compared to controls.

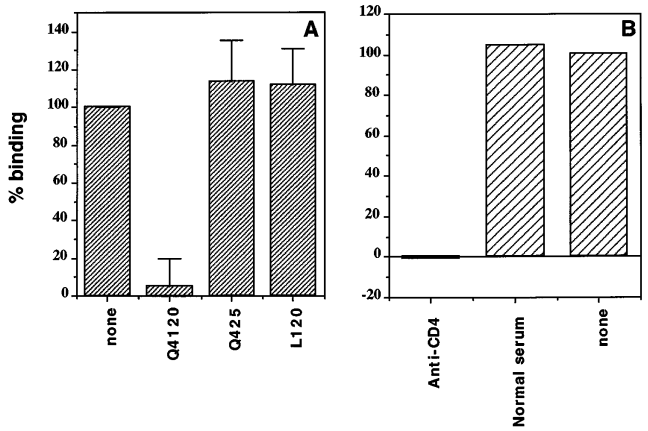

Figure 2. Inhibition of HIV-cell binding by CD4 mAbs and antisera. (*A*) A3.01 cells were preincubated with CD4 mAbs Q4120 (domain 1/CDR-1/2–like loops), Q425 (domain 3), or L120 (domain 4) at 20 μg/ml for 2 h at 4°C before addition of HIV-1 Hx10. Bound virus was detected as described for Fig. 1. (*B*) A3.01 cells were preincubated with either sCD4-specific sheep antiserum or normal sheep serum at a 1:90 dilution for 2 h at 4°C before addition of HIV-1 MN.

histograms. Despite a significant signal obtained on A2.01 cells, which represents CD4-independent binding of virus and HLA-DR–containing vesicular material, the signal obtained on A3.01 cells was substantially greater with both viruses, and all of the cells bound virus as demonstrated by the increased fluorescence of the entire population. The binding of both Hx10 and MN to A3.01 cells was dose dependent, although nonsaturating under the conditions used (Fig. 1, *C* and *D*), suggesting a low-avidity interaction between virions and cell-associated CD4. By contrast, the signal obtained with mock virus on A3.01 cells or virus on A2.01 cells did not substantially decrease with increasing dilution of the preparation, suggesting a saturating low level of CD4-independent binding. Overall, these results suggest that CD4 is required for efficient virus–cell binding. To confirm this, we preincubated the A3.01 cells with CD4 mAbs at a saturating concentration under the experimental conditions used, before addition of virus. The mAb Q4120, which binds to domain 1 of CD4 and competes for gp120 binding, inhibited Hx10 virus binding by 95% (Fig. 2). By contrast, neither Q425, which binds to CD4 domain 3 and interferes with HIV–cell fusion but not sgp120–cell binding, nor L120, which binds CD4 domain 4 and has little effect on HIV infection, interfered with HIV-1 binding. The lack of effect of Q425 on the virion–cell binding signal implies that HIV–cell fusion has little effect on the assay readout. Since we were obliged to use a mixture of three (nonbiotinylated) murine anti–HLA-DR mAbs to detect MN virus bound to the A3.01 cells to increase sensitivity, we were unable to carry out inhibition experiments with antibodies of murine origin. For this reason we used a polyclonal sheep anti-sCD4 antiserum at a dilution of 1:90, controlled by normal sheep serum at the same dilution, to inhibit MN binding. Fig. 2 *B* shows that under these conditions, virus binding was completely inhibited by the antiserum, whereas the control serum had no obvious effect.

sCD4 and CD4bs-specific gp120 mAbs Inhibit HIV–Cell Binding. The experiments with CD4 mAbs confirmed that HIV required CD4 on the target cell for specific binding. We next wished to investigate whether the binding of ligands to the virus was also able to inhibit HIV–cell binding in this assay. We initially tested sCD4 for its ability to interfere with virus binding and to neutralize virus infectivity, since it has previously been shown to compete with membrane CD4 for both sgp120 and virion binding. Fig. 3 shows the inhibition of HIV–cell binding and HIV infectivity for Hx10 and MN, respectively. The inhibition of virus binding paralleled that of neutralization with increasing sCD4 concentration, suggesting a relationship: an excellent and highly significant correlation was demonstrated after analysis of the results, and is summarized in Table 1 (r = 0.96 and 0.93 for Hx10 and MN, respectively). We next tested a panel of antibodies specific for the CD4 binding region of gp120 for their ability to neutralize and inhibit HIV–cell attachment. Fig. 4 shows the effect of Fab b12 and its IgG derivative, IgG1b12, mAb 21h on Hx10–cell binding and infectivity, and IgG1b12 on MN–cell binding

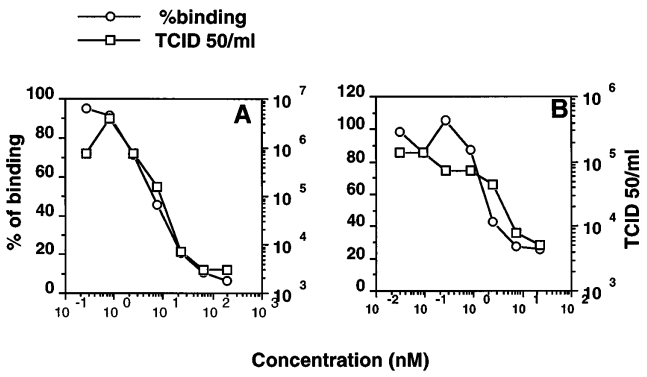

Figure 3. Inhibition of HIV–cell binding by sCD4. HIV Hx10 (*A*) or MN (*B*) virus was preincubated with increasing concentrations of sCD4 for 2 h at 37°C before addition of the A3.01 cells. The virus binding test was carried out as described for Fig. 1, and data are represented as percentage of binding. To measure virus infectivity, serial dilutions of a sample of the same sCD4-treated virus used for the binding test were incubated with A3.01 cells for 2 h at 37°C before culture for 10 d, and the viral titer calculated at peak p24 production in the culture supernatant. Neutralization is represented as reduction in infectivity ($TCID_{50}$).

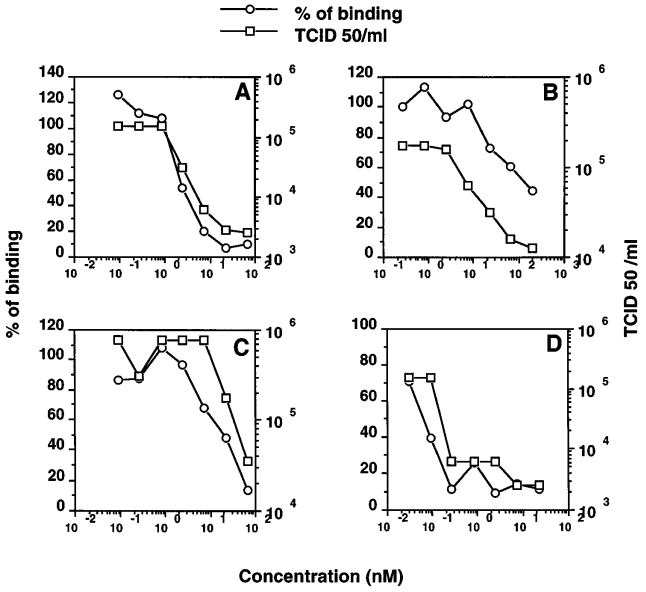

Figure 4. Inhibition of HIV-1 binding and infectivity by anti-gp120-CD4bs–specific mAbs. The following antibodies were tested for inhibition of binding and infectivity of Hx10 virus: (*A*) mAb IgG1b12; (*B*) Fab b12; (*C*) mAb 21 h. *D* represents IgG1b12 on MN virus. The experiment was carried out as described for Fig. 3, and results are represented as percentage binding versus reduction in infectivity.

and infectivity. The binding of both viruses to A3.01 cells was strongly inhibited by these anti–CD4 binding site–specific antibodies. Maximum inhibition of Hx10 binding was ~93, 55, and 86% for IgG1b12, Fab b12 and 21h, respectively, at 200 nM, and maximum inhibition of MN binding was 89% for IgG1b12 at the same concentration. These data confirm that a large part of the neutralizing activity of these antibodies is likely to be competition for virus–CD4 binding. The finding that Fab b12 neutralized and inhibited virus attachment demonstrates that cross-linking of the viral glycoproteins is not required for these functions. Further analysis of the relationship between inhibition of virion–cell attachment and neutralization for these antibodies revealed a strong to excellent and highly significant correlation for both viruses (Table 1).

Non-CD4bs Neutralizing Antibodies Inhibit HIV–Cell Binding. Inhibition of HIV-1–cell binding by CD4bs-specific mAbs was anticipated, since it has previously been shown that such antibodies are efficient inhibitors of sgp120 binding to sCD4 and $CD4^+$ cells. We next tested neutralizing mAbs specific for other gp120 epitopes for their ability to interfere with virus–cell binding. Several other confirmed TCLA virus neutralization epitopes have been identified: the hypervariable V2 and V3 loops, the CD4-induced (CD4i) epitopes recognized by mAbs 48d and 17b, and the unique 2G12 epitope. Fig. 5 shows the effect of V3 loop–specific mAbs on virion binding. Unexpectedly, these mAbs interfered with both Hx10– and MN–cell binding; maximum inhibition of virion attachment at 200 nM by 110.5, ICR41, and 447-52D was ~46, 47, and 85%, respectively, with Hx10. The effect of V3 loops on MN–cell binding was more pronounced than on Hx10; 447D, Loop 2, and the Fab fragment of Loop 2 inhibited MN–cell binding maximally at 200 nM by 96, 65, and 56%, respectively. Similarly, C108G, 48d, and 2G12 at the same concentration inhibited Hx10 binding by 83, 81, and 76%, respectively, and 48d reduced MN attachment by 79% (Fig. 6). Thus, all anti-gp120 neutralizing mAbs tested were able to interfere with the binding of two TCLA viruses to a $CD4^+$ T cell line. To determine whether the inhibition of virus-cell binding was general to all anti–envelope glycoprotein mAbs, we carried out binding and neutralization analyses with the neutralizing anti-gp41 mAb 2F5. By contrast with the results obtained with the anti-gp120 mAbs, 2F5 did not inhibit HIV–cell binding at any concentration tested, but potently interfered with HIV infection; at a concentration of 200 nM, 2F5 reduced Hx10 and MN infectivity by ~80- and 30-fold, respectively (Fig. 6).

The Relationship Between Inhibition of Virus–Cell Binding and HIV-1 Neutralization. As demonstrated in Figs. 3–6, all gp120-specific ligands, including sCD4, IgG molecules to diverse epitopes, and their Fab fragments, inhibited virion attachment to A3.01 cells. Moreover, analysis of the binding and neutralization curves revealed that the inhibition of virion binding correlated with neutralization in all cases ($r > 0.5$); only the gp41-specific ligand 2F5 showed no correlation (Table 1). However, inspection of the results summarized in Table 1 demonstrates a range in the strength of the correlations, from excellent and highly significant for sCD4, IgG1b12, and 48d on Hx10 and Loop 2 on MN, to correlated but not significant for several others. Although variability due to imprecision intrinsic to the methods used prevents any unequivocal interpretation of these differences, one possibility is that for some ligands, inhibition of virion attachment to cells may only partially mediate HIV neutralization, whereas other mechanisms may contribute to a greater or lesser extent. To analyze this in greater detail, we estimated the percentage of neutralization that is

Table 1. *Correlation of Inhibition of HIV-1–cell Attachment and Neutralization*

Specificity	Antibody/ligand	MN			Hx10		
		Correlation	Significance	% neutralization by binding inhibition	Correlation	Significance	% neutralization by binding inhibition
		r	~*P*		*r*	~*P*	
CD4/BS	21h	0.84*	0.02‡	20–80§	0.94	0.002	84–88
	IgG1b12	0.87	0.01	120	0.99	0.0001	66–90
	Fab b12	ND	ND	ND	0.92	0.004	52–62
	sCD4	0.93	0.002	20–120	0.96	0.001	82–100
V3 loop	447-52D	0.74	0.06	80–88	0.83	0.04	82–114
	ICR41	NB	NB	NB	0.63	0.1	70–140
	110.5	NB	NB	NB	0.79	0.04	32–42
	Loop 2	0.95	0.001	66–86	NB	NB	NB
	Fab Loop 2	0.83	0.01	88–100	NB	NB	NB
	19b	0.67	0.2	100–116	NB	NB	NB
Other gp120	2G12	NB	NB	NB	0.94	0.004	80–128
	48d	0.71	0.08	72–100	0.98	0.0002	6–68
	C108G	NB	NB	NB	0.83	0.02	78–102
gp41	2F5	0.32	0.5	0	−0.25	0.6	0

*Values indicate that the correlation between inhibition of HIV-1 cell binding is excellent ($r > 0.95$); strong ($r > 0.5$); or absent ($r < 0.5$).
‡~*P* values estimate significance of correlation between inhibition of HIV–cell binding and neutralization.
§Values represent the percentage of neutralization which is the result of the inhibition of virus–cell attachment. The range represents reading within a linear curve at two points; half the value for 0 ligand and half the value where 100% mean fluorescent intensity intersects the linear curve.
‖NB indicates no detectable mAb binding to virus and no detectable neutralization.

the result of inhibition of virus–cell attachment. This analysis was carried out by plotting the relative $TCID_{50}$ values (i.e., the postneutralization $TCID_{50}$ divided by the control values obtained in the absence of neutralizing ligand) as function of the percentage of virus binding. The percentage of virus binding values were read from fitted linear curves at half-maximal infectivity; two different values were obtained for each ligand depending on whether the

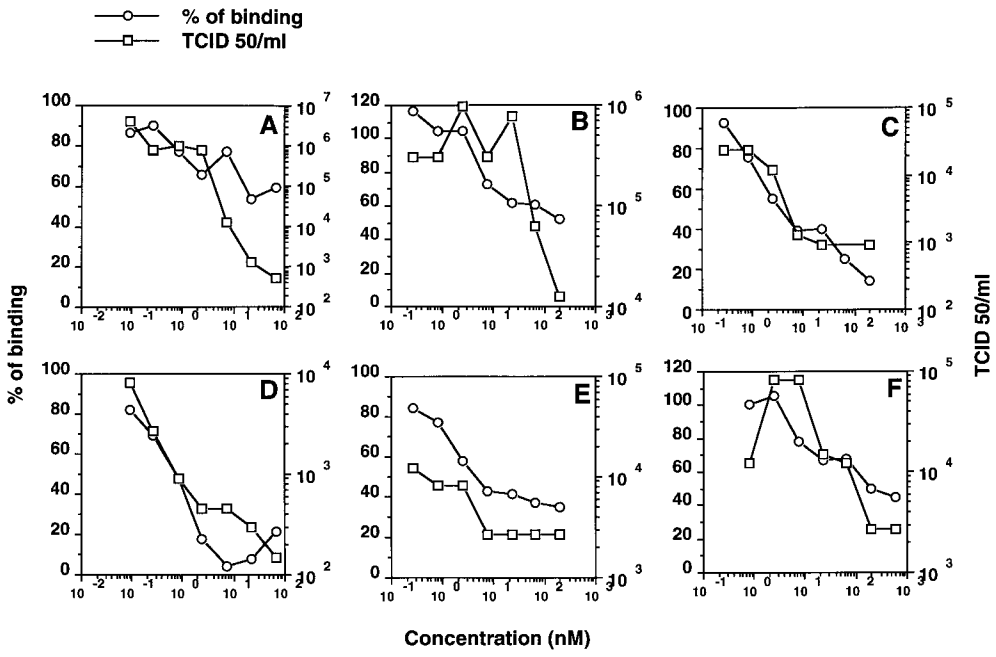

Figure 5. Inhibition of HIV-1 binding and infectivity by anti-gp120-V3 loop–specific mAbs. The following antibodies were tested for their ability to inhibit infectivity and cell binding of Hx10 virus; (*A*) 110.5, (*B*) ICR41.1, and (*C*) 447-52D or MN virus, (*D*) 447-52D, (*E*) Loop 2, and (*F*) Fab Loop 2. The experiment was carried out as described in Fig. 3, and results are represented as percentage of binding versus reduction in infectivity.

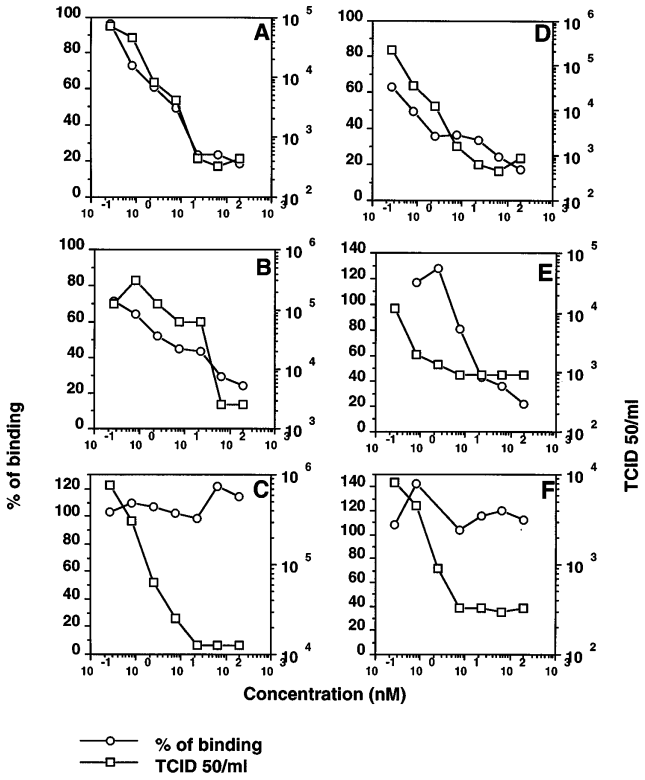

Figure 6. Inhibition of HIV-1 binding and infectivity by mAbs to other, non-CD4bs epitopes. The following antibodies were tested for their ability to inhibit infectivity and cell binding of Hx10 virus; (*A*) 48d, (*B*) 2G12, (*C*) anti-gp41 2F5, (*D*) C108G or MN virus, (*E*) 48d, and (*F*) 2F5. The experiment was carried out as described in Fig. 3, and results are represented as percentage of binding versus reduction in infectivity.

curves were read at 50% of the infectivity for 0 ligand or at 50% of the infectivity corresponding to 100% virus binding. These two readings represent a range of uncertainty in the estimates. Thus, the approximate fraction of virus that was prevented by ligand from binding to the cells at 50% neutralization could be calculated. If it is granted that virus that has not bound cannot infect, the unbound virus would correspond to the minimal fraction of the neutralization that was attributable to block of virus attachment. However, it should be noted that for the percentage of neutralization to be considered as due to a block of virus binding, it is assumed that the relationship between the mean fluorescence intensity values for bound virus and the numbers of infectious virions bound is linear in the relevant zone. The values presented in Table 1 indicate that for many of the ligands (21h, 447-52D, 2G12, and sCD4 for Hx10 and IgG1b12, Fab Loop 2, 447-52D, and 19b for MN) the likely contribution of inhibition of attachment to neutralization was >80%, suggesting that this is probably the dominant mechanism of neutralization. For others, the range was greater but did not exclude the possibility of a major role for inhibition of attachment in neutralization. Of the gp120-specific ligands, only 110.5 binding to Hx10 suggested a relatively minor role of inhibition of binding in neutralization, and the gp41-specific mAb 2F5 clearly neutralized both viruses by a mechanism unrelated to interference with HIV–cell attachment.

Inhibition of sgp120 to CD4$^+$ Cells and sCD4 to HIV-infected Cells. Previous studies have shown that V3 loop–specific mAbs are unable to inhibit sgp120 binding to CD4$^+$ cells. To confirm this, and to investigate the effects of other, non-CD4bs mAbs on sgp120–cell binding, we measured the association of antibody-pretreated sgp120 with A3.01 cells. The results are summarized in Table 2; all CB4bs antibodies and sCD4 inhibited both IIIB and MN sgp120–cell binding by 50% at concentrations ranging from 0.06 nM (IgG1b12 on MN) to 120 nM (Fab b12 on Hx10). By contrast, only three non-CD4bs mAbs achieved 50% inhibition of sgp120–cell binding; the anti–V3 loop mAbs ICR41 and Loop 2 on Hx10 and MN, respectively, and the CD4i-epitope specific mAb 48d with both viruses. Thus, the inhibition by neutralizing antibodies of sgp120 binding to CD4$^+$ cells is not representative of the inhibition of virus binding to the same cells. Since gp120 is in an oligomeric, gp41-associated form on the surface of infectious virions, it seemed important to investigate the effect of the neutralizing antibodies on the interaction between CD4 and virion-associated envelope glycoprotein. We therefore analyzed the ability of anti-gp120 neutralizing antibodies to inhibit the association of biotinylated sCD4 with HIV-infected (Hx10 or MN) cells. Similar to the results obtained for sgp120 binding to CD4$^+$ cells, the CD4bs-specific IgG molecules and sCD4 inhibited the sCD4-oligomeric gp120 interaction by 50% within a range of 0.7–6 nM, and the Fab b12 at 10–100 nM (Table 2). Thus, these ligands inhibit the gp120-CD4 interaction in all assays tested. However, in complete contrast to the virus–cell binding assay, antibodies to epitopes other than the CD4bs showed little or no inhibition of sCD4 binding to HIV-infected cells (Table 2); none of the antibodies achieved 50% inhibition at the highest dose tested (200 nM).

Discussion

We show here that mAbs to all confirmed HIV-1 gp120 neutralizing epitopes inhibit the binding of two TCLA viruses, one a molecular clone, the other a nonclonal isolate, to a CD4$^+$ T cell line, and that in all cases inhibition of virion attachment correlated with neutralization. These data strongly support the idea that inhibition of HIV–cell attachment is a major mechanism of TCLA HIV-1 neutralization. Thus, we confirm previous reports that mAbs specific for the gp120 CD4bs are potent inhibitors of sgp120–cell and virion–cell binding (7–11), but also demonstrate unequivocally that mAbs that react with the V2 and V3 loops and other independent structures (CD4i and 2G12 epitopes) also interfere with virus–cell binding. We have demonstrated this by the use of a novel virus–cell attachment assay based on the detection, with anti–HLA-DR antibodies, of HLA-DR$^+$ virus binding to CD4$^+$/HLA-DR$^-$ cells. In this way we have eliminated two potential artefacts of previously described HIV–cell binding assays that attempted to detect cell-bound virus using envelope glyco-

Table 2. *Inhibition of Virus and sgp120 Binding to CD4⁺ Cells, and sCD4 Binding to HIV⁺ Cells*

		Hx10			MN		
Specificity	Antibody/ligand	Virion	sgp120	sCD4	Virion	sgp120	sCD4
CD4/BS	IgG1b12	3.0*	20‡	6.0§	0.06	4.0	1.5
	Fab b12	120	30	10	ND	2.0	100
	21h	20	15	ND	ND	8.0	ND
	sCD4	6.0	15	0.7	2.0	2.0	0.7
V3	ICR41	200	200	>200	NB‖	NB	NB
	447-52D	0.4	>200	>200	0.7	>200	>200
	Loop 2	NB	NB	NB	4.0	30	>200
	Fab Loop 2	NB	NB	NB	200	>700	>200
	19b	NB	NB	NB	0.4	>200	>200
V2	C108G	0.8	ND	ND	NB	NB	NB
Other gp120	48d	7.0	30	>200	20	7.0	>200
	2G12	2.0	>200	>200	NB	>200	>200
gp41	2F5	>200	>200	>200	>200	>200	>200

*Represents concentration (nM) required for 50% inhibition of virus–A3.01 cell binding.
‡Represents concentration (nM) required for 50% inhibition of sgp120–A3.01 cell binding.
§Represents concentration (nM) required for 50% inhibition of sCD4–infected cell binding.
‖NB represents no detectable mAb binding to virus and no neutralization; absence of epitope or no epitope accessibility on the virion.

protein–specific antibodies: (*a*) the contamination of the virus–cell binding signal by shed sgp120 bound to cellular CD4, and (*b*) interference with detection of the bound virus by ligands prebound to the virus envelope glycoproteins.

The ability of neutralizing mAbs other than those specific for the gp120 CD4bs to inhibit virion–cell and, in some cases, sgp120 cell binding suggests that a mechanism other than direct competition for attachment to membrane CD4 is functioning. It may be that non-CD4bs mAbs interfere sterically with the gp120-CD4 interaction, particularly in the case where the gp120 molecules are tightly packed into a gp41-associated oligomer. Although we cannot rule this out as the mechanism of inhibition, the lack of interference of sCD4 binding to oligomeric gp120 on HIV-infected cells by non-CD4bs mAbs suggests that this explanation may not be sufficient. Moreover, the Fab fragment of the V3-specific mAb Loop2 interfered efficiently with MN virion–cell binding, further suggesting that steric considerations are unlikely to be dominant. Another possibility is that the binding of ligands to regions outside of the gp120 CD4bs induces conformational changes in the CD4 binding site, reducing its affinity for CD4. It is well established that the binding of sCD4 to virus or virus-infected cells can induce conformational changes in gp120 leading to increased exposure of spatially distinct epitopes such as the V3 loop (37, 61), and mAbs to epitopes including the CD4bs and the V2 and V3 loops have been reported to neutralize synergistically (11, 42, 62–66). However, the inability of most non-CD4bs–specific ligands to interfere in either sgp120 binding to CD4⁺ cells, or sCD4 binding to oligomeric gp120, again argues against this being the sole explanation. An additional factor which will influence HIV–cell binding is the number of intact envelope glycoprotein spikes per virion (67). Thus, mAbs which induce the dissociation of a critical number of gp120 molecules from gp41 will reduce virus–cell binding. This may be a factor in the reduction of Hx10-A3.01 binding, since 48d and V3 loop mAbs, such as 110.5 used in this study, have been shown to induce substantial gp120 dissociation (27). However, MN virus is much less sensitive to mAb-induced gp120 dissociation than Hx10, and sCD4 and 48d are the only ligands tested in this study that induce detectable loss of gp120 from the virion (results not shown). Moreover, if mAb-induced gp120 shedding was a major factor implicated in the reduction of HIV–cell binding, mAbs such as 110.5 and 48d would be expected to substantially inhibit sCD4 binding to virus-infected cells, which is not the case.

An intriguing possibility is that for HIV–cell binding of adequate avidity to allow virus–cell fusion, the virus may need to occupy binding sites on both CD4 and the appropriate coreceptor molecule. In this model, HIV may bind initially with relatively weak avidity to CD4, then rapidly recruit coreceptor molecules into the complex, or may bind only with sufficient avidity to remain attached to the cell if CD4 and the coreceptor are already established in a preformed complex. Thus, although the avidity of the HIV-1–CD4⁺ cell interaction has never been directly determined, measurement of virion–sCD4 binding suggests that it is likely to be substantially lower than that obtained for sgp120–CD4 (35, 36). In accord with this idea, analyses of virion adsorption to CD4⁺ cells strongly suggests that

this is an inefficient process (68), and a requirement for coreceptor molecules in the virus–cell attachment process has been postulated (69). Additionally, the finding that sCD4 triggers the exposure on gp120 of a binding site for CCR5 (29) suggests that this may be the sequence of events followed during virus–cell binding, and is consistent with the notion of two-site receptor binding. Moreover, it has been demonstrated that neutralizing mAbs to gp120 epitopes outside of the CD4bs inhibit the gp120–CCR5 interaction (28, 29), reinforcing the idea that for a successful entry into the target cell, gp120 binding to both receptor molecules is probably required.

Our results do not exclude the possibility that some of the mAbs tested also neutralize by mechanisms other than inhibition of virion–cell attachment; antibody-complexed virus escaping the block to attachment may nevertheless be neutralized at later steps in the life cycle. In this respect, the inhibition of virion–cell attachment may be considered as the most extreme end point resulting from a block to a critical number of virus–receptor interactions. The inhibition of a smaller number of virus–receptor interactions may not be sufficient to prevent virion attachment, but may inhibit the fusion process. Thus, for many of the antibodies tested here, inhibition of virus–receptor binding may be the dominant mechanism of neutralization, resulting in a block to virus–cell fusion, although only partial inhibition of attachment is observed. In another scenario, V3 loop–specific mAbs have been shown to neutralize cell-attached virus (24–26), and have been postulated to inhibit fusion or postfusion steps in the HIV-1 life cycle (26), and certain anti-CD4bs antibodies have been suggested to inhibit both HIV–cell membrane fusion and a postfusion step (70). However, alternative explanations for postattachment neutralization are that such mAbs either prevent the formation of a sufficient number of virus–receptor interactions to initiate fusion, or simply dissociate weakly adsorbed virus by interfering with a reversible binding step. These explanations seem reasonable if one considers that viral entry into certain $CD4^+$ T cell lines such as A3.01 is in the order of several h, as has been observed previously (71).

The data presented here are not consistent with recent studies that failed to demonstrate inhibition of HIV–cell binding with either sCD4 (72, 73) or the CD4bs-specific Fab b12 or its IgG derivative, IgG1b12 (70). The reasons for this are unclear. Demaria and co-workers (72, 73) detected virus binding using an anti–gp120-specific antiserum; pretreatment of virions with sCD4 or mAbs is therefore likely to modify the subsequent detection of bound virus. We have eliminated this possibility in our study by detecting bound virions using mAbs to a cell-derived molecule. We carried out the virus–cell binding at 37°C, whereas Demaria and co-workers (72, 73) and McInerney et al. (70) use 4°C for most experiments. However, this is unlikely to explain the differences observed, since McInerney et al. failed to find significant changes when their assay was run at either 4 or 37°C, and we find that both IgG1b12 and Fab b12 inhibit HIV–cell binding when carried out at 4°C (results not shown). We chose to carry out our virus–cell binding assay at 37°C as these conditions were most likely to represent the physiological conditions encountered by the virus during binding to its target cell. Virus–cell binding is temperature dependent, as has been shown in previous studies (35, 36) and it is clear from our own analyses that HIV–cell binding is much reduced at 4°C (results not shown). Although there was a risk that a small amount of virus–cell fusion might occur in our assay, it seems unlikely that this would strongly influence the results, since (*a*) fusion appears to be a relatively slow process, and 50% entry of a TCLA virus into A3.01 cells was shown to take 4 h (71), and (*b*) fused virus would probably leave an HLA-DR footprint at the cell surface which would be detected by antibody. Moreover, we found that pretreatment of the A3.01 cells with Q425, a CD4 mAb that inhibits HIV–cell fusion but not virus–cell binding, did not significantly modify the signal corresponding to bound virus, again implying that fusion is unlikely to alter the readout.

The studies we have described here have all been carried out using two TCLA viruses; it will now be important to determine whether cell attachment by primary isolates, particularly those using predominantly the CCR5 coreceptor, is also inhibited by anti–gp120-specific mAbs. Such experiments are not easy since the relatively high concentration of virus particles required to carry out the virus–cell binding assay described here is difficult to obtain for PBL or macrophage-grown viruses. However, in the absence of these data it seems reasonable to assume that since all HIV-1 viruses probably use a fundamentally conserved mechanism of binding and fusion, the inhibition of virus–cell binding is an important mechanism of antibody-mediated neutralization which requires further investigation using primary viruses.

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