Role of CD4 Epitopes outside the gp120-Binding Site during Entry of Human Immunodeficiency Virus Type 1

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Received 13 June 1996/Accepted 17 October 1996

CD4 is the primary receptor for human immunodeficiency virus (HIV). The binding site for the surface glycoprotein of HIV type 1 (HIV-1), gp120, has been mapped to the C′-C” region of domain 1 of CD4. Previously, we have shown that a mutant of rat CD4, in which this region was exchanged for that of human CD4, is able to mediate infection of human cells by HIV-1, suggesting that essential interactions between HIV and CD4 are confined to this region. Our observations appeared to conflict with mutagenesis and antibody studies which implicate regions of CD4 outside the gp120-binding site in postbinding events during viral entry. In order to resolve this issue, we have utilized a panel of anti-rat CD4 monoclonal antibodies in conjunction with the rat-human chimeric CD4 to distinguish sequence-specific from steric effects. We find that several antibodies to rat CD4 inhibit HIV infection in cells expressing the chimeric CD4 and that this is probably due to steric hinderance. In addition, we demonstrate that replacement of the rat CDR3-like region with its human homolog does not increase the affinity of the rat-human chimeric CD4 for gp120 or affect the exposure of gp41 following binding to CD4, providing further evidence that this region does not play a crucial role during entry of virus.

The primary receptor for human immunodeficiency virus type 1 (HIV-1) is CD4 (13, 27), a differentiation marker expressed on the surface of T lymphocytes, on a certain population of dendritic cells, and, to a lesser extent, on macrophages (44). CD4 is a member of the immunoglobulin (Ig)-like superfamily; its extracellular portion is comprised of four Ig domains (4, 45, 53). The binding site for HIV gp120 has been mapped to the second complementarity-determining region (CDR2)-equivalent region of the amino-terminal domain of CD4 by use of monoclonal antibodies (MAbs) (32, 47) and mutagenesis (1, 2, 6, 10, 33, 39, 49).

Binding of HIV to CD4 induces conformational changes in the envelope glycoproteins that lead to fusion of the virus and cellular membranes (35, 36, 48). Regions of CD4 outside the gp120-binding site have been implicated in these postbinding events. In particular, the F-G turn, or CDR3-equivalent region, of domain 1 (D1) has been implicated by numerous authors (3, 8, 26, 29, 30, 38, 52). However, the weight of evidence now suggests that this region is not involved in post-binding events (5, 34, 43, 51).

MAbs to regions of human CD4 distant from the gp120-binding site have been used in an effort to distinguish epitopes of CD4 that are involved in binding gp120 from epitopes that may be involved in postbinding events. Notably, MAbs against CD4 D1 (12, 52), D2 (7, 37), D3 (24), and D3D4 (23) have all been shown to inhibit virus infection, apparently with little or no effect on virus binding.

Previously we generated a mutant of rat CD4 which binds gp120 (49) and acts as an efficient receptor for HIV-1 (51), implying that regions of CD4 outside the gp120-binding site are unlikely to be essential for HIV interaction. In this study, we investigated the ability of a panel of anti-rat CD4 MAbs, none of which bind human CD4, to influence HIV infection of cells expressing this chimeric receptor. We show that MAbs which inhibit virus entry in this system bind epitopes of rat CD4 analogous to those of human CD4 bound by inhibitory anti-human CD4 MAbs. Furthermore, we show that although these MAbs have little effect on the binding of monomeric recombinant gp120 to cell surface CD4, the MAbs directed to D1 and D2 are unable to form a ternary complex with a soluble form of the receptor bound to oligomeric envelope protein (Env) on the surface of HIV-infected cells. We conclude that the principal mechanism by which D1 CDR3- and D2-directed MAbs inhibit HIV infection is by the inhibition of appropriate binding of CD4 to Env oligomers in the crowded environment of the virion and virus-infected cells.

MATERIALS AND METHODS

Cell lines. BHK-21 cells (31), HeLa cells (21), HeLa m6 cells (expressing the rat-human chimeric CD4 molecule mutant [m6] [51]), and the HIV-1 Env-expressing HeLa cell line H32 (obtained from Lee Bacheler, DuPont Co., Wilmington, Del.) were cultured in Dulbecco modified Eagle medium (Gibco BRL) containing 10% (vol/vol) fetal bovine serum (FBS) (Gibco BRL), 2 mM L-glutamine (Sigma), and antibiotics (50 U of penicillin per ml, 50 μg of streptomycin per ml, and 100 μg of neomycin per ml). In addition, HeLa m6 and H32 cells were maintained in 0.6 mg of Geneticin G418 (Sigma) per ml. C8166 cells (46) and H9 cells (40) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and were maintained in RPMI 1640 medium (Gibco BRL) with 10% FBS, L-glutamine, and antibiotics.

Preparation of soluble and truncated forms of CD4. Soluble forms of CD4 consisting of either D1 and D2 [sCD4(D1+2)], D3 and D4 [sCD4(D3+4)], or the four extracellular domains of rat CD4 (sCD4) were produced from Chinese...
hamster ovary (CHO) cell lines transfected with the modified cDNA for rat CD4 by using the glutamine synthetase selection system (4, 15).

Production of anti-rat CD4 MAbs. BALB/c mice were immunized with 30 mg of sCD4 in complete Freund’s adjuvant subcutaneously at an interval of 2 weeks. After six boost injections, mice were boosted with 60 mg of sCD4 in phosphate-buffered saline (PBS). For the final boost prior to taking the spleenocytes for fusion with NSO myeloma cells, a mixture of free sCD4 and sCD4 bound to cells was given intravenously. Anti-CD4 MAbs W3/25 (18 mg) was coupled to 0.1 ml of a 5% suspension of sheep erythrocytes by the chronic chloride method. After being washed, the cells were incubated with excess sCD4 (100 mg) and washed once, and a further 10 mg of sCD4 was added prior to intravenous injection. The spleen was taken for fusion with the myeloma NSO cells as described previously (51). The fusion was carried into four-well microtiter plates in the presence of irradiated thymocyte feeders, and hypoxanthine-aminopterin-thymidine selection was applied. Supernatants were assayed after 10 days for binding to cells or sCD4 immobilized on polystyrene plates by enzyme-linked immunosorbent assay.

Analysis of anti-CD4 MAb binding to sCD4 and fragments. The anti-CD4 MAbs were analyzed by binding to sCD4 or fragments immobilized by passive absorption to polystyrene plates and then reacting with [125I]-labeled rabbit anti-mouse (RAM) immunoglobulin G (IgG) polyclonal antiseraum as described previously (16, 54) or with alkaline phosphatase-conjugated RAM IgG followed by color development with the alkaline phosphatase substrate 4-nitrophenylphosphate. In addition to use of direct binding, various preparations of CD4 were tested for their ability to inhibit these binding assays.

Viruses. HIV-1M 
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4 (42) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and propagated by acute infection of C8166 cells. The cultures supernatants were clarified, filtered through a 0.45-μm-pore-size filters, treated with 40 U of DNase I (Sigma) per ml for 30 min at room temperature to remove contaminating proviral DNA, and stored in 1-ml aliquots. The virus was used for infection of C8166 cells. The supernatants were clarified, filtered through a 0.45-μm-pore-size filters, treated with 40 U of DNase I (Sigma) per ml for 30 min at room temperature to remove contaminating proviral DNA, and stored in 1-ml aliquots at −80°C.

Cocal virus (COC) (25) was obtained from Robert Shope (Yale Arbovirus Unit) and propagated in BHK-21 cells. COC(HIV) pseudotype virus was made by superinfecting acutely HIV-1M infected H9 cells with COC, and pseudotype plaque assays were performed essentially as described previously (51). These pseudotypes have the genome of COC and the spike glycoproteins of HIV, permitting HIV Env-mediated penetration of cells by COC, which leads to formation of COC plaques. The reaction of plaq. 120-mediated entry is calculated as the difference between the number of plaques produced when cells are challenged with virus neutralized with anti-COC antiseraum and the number of background plaques, which are challenged with COC(HIV) neutralized with both anti-COC antiseraum and anti-gp120 polyclonal antibody.

PCR-based HIV-1 entry assay. Entry of HIV-1 was detected by PCR and was quantitated as essentially as previously described (11). HeLa m6 cells were challenged with HIV-1M and incubated at 37°C for 2 h. The cells were then washed and incubated in fresh medium. Twenty hours after challenge, the cells were washed and lysed. Proximal DNA was amplified by PCR with the U3-3' U5' primer pair (with 3' U5' radio labelled with 32P and amplification products were electrophoresed on agarose gels and blotted onto Hybond N nylon (Amersham), which was then analyzed with a PhosphorImager. The bands were quantitated by using the ImageQuant program.

Flow cytometric analysis of MAb and gp120 binding to HeLa m6 cells. Binding of sCD4, gp120, and gp120 to HeLa m6 cells was analyzed by indirect flow cytometry with a FACSscan (Becton Dickinson and Co., San Jose, Calif.). Bacteriolytic-grown gp120 (HIV-1m strain) was obtained from the Medical Research Council AIDS-Related Programme. Cells were treated with gp120, and 5 × 105 cells were incubated with cell culture medium (at the appropriate concentration) or anti-rat CD4 MAb for 1.5 h or 1 h, respectively, on ice. After being washed in ice-cold PBS–0.5% bovine serum albumin (BSA), the cells were incubated for a further 45 min on ice with 50 μl of biotinylated anti-gp120 MAb (D47, which binds the V3 loop; a gift from Bob Bona, Doms), to detect gp120, or with fluorescein isothiocyanate-conjugated RAM Ig (RAM-FITC) (Serotec) to detect anti-rat CD4 MAbs. To detect bound gp120, cells were then washed and stained with streptavidin-FITC (Amersham). Formation of ternary complexes between sCD4, anti-rat CD4 MAbs, and oligomer Env on the surface of chronically HIV-1-infected H9 cells. Indirect flow cytometry was used to analyze ternary complex formation between anti-rat CD4 MAbs and a soluble form of m6 (sm6) (49) bound to H9 cells chronically infected with the HXB-2D strain of HIV-1. Half a million cells were washed twice in PBS–0.5% BSA by pelleting and resuspended in 50 μl of PBS–0.5% BSA containing sm6 at the appropriate concentration. The cells were incubated on ice for 2 h, washed, and resuspended with 50 μl of anti-rat CD4 MAb. After a further 1 h on ice, the cells were washed and stained with RAM-FITC for 30 min at 4°C, fixed with PBS–2% formaldeh dye overnight, and analyzed by flow cytometry.

Measurement of sCD4 binding and gp41 exposure on the surface of HIV-1-infected H9 cells. Chronically infected H9 cells were washed in RPMI containing 2% FCS and resuspended at 2 × 106 cells/ml in the presence of 20 μl of DNA microparticles of sCD4 (human rat, m6, or m11), at the appropriate concentration, was added to an equal volume of cells. Half of the cells were then incubated at 4°C to measure binding of sCD4, and the remaining half were incubated at 37°C to measure exposure of gp41. After four hours, the cells were pelleted and washed twice in PBS containing 1% FBS and 0.02% sodium azide. Bound sCD4 was detected by staining first with MAb L120 (D. Buck, Becton Dickinson) for human CD4 or with MRC-OX72 for rat CD4 and m6 and m11 and then with a phycoerythrin-conjugated anti-mouse Ig (Immunotech SA, Marseille, France). Exposure of gp41 was detected by staining with MAb (a gift from Zsolla-Panzer) followed by phycoerythrin-conjugated anti-human Ig (Immunotech SA). Cells were washed twice, fixed overnight in PBS–2% formaldehyde, and then analyzed by flow cytometry.

RESULTS

Anti-CD4 MAb binding to fragments of sCD4. The MAbs obtained were tested for their reactivities with sCD4(d1+2) and sCD4(d3+4), and a total of 11 were cloned for further use. The reactivities of these MAbs are shown in Table 1. As expected, those antibodies reactive with sCD4(d1+2) gave no reaction with sCD4(d3+4) and vice versa (Table 1). All of the new MAbs were shown to bind to cell surface CD4 by fluorescence-activated cell sorter analysis (data not shown).

Epitope mapping of anti-CD4 MAbs recognizing the aminoterminus domains of CD4. The determinants recognized by MAbs binding to D1 and D2 of rat CD4 generated in this and previous studies were characterized by using a series of mutations that had been produced in D1 and D2 in another project (49). Rat CD4 does not bind gp120 of HIV-1, but residues important in the HIV-1 binding site of human CD4 were substituted in the rat sequence in an attempt to make rat CD4 bind HIV-1, as illustrated in Fig. 1.

Mutants were prepared in the CDMS vector and transiently expressed in COS cells. MRC-OX35 and MRC-OX36 bound to m1 (data not shown) but not to m4 (Fig. 2A) indicating that epitopes in the B-C loop of D2 are required for binding of these MAbs. m11, which contained changes in the F-G loop in addition to changes in the C'-D region, caused the loss of MRC-OX64, -65, and -66 reactivity (Fig. 2B). As m6 contained only the C'-D region changes, the epitope for these MAbs must involve the F-G turn. In summary, three groups of MAbs were mapped: W3/25 (with MRC-OX37, -38, and -63) to the C'-C' region of D1 (data not shown) (14); MRC-OX64, -65, and -66 to the F-G turn in D1; and MRC-OX35 and -36 to the B-C region of D2.

Inhibition of viral entry by anti-rat CD4 MAbs. m6 binds gp120 with an affinity of two- to threefold less than that of human CD4 (49) and is an efficient receptor for HIV-1 when expressed on the surface of HeLa cells (51). HeLa m6 cells were preincubated with each of the m6-binding MAbs for 1 h

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**TABLE 1. Binding of anti-CD4 MAbs to domains of rat sCD4**

<table>
<thead>
<tr>
<th>MAb</th>
<th>IgG subclass</th>
<th>Binding to target protein*</th>
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<tr>
<td></td>
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<td>1</td>
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</table>

*Mean optical density at 405 nm for MAb binding to duplicate microtiter plate wells. No binding to any sCD4 preparation by an irrelevant IgG1 control MAb (OX21) was observed.
at 37°C. MRC-OX-63 was used as a positive control, as it binds the CDR2 region of rat CD4 and, consequently, does not bind m6, in which this region has been replaced with its human homolog. The cells were then challenged with HIV-1IIIB, and the level of virus entry was quantitated by using a PCR-based technique as described in Materials and Methods. (CD4 MAbs were present at all times during the course of the experiment, including during challenge with virus and after washing of cells.) The MAbs that bind the CDR3-equivalent region of CD4 D1 (MRC-OX-64, MRC-OX-65, and MRC-OX-66), the D2-binding MAbs (MRC-OX-35 and MRC-OX-36), and two of the D3D4-binding MAbs (MRC-OX-67 and MRC-OX-68) all significantly inhibited viral entry, while the control MAb and those binding other epitopes did not (Fig. 3A).

As a second measure of viral entry, HeLa m6 cells which had been preincubated with the MAbs at 37°C for 1 h were challenged with COC (HIV) pseudotype virus as described in Materials and Methods. The results confirm the PCR data, with the same set of MAbs inhibiting entry of pseudovirus (Fig. 3B).

Several anti-rat CD4 MAbs inhibit syncytium formation. HeLa m6 cells were preincubated with each of the anti-rat CD4 MAbs and then mixed with an equal number of HIV Env-expressing H32 cells. After overnight incubation in the continual presence of antibody, the cells were fixed and stained, and the number of syncytia was quantitated (Fig. 4). As expected, all of the MAbs that inhibited viral entry also inhibited syncytium formation. However, the D3D4-specific MAb, MRC-OX-71, also interfered with syncytium formation, although it had no effect on virus entry (Fig. 3). Very slight enhancement of syncytium formation was observed in the presence of MRC-OX-69.

Effect of the anti-rat CD4 antibodies on binding of monomeric gp120 to HeLa m6 cells. In order to determine whether binding of any of the anti-rat CD4 MAbs to HeLa m6 cells affected binding of recombinant monomeric gp120, a flow cytometry assay was developed. After a 1-h incubation of HeLa m6 cells with each of the anti-rat CD4 MAbs, gp120 was added to a final concentration of 10 μg/ml. This concentration is sufficient to saturate the gp120-binding sites on the surface of HeLa m6 cells (results not shown). The cells were incubated for a further 1.5 h on ice, and the amount of bound gp120 was determined by flow cytometry as described in Materials and Methods. The results presented in Fig. 5 demonstrate that binding of soluble, monomeric gp120 was not inhibited by any of the anti-rat CD4 MAbs.

Effect of gp120 on binding of anti-rat CD4 MAbs to HeLa m6 cells. In a reciprocal experiment, HeLa m6 cells were incubated with 50 μl of either PBS–0.5% BSA or gp120 (10 μg/ml) for 1.5 h at 4°C. These cells were then washed and stained with each of the m6-binding anti-rat CD4 MAbs and with MRC-OX-63 (as a negative control), as described in Materials and Methods. Prebinding of gp120 resulted in a reduction of at least 50% in binding of the D1 CDR3-directed MAbs (MRC-OX-64, MRC-OX-65, and MRC-OX-66) and of the D3-binding MAbs (MRC-OX-67 and MRC-OX-68) (Fig. 6). There was no significant reduction in binding of any of the other anti-rat CD4 MAbs.

The loss of binding of certain MAbs induced by monomeric gp120 was unexpected, as these MAbs did not affect binding of monomeric gp120 to CD4. One possible explanation is that gp120 induces a conformational change in CD4 that leads to a loss of the epitopes bound by these MAbs. In the case of the D1 CDR3-directed MAbs, this seems unlikely, as there would have to be a conformational change within an Ig domain, a structural motif known to be rigid. A more plausible explanation is that there is a conformational change in gp120 that leads to a masking of the D1 CDR3 loop. A gp120-induced conformational change in CD4 leading to the loss of the MRC-OX-67/68 epitope is conceivable, as this would require interdomain changes. The possibility of such conformational changes between the D1D2 and D3D4 halves of CD4, around the hinge region, has previously been proposed (24).

Numerous epitopes of m6 are masked after binding to cell surface, oligomeric Env. On the surface of virions, Env is oligomeric, and consequently, binding of CD4 to virions may differ from binding of CD4 to recombinant, monomeric gp120. Binding of a soluble form of m6 (sm6) to oligomeric Env was assessed by flow cytometry of chronically HIV-1IIIB-infected H9 cells (H9/HIV-1IIIB), as described in Materials and Methods.

The concentration of sm6 necessary to saturate H9/HIV-1IIIB cells (10 μg/ml) was determined as described in Materials and Methods (results not shown). This concentration is greater
FIG. 2. Epitope mapping of D1- and D2-directed MAbs. Fluorescence-activated cell sorter histograms of MAb tissue culture supernatants binding to COS cells transfected with the mutants of CD4 are shown, with dotted lines showing staining with the negative control MAb MRC-OX-21. (A) Localization of the epitopes for MRC-OX-35 and -36 to D2 of CD4 (m4). (B) Localization of the epitopes for MRC-OX-64, -65, and -66 to the F-G turn of D1 of CD4 (m11).
than that previously determined for human CD4 (48), and this is likely to reflect the lower affinity for gp120 of m6 compared to human CD4 (49).

Next, H9/HIV-1_{∆env} cells were saturated with sm6 and stained with each of the anti-rat CD4 MAbs as described in Materials and Methods. The epitopes for the D1 MAbs (MRC-OX-64, MRC-OX-65, and MRC-OX-66), the D2 MAbs (MRC-OX-35 and MRC-OX-36), and some of the D3D4 MAbs (MRC-OX-72 and MRC-OX-73) were masked (Fig. 7). There were various fluorescence intensities for all of the other MAbs (MRC-OX-67, MRC-OX-68, MRC-OX-69, MRC-OX-70, and MRC-OX-71), which may reflect various degrees of exposure of the epitopes bound by these MAbs or a variation in the valency of binding to sm6.

In order to address this point more specifically, we constructed a mutant (m11) in which the F-G turn of m6 was replaced with the equivalent residues of human CD4 (Fig. 1). The affinities of sm6 and sm11 for HIV-infected H9 cells and their abilities to induce exposure of gp41 were measured as described in Materials and Methods. The results presented in Fig. 8 demonstrate that the replacement of rat with human sequence in the CDR3 region of m6 has no effect either on its affinity for oligomeric Env or exposure of gp41 epitopes. Instead, we found that the degree of gp41 exposure could be directly correlated to the affinity of CD4 for gp120 by using a
panel of rat-human chimeric CD4 molecules with various gp120 affinities (results not shown).

**DISCUSSION**

In the work presented here, we have used a chimera of rat and human CD4, which acts as a functional receptor for HIV-1 (49, 51), to examine the role played by regions of CD4 distant from the gp120-binding site during the postbinding events that lead to membrane fusion. A panel of anti-rat CD4 MAbs, which bind this chimera but not human CD4, was used in an effort to determine the mechanisms by which anti-CD4 MAbs interfere with virus entry and syncytium formation without apparently blocking binding of virus (summarized in Fig. 6).

**D1.** The binding site for gp120 is contained within the CDR2-equivalent region of CD4 D1 (1, 2, 6, 10, 33, 39, 47, 49). Following binding to CD4, the spike glycoproteins of HIV undergo a series of conformational changes that lead to virus-cell fusion (35, 36, 48). It has become evident that these conformational changes cannot result from specific interactions of gp120 with the D1 CDR3 region of CD4 (5, 34, 43, 51), although subtle strain-specific effects have been reported (50). However, MAbs that bind this region of human CD4 inhibit virus replication and syncytium formation without apparently blocking binding of virus (summarized in Fig. 6).

**D2.** The anti-rat CD4 MAbs MRC-OX-35 and MRC-OX-36 bind a region of rat CD4 equivalent to that bound by the anti-human CD4 MAb 5A8, which was found to inhibit virus replication and syncytium formation (7). The mechanism by which the MAbs to this D2 epitope inhibit virus entry and syncytium formation appears to be similar, although not identical, to that of the anti-D1 CDR3 MAbs (34, 37, 52). MAbs that bind either of these epitopes reduce binding of sCD4 to membrane-bound oligomeric Env but do not affect binding of monomeric gp120 to CD4 (34, 37). In addition, we have shown that both of these epitopes are masked when sm6 has saturated the oligomeric Env sites on the surface of HIV-1-infected cells. However, the D2 epitopes differ from the D1 CDR3 epitope, as they are not masked following binding to monomeric gp120. A possible explanation for this is that the binding of multiple molecules of CD4 to oligomeric Env orients the epitope toward the interior of the complex, thereby concealing it from antibody.

**D3 and D4.** A role for the D3D4 moiety of CD4 in postbinding events has previously been implicated from work with anti-human CD4 MAbs (23, 24) and mutagenesis studies (22, 25), and such a role is also indicated by the involvement of the D3D4 epitope in the inhibition of virus entry and syncytium formation by the anti-D3 MAbs (Table 1). However, this role may be indirect, involving a region of CD4 close to the membrane that is involved in the postbinding events downstream of virus-cell attachment.

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**FIG. 5.** Effect of anti-rat CD4 MAbs on the binding of monomeric gp120 to HeLa m6 cells. HeLa m6 cells were incubated for 1 h at 4°C with each of the MAbs, washed, and then incubated for a further 1.5 h at 4°C with 10 µg of gp120 per ml. The cells were then washed and incubated with biotinylated anti-gp120 MAb for 45 min on ice. After further washing, the cells were stained with streptavidin-FITC and analyzed by flow cytometry. The graph shows the mean fluorescence channels with standard errors for duplicate samples. As a negative control (C), untransfected HeLa cells were used, and as a positive control, cells preincubated with MRC-OX63 were used. Each of the MAbs is indicated by its MRC-OX number.

**FIG. 6.** Effect of monomeric gp120 on the binding of anti-rat CD4 MAbs to HeLa m6 cells. HeLa m6 cells were incubated for 1.5 h at 4°C with either PBS–0.1% BSA (filled bars) or 10 µg of monomeric gp120 per ml (hatched bars). The cells were then washed and incubated with each of the anti-rat CD4 MAbs (shown by their MRC-OX numbers) for a further 1 h. After further washing, the cells were stained with RAM-FITC and analyzed by flow cytometry. The graph shows the mean fluorescence channels for duplicate determinations with standard errors.
41). The MAbs against D3D4 of rat CD4 can be placed in distinct functional groups as a result of our experiments. MRC-OX-67 and MRC-OX-68 form the first group. These MAbs partially inhibit cell-free virus entry and syncytium formation, and their epitopes are partially masked by pre-binding of monomeric gp120. However, in contrast to the D1 CDR3 and D2 antibodies, they bind normally to sm6 complexed with oligomeric Env. In these respects, they appear to be analogous to the anti-human CD4 MAb Q425, which binds close to the top of D3, near the hinge region (24). Studies with the Q425 MAb have led to the suggestion that flexion about the hinge of CD4 may be involved in the post-binding events that lead to fusion (24).

The second group of D3D4 MAbs comprises MRC-OX-69 and MRC-OX-71, both of which modulate syncytium formation, albeit modestly, but have no effect on entry of cell-free virus. This suggests that there may be differences in the mode of interaction between CD4 and gp120 during syncytium formation and entry of cell-free virus. However, a modest alteration in the kinetics of fusion caused by binding of MRC-OX-69 or MRC-OX-71 could explain this observation, given that syncytium formation requires more interactions between Env and CD4 than does entry of cell-free virus.

A third group of D3D4 MAbs includes MRC-OX-72 and MRC-OX-73, which do not interfere with HIV infection or syncytium formation and yet have epitopes that are masked by interaction with oligomeric but not monomeric gp120. This shows that the results obtained from this system to examine binding of CD4 to oligomeric Env, although more reliable than those obtained from investigating monomeric interactions, must still be treated with caution.

The system utilized for analyzing CD4-gp120 interactions is critical, particularly when assessing the effects of MAbs on this interaction. Although analysis of the binding of recombinant, monomeric gp120 to sCD4 or cell surface CD4 reveals important clues, the true physiological picture represents an even greater level of complexity, since both the Env glycoproteins and CD4 will be in an oligomeric, membrane-bound form. The packing of gp120-CD4 complexes is therefore more constrained in a virus-cell interaction than in monomeric gp120-CD4 interactions, and consequently, a MAb that has little or
no effect on formation of this complex when the constituents are monomeric may well interfere with appropriate binding in the oligomeric system.

In summary, anti-CD4 MAbs affect virus entry and syncytium formation of m6-expressing cells in a way analogous to that of MAbs against human CD4. For example, the CDR3-directed mAbs, MRC-OX-64, -65, and -66, may be analogous to the anti-human CD4 MAB L71 (52); the D2-directed MAbs, MRC-OX-35 and -36, may be analogous to 5A8 (7); and the D3 MAbs, MRC-OX-68 and -69, may be analogous to Q425 (24). This conservation of function shows that while the primary sequence of CD4 epitopes outside the gp120-binding site does not appear to influence cell-virus fusion, binding of MAbs to some of these regions does. One mechanism by which these inhibitory MAbs may act is by sterically interfering with packing of CD4 molecules to oligomers of Env in the crowded environment on the virion surface. In addition, some MAbs may sterically hinder molecular rearrangements induced by the CD4-gp120 interaction either in gp120-gp41 oligomers or around the hinge of CD4.

Recently, several HIV-1 fusion cofactors have been identified (9, 17–20). The relationship of these cofactors to the Env-CD4 interaction remains to be elucidated at the molecular level. As there is little sequence identity between m6, a functional (9, 17–20). The relationship of these cofactors to the Env-CD4 interaction either in gp120-gp41 oligomers or around the hinge of CD4.

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ACKNOWLEDGMENTS

This work was funded by the MRC AIDS-Directed Programme; by the ANRS, CNRS, and INSERM of France; and by the EC Concerted Action “Virus-cell membrane interactions.”

We thank Simon Davis and John Moore for useful discussions and Vinod Achen and Harsh Sharma for help in preliminary characterization of the CD4 MAbs.

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