Differential Inhibition of Human Immunodeficiency Virus Type 1 Fusion, gp120 Binding, and CC-Chemokine Activity by Monoclonal Antibodies to CCR5

WILLIAM C. OLSON,1 GWÉNAËL E. E. RABUT,2 KIRSTEN A. NAGASHIMA,1 DIEP N. H. TRAN,1 DEBORAH J. ANSELMA,1 SIMON P. MONARD,2 JEREMY P. SEGAL,2 DANIAH A. D. THOMPSON,2 FRANCIS KAJUMO,2 YONG GUO,2 JOHN P. MOORE,2 PAUL J. MADDO,1, and TATJANA DRAGIC2*

Aaron Diamond AIDS Research Center, The Rockefeller University, New York, New York 10016,2 and Progenics Pharmaceuticals, Inc., Tarrytown, New York 105911

Received 28 December 1998/Accepted 12 February 1999

The CC-chemokine receptor CCR5 mediates fusion and entry of the most commonly transmitted human immunodeficiency virus type 1 (HIV-1) strains. We have isolated six new anti-CCR5 murine monoclonal antibodies (MAbs), designated PA8, PA9, PA10, PA11, PA12, and PA14. A panel of CCR5 alanine point mutants was used to map the epitopes of these MAbs and the previously described MAb 2D7 to specific amino acid residues in the N terminus and/or second extracellular loop regions of CCR5. This structural information was correlated with the MAbs' abilities to inhibit (i) HIV-1 entry, (ii) HIV-1 envelope glycoprotein-mediated membrane fusion, (iii) gp120 binding to CCR5, and (iv) CC-chemokine activity. Surprisingly, there was no correlation between the ability of a MAb to inhibit HIV-1 fusion-entry and its ability to inhibit either the binding of a gp120-soluble CD4 complex to CCR5 or CC-chemokine activity. MAbs PA9 to PA12, whose epitopes include residues in the CCR5 N terminus, strongly inhibited gp120 binding but only moderately inhibited HIV-1 fusion and entry and had no effect on RANTES-induced calcium mobilization. MAbs PA14 and 2D7, the most potent inhibitors of HIV-1 entry and fusion, were less effective at inhibiting gp120 binding and were variably potent at inhibiting RANTES-induced signaling. With respect to inhibiting HIV-1 entry and fusion, PA12 but not PA14 was potently synergistic when used in combination with 2D7, RANTES, and CD4-immunoglobulin G2, which inhibits HIV-1 attachment. The data support a model wherein HIV-1 entry occurs in three stages: receptor (CD4) binding, coreceptor (CCR5) binding, and coreceptor-mediated membrane fusion. The antibodies described will be useful for further dissecting these events.

Human immunodeficiency virus type 1 (HIV-1) induces viral-to-cell membrane fusion to gain entry into target cells (9, 15, 63). The first high-affinity interaction between the virion and the cell surface is the binding of the viral surface glycoprotein gp120 to the CD4 antigen (13, 28, 37, 38). This in turn induces conformational changes in gp120, which enable it to interact with one of several chemokine receptors (5, 6, 19, 33). The CC-chemokine receptor CCR5 is the major coreceptor for macrophage-tropic (R5) strains and plays a crucial role in the transmission of HIV-1 (5, 6, 19, 33). T-cell line-tropic (X4) viruses use CXCR4 to enter target cells and usually, but not always, emerge late in disease progression or as a consequence of virus propagation in tissue culture (5, 6, 19, 33). Some primary HIV-1 isolates are dualtropic (R5X4) since they can use both coreceptors, though not always with the same efficiency (12, 53). Mutagenesis studies coupled with the resolution of the gp120 core crystal structure have demonstrated that the coreceptor-binding site on gp120 includes several highly conserved residues (30, 49, 62).

We and others have demonstrated that tyrosines and negatively charged residues in the amino-terminal domain (Nt) of CCR5 are essential for gp120 binding to the coreceptor and for HIV-1 fusion and entry (7, 16, 18, 20, 25, 29, 48, 50). Residues in the extracellular loops (ECLs) 1 to 3 of CCR5 were dispensable for coreceptor function, and yet the CCR5 interdomain configuration had to be maintained for optimal viral fusion and entry (22). This led us to conclude either that gp120 forms interactions with a diffuse surface on the ECLs or that the Nt is maintained in a functional conformation by bonds with residues in the ECLs. Studies with chimeric coreceptors and anti-CCR5 monoclonal antibodies (MAbs) have also shown the importance of the ECLs for viral entry (6, 50, 60).

Molecules that specifically bind to CCR5 and block interactions with its ligands are a powerful tool to further probe the structure-function relationships of this coreceptor. Characterizing such compounds could also assist in designing effective therapeutic agents that target coreceptor-mediated steps of viral entry. Inhibitors of CCR5 or CXCR4 coreceptor function identified to date are diverse in nature and include small molecules, peptides, chemokines and their derivatives, and MAbs. No small molecule that specifically inhibits only CCR5-mediated fusion has been described, although a distamycin analogue has been reported to inhibit HIV-1 entry and to bind CCR5, CXCR4, and other chemokine receptors (26). Inhibition of HIV-1 entry by CC-chemokines is mediated by at least two distinct mechanisms: blockage of the gp120-coreceptor interaction and internalization of the chemokine-receptor complex (1, 4, 24, 55, 59). The variant AOP-RANTES also inhibits recycling of CCR5 to the cell surface (36, 52). Variants such as RANTES 9-68 and Met-RANTES only prevent the gp120-CCR5 interaction and do not down-regulate CCR5 (64). Three sets of anti-CCR5 MAbs have been previously described (25, 46, 60, 61). Of the approximately 25 MAbs generated, only 2D7 has been shown to inhibit efficiently HIV-1 entry and CC-chemokine-induced calcium mobilization.
(60). The 2D7 epitope is located in ECL2, which also contains the CC-chemokine binding site (51). Several anti-CCR5 MAbs were used to probe differences in epitope presentation when CCR5 is expressed on different cell types or mutated in its Nt region. The patterns of reactivity observed suggested cell-type-specific alterations in CCR5 structure (25). Only one anti-CCR4 MAb, 12G5, has been extensively characterized for its antiviral properties. The efficiency of 12G5 inhibition of viral entry has been reported to be both cell and isolate dependent (59, 54). This MAb binds to ECL2 of CCR4, but the mechanism of HIV-1 entry when this entry is unknown (8).

Using a novel screening procedure that selects for HIV-1 inhibitory activity, we have isolated and characterized a panel of six murine MAbs, designated PA8, PA9, PA10, PA11, PA12, and PA14. All six MAbs specifically bound to CCR5+ cells but with different efficiencies that were cell type dependent. Epitope mapping studies identified the residues that are important for MAb binding and also revealed information about the folding and interactions of the CCR5 extracellular domains. Surprisingly, MAb inhibition of HIV-1 entry and fusion did not correlate with inhibition of either the binding of a gp120-soluble CD4 (sCD4) complex to CCR5 or CC-chemokine activity. Potent synergetic inhibition of HIV-1 entry and fusion was observed when certain CCR5 MAbs were used in combination with other HIV-1 attachment and fusion inhibitors. It is the primary screen antibodies were tested for their ability to inhibit HIV-1 entry and binding, and coreceptor-mediated membrane fusion.

MATERIALS AND METHODS

Reagents. MAb 2D7 was produced from Pharmigen (San Diego, Calif.), and CC- and CXC-chemokines were obtained from R&D Systems (Minneapolis, Minn.), CD4-immunoglobulin G2 (IgG2) (2), sCD4 (3), and recombinant HIV-1Fr-ER gp120 and HIV-1nt-1 gp120 (55) were produced by Progenics Pharmaceuticals, Inc. CD4-IgG2 is an antibody-like recombinant fusion protein in which the D1 and D2 domains of human CD4 are linked to the heavy and light chain constant regions of human IgG2. sCD4 contains the extracellular domains D1 to D4 of CD4.

Isolation and purification of anti-CCR5 MAbs. Murine L1-2-CCR5+ cells (59) were infected with 108 M13 or M15 containing E. coli 1, a plasmid that encodes for the expression of the CD4 protein, and recombinant mouse IgG1. The MAb binding was detected with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG1 (Caltag, Burlingame, Calif.) diluted 1:50. The extent of binding of a MAb to mutant CCR5 was determined by the equation (mutant CCR5 PE MFI/wild-type CCR5 PE MFI)/(mutant CCR5 PE MFI/wild-type CCR5 PE MFI) = 100%. This normalizes MAb binding to the wild-type cell but allows for discrimination of differences in MAb binding to mutants that are due to MAb differences as well as to differences in the cells. The MFI of wild-type CCR5 as measured by the FITC-labeled mouse anti-HA MAb (BabCo, Richmond, Calif.) per ml for intracellular labeling. Finally, cells were washed once with binding buffer and once with DPBS, resuspended in 1% formaldehyde in PBS, and analyzed by flow cytometry. The extent of binding of a MAb to mutant CCR5 was determined by the equation (mutant CCR5 PE MFI/wild-type CCR5 PE MFI)/(mutant CCR5 FITC MFI/wild-type CCR5 FITC MFI) × 100%. This normalizes MAb binding for mutant coreceptor expression levels.

CD4-binding assay. sCD4 and scD4 were biotinylated with N-hydroxysuccinimide–biotin (Pierce, Rockford, Ill.) according to the manufacturer’s instructions, and uncoupled biotin was removed by dialfiltration. Sodium butyrate-treated L1-2-CCR5+ cells were incubated with varying dilutions of the individual biotinylated proteins. A 1:1 equimolar mixture of sCD4 and biotinylated gp120, or 1.25 μg of sCD4 per ml and 2.5 μg of biotinylated gp120 per ml in the presence of varying concentrations of anti-CCR5 MAbs PA5 to PA12, PA14, or 2D7 or a non-specific murine IgG1, for 1 h at room temperature in 0.1% NaCl in DPBS. Cells were washed with the incubation buffer and incubated with streptavidin-PE (Becton Dickinson) diluted 1:50, for 1 h at room temperature. Finally, cells were washed with binding buffer and analyzed with a fluorescence plate reader (PerSeptive Biosystems, Framingham, Mass.).

RESULTS

Isolating anti-CCR5 MAbs PA8, PA9, PA10, PA11, PA12, and PA14. We have found that peptides corresponding to the extracellular domains of CCR5 are inefficient at raising specific, high-titer antibody against the native, cell surface receptor (45, 46). BALB/c mice were immunized, therefore, with murine L1-2-CCR5+ cells, and hybridoma culture supernatants were tested for their ability to inhibit JR-FL envelope-
mediated membrane fusion with CD4+ CCR5+ PM1 cells in the RET assay (17, 35). Of 10,000 hybridoma supernatants screened, well over 100 inhibited fusion by >50%, but only 6—designated PA8, PA9, PA10, PA11, PA12, and PA14—specifically and intensely stained L1.2-CCR5± but not the parental L1.2 cells, as demonstrated by flow cytometry (data not shown). Based on previous experience, we assume that the other MAbs capable of inhibiting fusion were probably directed against cell surface adhesion molecules such as LFA-1 (34). Hybridomas PA8 to PA12 and PA14 were determined by isotyping enzyme-linked immunosorbent assay (Cappel, Durham, N.C.) to secrete IgG1 MAbs. Ascites fluids were prepared from BALB/c mice that were injected with the six hybridomas, and the IgG1 fractions were purified. PA8, PA9, PA11, PA12, and PA14 exhibited distinct isoelectric focusing profiles, whereas PA10 had a profile very similar to that of PA9 and therefore may be a second isolate of the same MAb (data not shown).

**MAB binding to CCR5 transfectants and CD4+ lymphocytes.** Nonspecific mouse IgG1 and IgG2a did not stain either 25%, and PA9 to PA12 stained 6 to 12% (Fig. 1i to o). The PA11, PA12, and PA14 exhibited distinct isoelectric focusing shifts do not reflect differences in MAb affinities for CCR5 as other MAbs remained constant over a wide range of antibody concentrations (data not shown), suggesting that the other shifts do not reflect differences in MAb affinities for CCR5 as expressed on these cells.

Compared with L1.2-CCR5+ cells, 7-day-old CD4+ lymphocytes purified from mitogen-stimulated PBMC exhibited different patterns of staining by the anti-CCR5 MAbs. PA8 did not stain CD4+ lymphocytes whereas 2D7 and PA14 stained >50%, and PA9 to PA12 stained 6 to 12% (Fig. 1i to o). The MFI of the stained CD4+ lymphocytes were low but still higher than those for the PM1 cell line (Fig. 1p). Their rank order was (2D7 > PA14 > PA12 > PA11 > PA10 > PA9 > PA8). This differed somewhat from the order of reactivities observed on CCR5 transfectants. The difference between PA9 MFI and PA14 MFI was fivefold. Other groups have observed similar differences in the ability of anti-CCR5 MAbs to stain stable, CCR5+ cell lines versus PBMC (25). This may be due to cell-specific differences in CCR5 conformation, posttranslational modification, or oligomerization. Alternatively, association with other cell surface molecules may differ between cells. Since an obvious choice for such a molecule would be the CD4 cell surface antigen, which is absent from L1.2-CCR5+ cells and present on PBMC, we also tested the ability of PA8 to PA12, PA14, and 2D7 to stain HeLa cells transiently expressing CCR5 alone or with CD4. We observed no differences in the ability of any of the MAbs to stain cell surface CCR5 in the presence of CD4 (data not shown). If there is an association between these two proteins, it does not involve epitopes recognized by the anti-CCR5 MAbs available to us. Alternatively, an association between CCR5 and CD4 might occur only on primary lymphocytes.

**MAB binding to CCR5 transfectants and CD4+ lymphocytes.** Nonspecific mouse IgG1 and IgG2a did not stain either 25%, and PA9 to PA12 stained 6 to 12% (Fig. 1i to o). The PA11, PA12, and PA14 exhibited distinct isoelectric focusing profiles, whereas PA10 had a profile very similar to that of PA9 and therefore may be a second isolate of the same MAb (data not shown).

**Epitope mapping of the MAbs with CCR5 aline mutants.** None of the antibodies was able to detect reduced and denatured CCR5 protein by Western blotting, indicating that they recognize conformationally sensitive epitopes (data not shown). MAB epitope mapping studies were performed with a panel of 70 aline point mutants of residues in the Nt and ECLs of CCR5. HeLa cells were transfected with mutant or wild-type CCR5 coding sequences appended with C-terminal HA tags and infected with VTF7-3 to boost coreceptor expression. The cells were then incubated with the anti-CCR5 MAbs, binding of which was revealed by a PE-labeled goat anti-mouse IgG (21). A second, intracellular stain was performed with an FITC-labeled anti-HA MAb (BabCo). This internal control allowed us to directly normalize staining by the anti-CCR5 MAbs for mutant coreceptor expression levels on the cell surface. MAB binding to each mutant is expressed as a percentage of binding to wild-type CCR5 (Fig. 2). The expression levels of the CCR5 mutants measured by this technique are equivalent to those detected by dot blotting of plasma membrane extracts of CCR5-expressing cells (18, 22, 48).

Certain point mutations reduced by >50% the binding of most of the antibodies to CCR5. In general, PA8 to PA12 were the most affected and PA14 and 2D7 were the least affected by this class of mutants, which included the cysteine pair C101A and C178A; the Nt mutants Y10A, D11A, and K25A; the ECL1 mutant D95A; the ECL2 mutants K171A/E172A, Q188A, and K191A/N192A; and the ECL3 mutants F263A and F264A (Fig. 2). These mutations probably cause conformational perturbations that have a common effect on the binding of all MAbs. However, we cannot exclude that some of these residues are part of the epitopes of some of the MAbs. We assumed that if a mutation lowered binding of an individual MAb by >75% and did not also lower binding of most of the other antibodies, the residue was probably a direct contributor to the epitope recognized by the MAb. Using these stringent guidelines, we concluded that the seven anti-CCR5 MAbs recognize overlapping but distinct epitopes (Fig. 2). MAB PA8 binding to CCR5 depended on N13 and Y15 in the Nt. MAbs PA9 and PA10 required D2, Y3, Q4, P8, and N13 in the Nt and Y176 and T177 in ECL2. MAbs PA9 also required S7 in the Nt. MAbs PA11 and PA12 binding depended on Q4 in the Nt. PA14 required D2 in the Nt and R168 and Y176 in ECL2. Finally, MAB 2D7 required Q170 and K171/E172 in order to bind to CCR5.

**Chemokine signaling in the presence of anti-CCR5 MAbs.** Chemokine receptor-binding agents can be antagonists or, more rarely, agonists of receptor-mediated intracellular signaling. Alternatively, they could have no effect on signaling. CCR5 is able to bind three CC-chemokines, RANTES, MIP-1α, and MIP-1β, and transduce a signal that modulates cytosolic calcium levels. We therefore tested the agonist-antagonist activity of various concentrations of MAbs PA8 to PA12, PA14, and 2D7. Changes in intracellular calcium concentrations, [Ca2+]i, were measured in Indo-1-loaded L1.2-CCR5± cells. None of the MAbs stimulated a change in [Ca2+]i, indicating that they are not agonists for CCR5. PA8 to PA12 were also unable to inhibit Ca2+ fluxes induced by RANTES (Fig. 3a and data not shown), even at concentrations as high as 100 μg/ml, showing that they are not antagonists either. These concentrations provide saturating binding of the MAbs to L1.2-CCR5± cells, as shown by flow cytometry and the gp120-CCR5 binding assay (Fig. 3d and data not shown). MAbs PA14 and 2D7, however, blocked calcium mobilization induced by RANTES, although with different potencies (Fig. 3a and b). The 50% inhibitory concentration (IC50) for PA14 calcium influx inhibition was 45 μg/ml, which was approximately eight-
fold higher than the IC₅₀ for 2D7 (Fig. 3b). RANTES-, MIP-1α-, and MIP-1β-induced calcium fluxes were each inhibited by similar concentrations of PA14 (data not shown). None of the MAbs affected SDF-1-induced calcium mobilization in L1.2-CCR5⁺ cells, which endogenously express murine CXCR4 (data not shown). Finally, neither MAbs nor CC-chemokines affected cytosolic calcium levels in parental L1.2 cells (data not shown).

Inhibition of CCR5 coreceptor function by the MAbs. MAbs PA8 to PA12 and PA14 were initially selected on the basis of their ability to inhibit HIV-1 envelope-mediated membrane fusion. This activity was confirmed and quantified for the purified MAbs. As expected, all six MAbs, as well as MAb 2D7, blocked fusion between CD4⁺ CCR5⁺ PM1 cells and HeLa-EnvJR-FL cells in the RET assay. The rank order of potency was 2D7 > PA14 > PA12 > PA11 > PA10 > PA9 > PA8 (Fig. 4a). IC₅₀ for PA14 and 2D7 were 1.7 and 1.6 μg/ml, respectively; for PA11 and PA12, these were 25.5 and 10.0 μg/ml, respectively (Table 1). PA8, PA9, and PA10 inhibited fusion by only 10 to 15% at 300 μg/ml. None of the MAbs affected fusion between PM1 cells and HeLa-EnvLAI cells, which express the full-length envelope protein from an X4 virus (data not shown).

We also tested the ability of the different anti-CCR5 MAbs
to inhibit entry of an R5 virus, JR-FL, and an R5X4 virus, Gun-1, in a single-round replication, luciferase-based entry assay. We typically measured 10,000 to 20,000 RLU in the absence of antibody and 1 to 5 RLU in the absence of virus. The rank order of potency in the entry assay was similar to the one determined in the fusion assay (Fig. 4b). We were unable to obtain 50% inhibition of JR-FL or Gun-1 entry with PA8 to PA11. The IC50 for PA12 was 2.5 μg/ml; however, we were unable to inhibit entry by 60% with this MAb. The discrepancies between fusion and entry data are probably due to cell type-specific differences such as coreceptor density. The IC50 for PA14 and 2D7 inhibition of JR-FL entry were determined to be 0.024 and 0.026 μg/ml, respectively (Table 2), and were 60-fold lower than those obtained in the fusion assay. Entry of dualtropic Gun-1 was two- to threefold more sensitive to inhibition by anti-CCR5 MAbs than was JR-FL entry (data not shown).

Anti-coreceptor MAbs might inhibit envelope-mediated fusion either by directly affecting the gp120-CCR5 interaction or...
MAbs. L1.2-CCR5 shown). sCD4 was gp120 and CCR5 dependent (Fig. 4c and data not shown). Similarly, binding of biotinylated L1.2-CCR5 interaction. For this, we used an assay that detects binding to the ability of the different MAbs to block the gp120-CCR5 of viral fusion and entry by PA8 to PA12 and PA14, we tested active fusion complex. To examine the mechanism of inhibition by impeding postbinding steps involved in the formation of an

![Image](http://jvi.asm.org/) by Tryptol-Schnapp concentration dependent on residue Q4 in the Nt.

With the exception of PA8, all MAbs abrogated gp120-sCD4 binding to L1.2-CCR5+ (Fig. 3d). MAbs PA9, PA10, PA11, and PA12 inhibited binding with IC_{50} of 0.24, 0.13, 0.33, and 0.24 µg/ml, respectively (Table 1). Surprisingly, MAbs PA14 and 2D7 were among the least efficient inhibitors of gp120-sCD4 binding, with IC_{50} of 1.58 and 1.38 µg/ml, respectively (Table 1). Therefore, there was no correlation between the ability of a MAb to inhibit CCR5-mediated membrane fusion and entry and its ability to block gp120-sCD4 binding to the coreceptor. Inhibition by PA8 saturated at ~40%. Taken together with the flow cytometry data of Fig. 1, this result suggests that PA8 binds to only a subset of CCR5 molecules as expressed on L1.2 transfectants, although other interpretations are possible.

Synergistic inhibition of HIV-1 fusion by combinations of anti-CCR5 MAbs and other viral entry inhibitors. Coreceptor-specific agents may act at multiple stages of the entry process and exhibit nonadditive effects when used in combination. From a clinical perspective, it is important to determine the interactions of coreceptor-specific drug candidates with endogenous chemokines, which may afford some level of protection against disease progression. CCR5 MAbs were therefore tested in combination with each other or with RANTES, or with CD4-IgG2, which binds to gp120 to inhibit HIV-1 attachment to target cells. Dose-response curves were obtained for the agents used individually and in combination in viral fusion and entry assays. Data were analyzed by the median effect principle (10). The concentrations of single agents or their mixtures required to produce a given effect were quantitatively compared in a term known as the combination index (CI). A CI value greater than 1 indicates antagonism, CI = 1 indicates an additive effect, and CI < 1 indicates a synergistic effect wherein the presence of one agent enhances the effect of another.

Combinations of PA12 and 2D7 were the most potently synergistic, with CI values ranging between 0.02 and 0.29, depending on the ratio of the antibodies (Fig. 5 and Table 2). The degree of synergy is known to vary with the stoichiometry of the agents. The viral entry and fusion assays were generally consistent in identifying MAb combinations that are highly synergistic (PA12 and 2D7), moderately synergistic (PA12 and PA14), additive (PA11 and PA12), and weakly antagonistic (PA14 and 2D7). The lack of synergy between PA14 and 2D7 is not surprising given that these MAbs cross-compete for binding to CCR5+ cells as determined by flow cytometry (data not shown). The additive effect observed for the combination of PA11 and PA12 is also consistent with their binding to similar epitopes in CCR5, including a shared dependency on residue Q4 in the Nt.

We also tested the ability of MAbs PA12, PA14, and 2D7 to synergize with RANTES in blocking cell-cell fusion. PA12 and RANTES combinations exhibited moderate synergy (Table 2). PA14 and 2D7 exhibited no synergy with RANTES, which is consistent with these MAbs being inhibitory of RANTES binding and signaling (Fig. 3a and b). Finally, we tested synergy between MAbs PA12, PA14, and 2D7 and CD4-IgG2, which interacts with gp120. We observed moderate synergy between PA12 and CD4-IgG2 over a broad range of concentrations but no synergy between PA14 or 2D7 and CD4-IgG2 when used at concentrations near their IC_{50} (Table 2).

**DISCUSSION**

We have isolated and characterized six murine anti-CCR5 IgG1 MAbs. Whereas PA8, PA9, PA11, PA12, and PA14 are distinct molecular species, PA9 and PA10 are practically indis-
tinguishable by our analyses and therefore are probably the same MAb. All of the MAbs that we isolated recognize complex conformational epitopes, as is often the case with MAbs raised against native, cell surface proteins. Epitope mapping was performed with a panel of CCR5 alanine point mutants. Mutations that affected binding of all MAbs similarly were assumed to cause conformational perturbations in the coreceptor, though we cannot formally exclude that they participate in some of the MAb epitopes. The latter would be especially true if some of these residues were immunodominant. Only two of these residues, Y10 and D11, have been shown to affect HIV-1 entry by anti-CCR5 MAbs was tested in a single-round replication luciferase-based entry assay (b). U87-CΔ4+ CCR5+ cells were infected with NLLuc+ Env+ reporter virus carrying the JR-LF envelope in the presence of 0 to 25 μg of PA8 to PA12 per ml or 0 to 25 μg of PA14 or 2D7 per ml. Luciferase activity (RLU) was measured in cell lysates 72 h postinfection. Results are from a representative experiment and are expressed as percent inhibition of entry = [1 − (RLU in the presence of MAb/RLU in the absence of MAb)] × 100%. Shown is binding of biotinylated (b) gp120, sCD4, and b-gp120-sCD4 complexes to L1.2-CCR5+ cells (c). Strong binding is observed when gp120 derived from the R5 virus HIV-1JR-LF is complexed with an equimolar amount of sCD4. No binding is observed in the absence of sCD4 or for gp120 derived from the X4 virus HIV-1LAI. Background binding to CCR5-L1.2 cells has been subtracted from all curves. Inhibition of gp120-sCD4 binding to L1.2-CCR5+ cells was tested in the presence of varying concentrations of each antibody (d). Cells were preincubated in 96-well plates with an anti-CCR5 MAb followed by an incubation with a saturating concentration of biotinylated gp120-sCD4. Finally, binding of PE-labeled streptavidin to cells was measured with a fluorescence plate reader. Results are from a representative experiment and are expressed as percent inhibition of gp120-sCD4 binding = [1 − (MFI in the presence of MAb/MFI in the absence of MAb)] × 100%.

FIG. 4. Inhibition of CCR5 coreceptor function by anti-CCR5 MAbs. Inhibition of cell-cell fusion by anti-CCR5 MAbs was tested in the RET assay (a). A total of 0 to 250 μg of PA8 to PA12 per ml or 0 to 25 μg of PA14 or 2D7 per ml was added to a mix of HeLa-EnvJR-LF+ and PM1 cells. Results are mean RET values from three independent experiments and are expressed as percent inhibition of fusion = [1 − (% RET in the presence of MAb/RET in the absence of MAb)] × 100%. Inhibition of HIV-1 entry by anti-CCR5 MAbs was tested in a single-round replication luciferase-based entry assay (b). U87-CΔ4+ CCR5+ cells were infected with NLLuc+ Env+ reporter virus carrying the JR-LF envelope in the presence of 0 to 250μg of PA8 to PA12 per ml or 0 to 25 μg of PA14 or 2D7 per ml. Luciferase activity (RLU) was measured in cell lysates 72 h postinfection. Results are from a representative experiment and are expressed as percent inhibition of entry = [1 − (RLU in the presence of MAb/RLU in the absence of MAb)] × 100%. Shown is binding of biotinylated (b) gp120, sCD4, and b-gp120-sCD4 complexes to L1.2-CCR5+ cells (c). Strong binding is observed when gp120 derived from the R5 virus HIV-1JR-LF is complexed with an equimolar amount of sCD4. No binding is observed in the absence of sCD4 or for gp120 derived from the X4 virus HIV-1LAI. Background binding to CCR5-L1.2 cells has been subtracted from all curves. Inhibition of gp120-sCD4 binding to L1.2-CCR5+ cells was tested in the presence of varying concentrations of each antibody (d). Cells were preincubated in 96-well plates with an anti-CCR5 MAb followed by an incubation with a saturating concentration of biotinylated gp120-sCD4. Finally, binding of PE-labeled streptavidin to cells was measured with a fluorescence plate reader. Results are from a representative experiment and are expressed as percent inhibition of gp120-sCD4 binding = [1 − (MFI in the presence of MAb/MFI in the absence of MAb)] × 100%.

VOL. 73, 1999 DIFFERENTIAL INHIBITION OF HIV-1 FUSION BY MAbs 4151

MABS PA8 to PA12 and PA14 stained CCR5+ cells with different intensities and in a cell type-dependent manner. All of the MAbs that we isolated recognize complex conformational epitopes, as is often the case with MAbs raised against native, cell surface proteins. Epitope mapping was performed with a panel of CCR5 alanine point mutants. Mutations that affected binding of all MAbs similarly were assumed to cause conformational perturbations in the coreceptor, though we cannot formally exclude that they participate in some of the MAb epitopes. The latter would be especially true if some of these residues were immunodominant. Only two of these residues, Y10 and D11, have been shown to affect HIV-1 entry by anti-CCR5 MAbs was tested in a single-round replication luciferase-based entry assay (b). U87-CΔ4+ CCR5+ cells were infected with NLLuc+ Env+ reporter virus carrying the JR-LF envelope in the presence of 0 to 250μg of PA8 to PA12 per ml or 0 to 25 μg of PA14 or 2D7 per ml. Luciferase activity (RLU) was measured in cell lysates 72 h postinfection. Results are from a representative experiment and are expressed as percent inhibition of entry = [1 − (RLU in the presence of MAb/RLU in the absence of MAb)] × 100%. Shown is binding of biotinylated (b) gp120, sCD4, and b-gp120-sCD4 complexes to L1.2-CCR5+ cells (c). Strong binding is observed when gp120 derived from the R5 virus HIV-1JR-LF is complexed with an equimolar amount of sCD4. No binding is observed in the absence of sCD4 or for gp120 derived from the X4 virus HIV-1LAI. Background binding to CCR5-L1.2 cells has been subtracted from all curves. Inhibition of gp120-sCD4 binding to L1.2-CCR5+ cells was tested in the presence of varying concentrations of each antibody (d). Cells were preincubated in 96-well plates with an anti-CCR5 MAb followed by an incubation with a saturating concentration of biotinylated gp120-sCD4. Finally, binding of PE-labeled streptavidin to cells was measured with a fluorescence plate reader. Results are from a representative experiment and are expressed as percent inhibition of gp120-sCD4 binding = [1 − (MFI in the presence of MAb/MFI in the absence of MAb)] × 100%.

FIG. 4. Inhibition of CCR5 coreceptor function by anti-CCR5 MAbs. Inhibition of cell-cell fusion by anti-CCR5 MAbs was tested in the RET assay (a). A total of 0 to 250 μg of PA8 to PA12 per ml or 0 to 25 μg of PA14 or 2D7 per ml was added to a mix of HeLa-EnvJR-LF+ and PM1 cells. Results are mean RET values from three independent experiments and are expressed as percent inhibition of fusion = [1 − (% RET in the presence of MAb/RET in the absence of MAb)] × 100%.
One explanation for cell type-specific staining by MAbs would be that activated PBMC (and monocytes) secrete CC-chemokines that bind to cell surface CCR5, masking some MAb epitopes. However, one would expect this to be especially true for PA14 and 2D7, which are antagonists of chemokine-induced calcium mobilization and presumably compete with CC-chemokines for binding to CCR5. Yet these MAbs stain CD4+ lymphocytes the most intensely. Alternatively, differential CCR5 epitope exposure may reflect cell type-specific receptor oligomerization, association with other cell surface molecules, or different posttranslational modifications such as glycosylation. We have shown that differences in MAb binding probably do not reflect cell type-specific differences in CD4-CCR5 interactions.

A >90% inhibition of fusion could be attained with PA11, PA12, and PA14, and >90% inhibition of entry could be attained with PA14. The most potent of the six MAbs in blocking fusion and entry was PA14, which was slightly more effective than 2D7. Surprisingly, PA14 and 2D7 were among the least potent inhibitors of gp120-sCD4 binding to L1.2-CCR5+ cells, whereas PA9 to PA12 blocked with similar potencies, and PA8 was unable to block >90% of gp120-sCD4 binding.

However, even at antibody concentrations of 300 μg/ml, PA8, PA9, and PA10 blocked cell-cell fusion by <15% and viral entry by <40%. It is thus somewhat puzzling that these hybridomas came to be selected during primary screening, which employed the identical cell-cell fusion assay. One possibility is that the secreted MAbs acted synergistically with chemokines or other factors in the hybridoma supernatants. Another possibility is that their potency was diminished during subcloning and purification.

Inhibition of cell-cell fusion required in some cases almost 2 orders of magnitude more antibody than what was needed to block viral entry. Presumably, more gp120-CD4-CCR5 interactions as well as interactions between adhesion molecules are established and act cooperatively during cell-cell fusion, compared to virus-cell fusion, making it more difficult to inhibit. This is commonly observed with antibodies to LFA-1 or to the HIV-1 envelope glycoprotein (41, 47).

The low staining of CD4+ lymphocytes and the partial inhibition of fusion and entry by some of our MAbs suggest that they are able to bind to only a subset of CCR5 molecules expressed on primary CD4+ lymphocytes, PM1 and U87MG-CD4+ CCR5+ cell lines. Yet, other than PA8, all MAbs are still unable to detect gp120-sCD4 binding to them (data not shown). Overexpression of CCR5 on L1.2, along with other cell-specific factors, therefore might favor a coreceptor conformation that prominently exposes the Nt, making it more accessible to both MAbs and gp120. Such a conformation might be induced by receptor oligomerization, by diminished or altered associations with cell surface proteins, or by receptor interactions with G proteins (23, 58). Do multiple conformations of CCR5 coexist on the cell surface, and are they permissive for viral entry? The patterns of MAb reactivity would
suggest so, since HIV-1 entry and fusion can occur, albeit at reduced levels, in the presence of MAb concentrations that saturate epitopes required for gp120 binding to L1.2-CCR5+ cells. We favor the hypothesis that the coreceptor molecules present on L1.2-CCR5+ cells possess one HIV-1 entry-competent conformation whereas CCR5 molecules on PBMC, PM1, and CCR5+ U87MG exist in multiple entry-competent states that display different MAb reactivities. Whereas PA14 and 2D7 may recognize all conformations, other MAbs may not. Why L1.2 cells are conducive to a particular coreceptor conformation remains to be determined.

No obvious correlation was observed between the HIV-1 and CC-chemokine inhibitory activities of the MAbs. PA8 to PA12 did not inhibit CC-chemokine induced calcium mobilization in L1.2-CCR5+ cells, nor did they mediate signaling through CCR5. Compared with 2D7, PA14 is equipotent at inhibiting HIV-1 fusion and entry but 10-fold less potent at inhibiting RANTES-induced signaling. Whereas 2D7 and RANTES binding maps primarily to ECL2, the PA14 epitope maps to both ECL2 and the Nt domain and may have less potential for steric overlap. These data demonstrate the feasibility of developing chemokine receptor-specific HIV-1 inhibitors that do not block normal receptor activity, an observation with considerable therapeutic implications.

Synergy between CCR5 MAbs and other viral entry inhibitors may reflect their interactions with distinct epitopes that are involved in interdependent, consecutive steps of HIV-1 entry. The degree of synergy observed between PA12 and 2D7 (CI < 0.1 under many circumstances) is extraordinary since CI values <0.2 are rarely observed for combinations of anti-HIV-1 antibodies (31, 32, 57), reverse transcriptase inhibitors (27), or protease inhibitors (40). Because of its potency, the PA12-2D7 combination was examined in multiple assay formats and concentration ratios, for which consistently high levels of synergy were observed. The PA12-2D7 combination did not act as a CCR5 agonist. Moreover, the presence of PA12 had no effect on the ability of 2D7 to block RANTES signaling in L1.2-CCR5+ cells (data not shown), indicating that the mechanism of synergy does not involve receptor down-regulation.

Important synergies were also observed when PA12 was used in combination with RANTES, but we do not know to what extent RANTES-induced CCR5 down-regulation contributed to this phenomenon. Moderate synergy was observed for PA12 combined with PA14. Agents that bind similar regions on CCR5 (PA11-PA12 or PA14-2D7-RANTES) generally displayed additive or antagonistic effects when used in combination. We also observed synergy between PA12 and CD4-IgG2. The CD4-gp120 complex is metastable, and if it is unable to interact with a coreceptor, it decays into a nonfusogenic state (41–44). Synergy would be expected if PA12 prevented the activated virus from interacting with CCR5 prior to decay. The lack of synergy between MAbs PA14 and CD4-IgG2 suggests that they act on two nonconsecutive and independent steps of viral entry.

We and others have recently demonstrated that determinants of gp120 binding reside in the first 20 residues of the CCR5 Nt domain and center on a region spanning Y10 to E18 (18, 20, 22, 48). MAbs that map to this region potently block the gp120-CCR5 interaction but are not nearly as efficient at inhibiting HIV-1 fusion and entry into target cells as are PA14 and 2D7, whose epitopes lie outside this region. PA14 recognizes the tip of the Nt and residues in ECL2, whereas the 2D7 epitope seems to be located exclusively in ECL2. At present, we can only speculate about the mechanism of action of these MAbs. It may be that their binding to the first few residues of ECL2 induces conformational changes in the coreceptor that prevent membrane fusion. Alternatively, obstruction of ECL2 epitopes might impede coreceptor oligomerization and the formation of a fusion-competent protein complex. Yet another possibility is that residues in ECL2 line the fusion pore and binding of the MAbs impedes gp41 from inserting the fusion peptide into the plasma membrane. In contrast, MAbs PA8 to PA12 probably inhibit fusion and entry only by directly competing for binding with gp120-CD4 complexes. We do not know if parameters other than epitope exposure and affinity for CCR5 determine the antiviral activity of these MAbs. It is unclear why inhibiting steps subsequent to the gp120-coreceptor interaction would be more efficient than directly blocking that interaction. One possibility is that the off rate of gp120 binding to CCR5 is much lower than the on rate of MAb binding to CCR5. Thus, every time a MAb detaches itself from a coreceptor molecule, a virion-associated gp120 molecule replaces it in a quasi-irreversible fashion since this interaction leads to membrane fusion. Another possibility is that the interactions of CCR5 with oligomeric gp120-gp41 are more complex than that with monomeric gp120.

Our observations are consistent with a model wherein HIV-1 entry occurs in three distinct, sequential steps involving receptor binding, coreceptor binding, and coreceptor-mediated membrane fusion. Separate coreceptor binding and fusion events are suggested by the lack of correlation between the MAbs’ abilities to block gp120 binding and HIV-1 entry-fusion. The chronology of events during fusion is further suggested by the patterns of synergies observed. MAbs that potently inhibit the middle step of the process, namely, gp120 binding, act synergistically with inhibitors of prior and subsequent steps. In keeping with this model, no or weak synergies were observed between agents, such as 2D7 and CD4-IgG2, that act at the first and third steps. The antibodies described herein provide tools for probing these hypotheses.

ACKNOWLEDGMENTS

This work was supported by grants AI43847-01, AI41420-01, and AI40810-01. We thank Alexandra Trkola and Tom Sakmar for helpful advice on experiments.

REFERENCES

Clapham. 1996. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. J. Virol. 70:8355–8360.