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Antigenically Distinct Conformations of CXCR4

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The major human immunodeficiency virus type 1 (HIV-1) coreceptors are the chemokine receptors CCR5 and CXCR4. The patterns of expression of the major coreceptors and their use by HIV-1 strains largely explain viral tropism at the level of entry. However, while virus infection is dependent upon the presence of CD4 and an appropriate coreceptor, it can be influenced by a number of factors, including receptor concentration, affinity between envelope gp120 and receptors, and potentially receptor conformation. Indeed, seven-transmembrane domain receptors, such as CCR5, can exhibit conformational heterogeneity, although the significance for virus infection is uncertain. Using a panel of monoclonal antibodies (MAbs) to CXCR4, we found that CXCR4 on both primary and transformed T cells as well as on primary B cells exhibited considerable conformational heterogeneity. The conformational heterogeneity of CXCR4 explains the cell-type-dependent ability of CXCR4 antibodies to block chemotaxis to stromal cell-derived factor 1α and to inhibit HIV-1 infection. In addition, the MAb most commonly used to study CXCR4 expression, 12G5, recognizes only a subpopulation of CXCR4 molecules on all primary cell types analyzed. As a result, CXCR4 concentrations on these important cell types have been underestimated to date. Finally, while the factors responsible for altering CXCR4 conformation are not known, we found that they do not involve CXCR4 glycosylation, sulfation of the N-terminal domain of CXCR4, or pertussis toxin-sensitive G-protein coupling. The fact that this important HIV-1 coreceptor exists in multiple conformations could have implications for viral entry and for the development of receptor antagonists.

The discovery of the receptors used by human immunodeficiency virus type 1 (HIV-1) to infect cells, coupled with a greater understanding of the membrane fusion-inducing conformational changes undergone by the viral envelope protein (Env) upon receptor binding, has identified several promising drug and vaccine targets (reviewed in reference 12). The viral Env protein binds cell surface CD4 with a high affinity, resulting in conformational changes that enable Env to bind a coreceptor (32, 54, 56). Coreceptor binding is thought to trigger the ability of Env to mediate fusion between the viral and cellular membranes. The major HIV-1 coreceptors are the chemokine receptors CCR5 and CXCR4 (reviewed in reference 11). The R5 virus strains that use CCR5 with CD4 to infect cells are largely responsible for virus transmission and are typically macrophage tropic. The accrual of mutations in Env can lead to X4 virus strains that use CXCR4 in place of CCR5 or R5X4 virus strains that can use both receptors. While X4 virus strains do not always evolve in infected individuals, their emergence is a harbinger of progression to AIDS (51, 52).

While virus infection is dependent upon the presence of CD4 and an appropriate coreceptor, it can be influenced by receptor concentration (21, 29, 30, 43, 48), affinity between Env and receptors (28), and potentially receptor conformation (33). Generally, the efficiency of virus entry falls as coreceptor levels fall, although some infection is still observed even when coreceptor levels are very low. The affinity between Env and coreceptors may also prove to be important. In at least one case, changes in a viral Env protein associated with increased pathogenicity have been associated with increased coreceptor affinity (28). Finally, seven-transmembrane domain receptors, such as CCR5, can exhibit conformational heterogeneity, although the significance for virus infection is uncertain (1, 33, 35).

Small-molecule inhibitors of both CCR5 and CXCR4 have been described (1, 14–16, 39). An effective coreceptor inhibitor could prevent virus infection by down-regulating the coreceptor or by directly interfering with Env-receptor interactions, thus effectively reducing coreceptor concentration. The CXCR4 inhibitors described to date appear to directly block Env-CXCR4 interactions and to do so without inducing receptor down-regulation (1, 14, 16, 39). Small-molecule inhibitors could block virus infection by altering receptor conformation, either inhibiting Env-coreceptor binding or reducing the affinity of the interaction (20). The small-molecule inhibitor of CCR5, TAK779, may fall into this category. TAK779 likely binds to a hydrophobic pocket formed largely by the transmembrane domain helices of CCR5, a region that until now has not been directly implicated in coreceptor function (20). Nonetheless, it effectively blocks Env-CCR5 binding (20). It is clear that a greater appreciation of coreceptor expression, conformation, and Env-coreceptor interactions is needed to fully understand the mechanism by which existing receptor inhibitors function and to develop more effective receptor antagonists.

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In this study, we have produced a panel of monoclonal antibodies (MAbs) to CXCR4 and have used these to study CXCR4 expression and conformation on cell lines and primary T and B cells. We found that CXCR4 on both primary and transformed T cells and on freshly isolated B cells often exhibits conformational heterogeneity, while CXCR4 on most B-cell lines tested does not. The conformational heterogeneity of CXCR4 explains the cell-type-dependent ability of CXCR4 antibodies to block stromal cell-derived factor 1α (SDF-1α)-induced chemotaxis and HIV-1 infection. In addition, the MAb most commonly used to study CXCR4 expression, 12G5, recognizes only a subpopulation of CXCR4 molecules on T cells and freshly isolated B cells. As a result, CXCR4 concentrations on these important cell types have often been underestimated to date. While the factors responsible for altering CXCR4 conformation are not known, the fact that this important HIV-1 coreceptor exists in multiple conformations could have implications for viral entry and for the development of receptor antagonists.

MATERIALS AND METHODS

Cell lines. The following cell lines were obtained from the American Type Culture Collection: Jurkat $CD4^+$ (TIB-152; T lymphocytes), Jurkat $CD4^-$ (CRL-10915; T lymphocytes), RS4:11 (CRL-1873; pro-B-cell line), HS-Sultan (CRL-1484; mature B-cell line), and Reh (CRL-8286; pro-B-cell line). The following cell lines were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program: PM1 (T-cell line), Hut78 (T-cell line), and CMX174 (hybrid human B-cell–T-cell line). The following cell lines were provided by J. A. Hoxie: SupT1 (non-Hodgkin's T-cell lymphoma), BC7 ($CD4^-$ SupT1 clone), Nalm6 (pre-B-cell line), and C8166 (T-cell line). Blin1 (pre-B-cell line) were a courtesy of M. Z. Ratajczak. MAbs 1, 2, 8, 12, 16, 17 and 18 were generated by immunizing BALB/c mice with syngeneic mouse 3T3 fibroblasts stably transfected to express the full-length human CXCR4 sequence (R&D Systems, Minneapolis, Minn.). Primary peripheral blood lymphocytes (PBLs) were purified by Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) and kept with interleukin-2 (IL-2; 20 U/ml) alone or with IL-2 and phytohemagglutinin (PHA; 10 μg/ml). All cell lines and primary cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Antibodies and reagents. The following purified antibodies were used in this study: anti-CXCR4 mouse MAbs 1, 2, 8, 12, 16, 17, and 18—clones 44701.111 (immunoglobulin G2b [IgG2b]), 44702.111 (IgG2b), 44708.111 (IgG2a), 44712.111 (IgG2a), 44716.111 (IgG2b), 44717.111 (IgG2b), and 44718.111 (IgG2b), respectively (R&D Systems); 12G5 (IgG2a) (kindly provided by J. A. Hoxie); 4G10 (kindly provided by C. C. Broder); anti-CD4 mouse MAb Leu3A (Becton Dickinson); mouse immunoglobulins (mIgs) (Sigma, St. Louis, Mo.); fluorescein isothiocyanate-conjugated anti-CD4 (Caltag, Burlingame, Calif.); phycoerythrin (PE)- or allophycocyanin-conjugated anti-CD8 (Caltag); Tricolor-conjugated anti-CD19 (Caltag); PE-conjugated goat anti-mouse Fab fragments (Caltag); PE-conjugated horse anti-mouse serum (Vector Laboratories, Inc.); PE-conjugated 12G5 (FAB170P; R&D Systems); and PE-conjugated MAb 17 (FAB173P; R&D Systems). Human SDF-1α was obtained from Peprotech, Inc.

Flow cytometry. 293T cells were transfected (via $CaPO_4$ precipitation) with various constructs and allowed to express for 48 h. Prior to primary antibody staining, transfected 293T cells were lifted off and washed with fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline supplemented with 3% fetal calf serum and 0.05% sodium azide). Lymphocytic cell lines were used in logarithmic growth phase. Antibodies were added at a final concentration of 10 μg/ml, except for 4G10 (25 μg/ml), and incubated for 30 min on ice. The samples were washed, incubated with PE-conjugated goat anti-mouse Fab fragments (1:100 dilution) or PE-conjugated horse anti-mouse serum (1:100 dilution) for 30 min on ice, washed, and fixed in 1% paraformaldehyde. For whole blood, 200 μl was stained in three steps of 30 min each on ice. In the first step anti-CXCR4 was added, in the second step PE-conjugated goat anti-mouse Fab fragments were added, and in the third step fluorescein isothiocyanate-conjugated anti-CD4, Tricolor-conjugated anti-CD19, or allophycocyanin-conjugated anti-CD8 was used. This last step was carried out in the presence of 20 μg of mIgs/ml. Red blood cells were lysed using CAL-Lyse (Caltag) according to the manufacturer's instructions. Pertussis toxin was used at a final concentration of 100 ng/ml and was incubated with cells for 8 to 16 h before processing for flow cytometry was done.

Plasmids. Plasmids expressing CXCR4/CXCR2 or rat-human CXCR4 chimeras were previously described (6, 18). The CXCR4 N-terminal truncations, i.e., Δ12-, Δ15-, and Δ23-CXCR4, were constructed by PCR using the following primers: 5′-CGG AAT TCT TAG CTG GAG TGA AAA CTT GAA GAC-3′ for all three constructs; 5-GGG GTA CCA TGA CTT CAG ATA ACT ACA CCG AGG AA-3′ for Δ12-CXCR4; 5′-GGG GTA CCA TGG GCT CAG GGG ACT ATG ACT-3′ for Δ15-CXCR4; and 5′-GGG GTA CCA TGA AGG AAC CCT GTT TCC GTG A-3′ for Δ23-CXCR4. The resulting PCR products were digested with *Eco*RI and *Kpn*I and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, Calif.). The constructs were verified by sequencing before use in transfection experiments. The CXCR4 alanine scanning mutants and the rat and feline CXCR4 constructs have all been previously described (5, 6, 55).

Infection assays. The generation of an HIV-1 NL4-3 replication-competent luciferase reporter virus has been described previously (44). Briefly, a 2-kb *Bam*HI/*Xho*I restriction fragment derived from pNL4-3.Luc.R^-E^- (9), containing the 3′end of the viral genome and a luciferase reporter gene in place of *nef*, was inserted into a modified pBR322 vector containing the full-length HIV-1 NL4-3 provirus. This construct, named pBRNL4-3dnefluc, yields replication-competent HIV-1 luciferase reporter viruses after transient transfection of 293T cells. For infection, 30 μl of virus was used with 10^5 cells in the presence of 4 μg of Polybrene/ml. At 48 h after infection, cells were lysed with 0.5% Triton X-100 in phosphate-buffered saline, and an appropriate aliquot was analyzed for luciferase activity. For blocking experiments, cells either were incubated with various antibodies at 20 μg/ml for 30 min at 37°C before infection or were washed in culture medium, incubated with various antibodies at 20 μg/ml for 30 min at 37°C, and then infected.

Chemotaxis assay. Cells growing in logarithmic phase were harvested, washed twice, and resuspended in RPMI 1640 medium supplemented with 0.5% bovine serum albumin at a concentration of 10^7 cells/ml. A 100-μl quantity of cell suspension was placed on top of 600 μl of RPMI 1640 medium supplemented with 0.5% bovine serum albumin in a 24-mm Transwell (Corning Costar, Cambridge, Mass.). SDF-1α at a concentration of 100 ng/ml was used as a chemotactic agent. The cells were allowed to undergo chemotaxis overnight, and the bottom chamber was harvested. A 15-μl quantity of Polybead polystyrene 15-μm microspheres (135,000 beads; Polysciences, Inc) was added for standardization, and the samples were analyzed by flow cytometry. For blocking of chemotaxis, the cells were incubated with various antibodies at 10 μg/ml for 30 min at 37°C and then used in the chemotaxis assay.

RESULTS

Generation and characterization of MAbs to CXCR4. To determine if CXCR4 exists in distinct conformations, we immunized mice with murine 3T3 cells overexpressing human CXCR4. We have found that this approach typically results in the production of conformation-dependent antibodies that efficiently recognize seven-transmembrane domain receptors on the surface of cells (33). Seven MAbs were generated that specifically recognized 293T cells expressing CXCR4 in FACS analysis (data not shown). The MAbs did not recognize other seven-transmembrane domain receptors tested, including CCR1, CCR2, CCR4, CCR5, CCR8, CXCR1, CXCR2, GPR1, GPR15, ChemR23, STRL33, APJ, or CX3CR1. Coexpression of CD4 with CXCR4 in 293T cells did not affect MAb reactivity with CXCR4 (data not shown). In addition to MAbs 1, 2, 8, 12, 16, 17, and 18, we also used 12G5, a conformation-dependent MAb that recognizes a determinant in the first and second extracellular loops of CXCR4 (6, 22), and MAb 4G10, which was generated against the N-terminal domain of CXCR4 (57). The specificity of the newly developed MAbs was also confirmed by treating Jurkat cells with the CXCR4 inhibitor T22 (Fig. 1) (40) or the CXCR4 ligand SDF-1α (data not shown) (4) for 20 min at 4°C prior to the addition of antibody. These treatments largely abrogated the binding of CXCR4

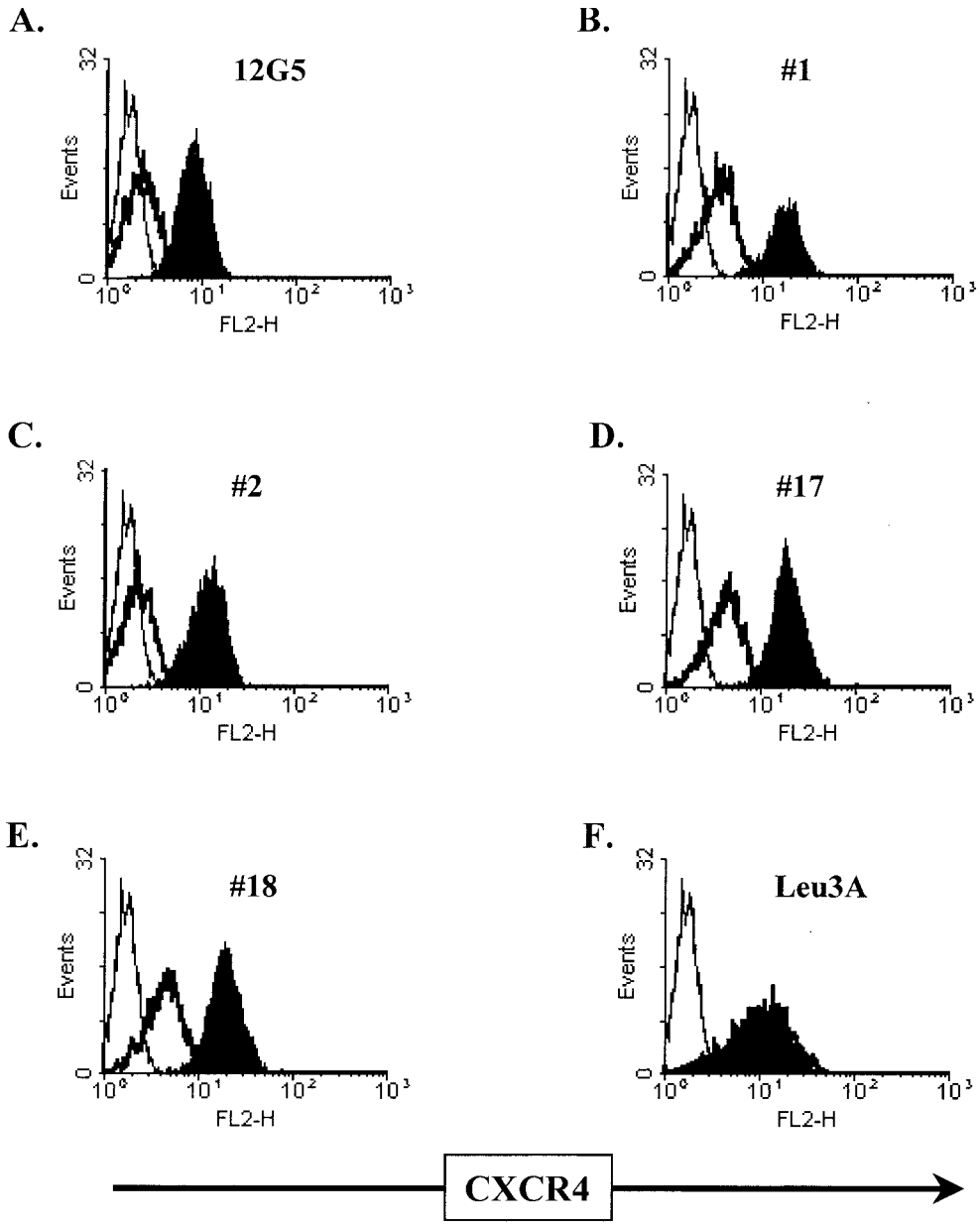
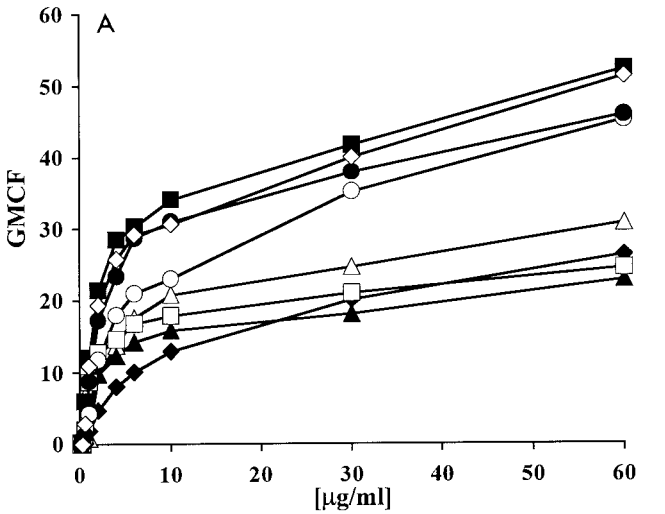


FIG. 1. Binding of CXCR4 MAbs to Jurkat CD4$^+$ cells is inhibited by T22. Binding of MAbs 12G5, 1, 2, 17, 18, and Leu3A to Jurkat CD4$^+$ cells in the presence (thick line) or in the absence (solid black area) of T22 (40) was examined. The background binding measured with mIgGs (thin line) is also shown. The percentages of inhibition obtained in the presence of T22 in this experiment were 81.2, 88.4, 85.2, 86.5, 86.8, and 0% for 12G5, 1, 2, 17, 18, and Leu3A, respectively. FL2-H, staining obtained with the indicated MAb. A representative experiment out of three done is shown.

MAbs to Jurkat CD4$^+$ cells, reducing staining intensity by more than 80% for each antibody. Leu3A, a CD4-specific antibody, was not affected by the treatment (Fig. 1).

CXCR4 exists in multiple conformational states. Having defined the specificity of the CXCR4 MAbs, we used these to determine if CXCR4 exists in antigenically distinct states. To do this, CXCR4-positive cells need to be stained with saturating levels of an MAb and for a time sufficient to achieve maximum reactivity. Since differences in staining could potentially arise from differences in MAb affinity for CXCR4, we stained Jurkat CD4$^+$ cells expressing CXCR4 with different concentrations of each MAb to determine 50% effective concentrations (EC_{50}s) (Fig. 2). First, we determined that all MAbs reached steady-state binding conditions by 15 min. Then, to measure EC_{50}s, cells were stained with MAbs at different concentrations for 1 h before processing for FACS analysis (Fig. 2A). We found that all of the CXCR4 MAbs exhibited similar affinities for CXCR4, with EC_{50}s varying by no more than 2.5-fold (Fig. 2B). Therefore, in subsequent experiments, cells were stained for 1 h with 10 μg of each MAb/ml.

Using the saturating conditions defined above, we stained a number of commonly used human lymphoid cell lines with the panel of MAbs. As shown in Fig. 3A, the MAbs recognized



B

mAb	nEC_{50} [μg/ml]	%12G5 nEC_{50}
12G5	1.9±0.5	100
1	5.0±1.1	263
2	3.9±0.9	205
8	3.5±0.8	184
12	4.1±0.8	215
16	4.2±0.8	221
17	4.9±1.5	257
18	4.6±1.7	242

FIG. 2. Equilibrium binding of anti-CXCR4 MAbs. (A) Jurkat $CD4^+$ cells were stained with the indicated concentrations of CXCR4 MAbs 12G5, 1, 2, 8, 12, 16, 17, and 18 for 1 h followed by PE-coupled anti-mouse antibody and analyzed by flow cytometry as described in Materials and Methods. A representative experiment out of three done is shown. (B) Relative affinity for each antibody presented as normalized EC_{50} (nEC_{50}). The EC_{50} of each antibody was the concentration of antibody that gave a half-maximal GMCF value, based upon flow cytometric analysis using serial dilutions of the MAbs on Jurkat $CD4^+$ and SupT1 cells. The results are the mean and standard error of the mean obtained from three experiments with each cell type.

approximately the same levels of CXCR4 on Blin1, Nalm6, and Reh cells (two pre-B-cell lines and one pro-B-cell line, respectively) (26), although the absolute levels of CXCR4 varied by two- to threefold between cell types. When RS4:11 cells (pro-B-cell line) (26) were used, MAbs 1 and 18 reacted more strongly than the other MAbs. In contrast, a different but consistent staining pattern was obtained when T-cell lines were examined (Fig. 3B and C). Once again, CXCR4 levels varied between cell types (up to fourfold between PM1 and Bc7 cells), but generally MAbs 1, 16, 17, and 18 gave higher (and similar) geometric mean channel fluorescence (GMCF) values than MAbs 12G5, 2, 8, and 12. This striking difference in reactivity patterns is most likely due to antigenically distinct populations of CXCR4, since the MAbs were specific for CXCR4 and exhibited similar binding constants.

CXCR4 conformations on primary cell types. To determine if CXCR4 exists in antigenically distinct conformations on primary cell types, human PBLs were stained with saturating concentrations of CXCR4 MAbs after being cultured for 4 days in the presence of IL-2 (Fig. 4A) or PHA–IL-2 (Fig. 4B). The data shown are a summary of six different experiments with two or three different donors in each. The staining patterns observed on these primary cell types reflected what was observed on the T-cell lines. MAbs 1, 16, 17, and 18 recognized a much greater proportion of cell surface CXCR4 on both $CD4^+$ and $CD8^+$ T cells than did MAbs 12G5, 2, 8, and 12, irrespective of culture conditions. The differences between the two groups of MAbs varied depending on the donor, with GMCF values varying by two- to fivefold between the two reactivity groups. The addition of SDF-1α to cells at 4°C prior to the addition of MAbs largely prevented binding (>80%), confirming the specificity of these MAbs on human PBLs (data not shown) (4). This experiment was repeated with fresh blood, with similar results (data not shown). To examine CXCR4 conformations on primary B cells ($CD19^+$ cells), cells from eight different donors were stained within 1 h of collection. Differential reactivity of the MAbs was once again observed (Fig. 5). Therefore, we conclude that CXCR4 exists in antigenically distinct populations on primary T and B cells and on some but not all lymphoid cell lines that we tested.

Differential staining correlates with epitope mapping. CXCR4 possesses four extracellular domains: an N-terminal region and three extracellular loops (ECL1, ECL2, and ECL3). To further identify the domain or domains recognized by each of the newly developed CXCR4 MAbs and to determine if the differential staining of CXCR4 by the panel of MAbs correlated with specific antigenic epitopes, we tested the MAbs for the ability to recognize receptor chimeras, point mutants, and CXCR4 molecules from other species. None of the MAbs, with the exception of the N-terminal MAb 4G10, recognized CXCR4 on Western blots, indicating that they bound to conformation-dependent epitopes (data not shown). Therefore, we used FACS analysis to monitor reactivity with receptor mutants and chimeras.

Using a panel of receptor chimeras generated between CXCR4 and CXCR2, we found that only MAb 4G10 recognized the 4222 receptor chimera, which contains the N-terminal domain of CXCR4 in a CXCR2 background (Table 1). This result was expected, given that this MAb was obtained as a result of immunization with a peptide equivalent to the N terminus of CXCR4 (57). In contrast, the reciprocal chimera (2444) was recognized by the entire panel of MAbs save for the N-terminal MAb 4G10. Likewise, all of the MAbs recognized chimera 4442, indicating that the determinants recognized by the conformation-dependent MAbs reside in ECL1 and ECL2. Additional receptor chimeras were expressed inefficiently. Therefore, we tested two rat-human CXCR4 chimeras (6). While none of the MAbs recognized rat CXCR4, the conformation-dependent MAbs all bound to a chimera carrying ECL2 of human CXCR4 in a rat CXCR4 background

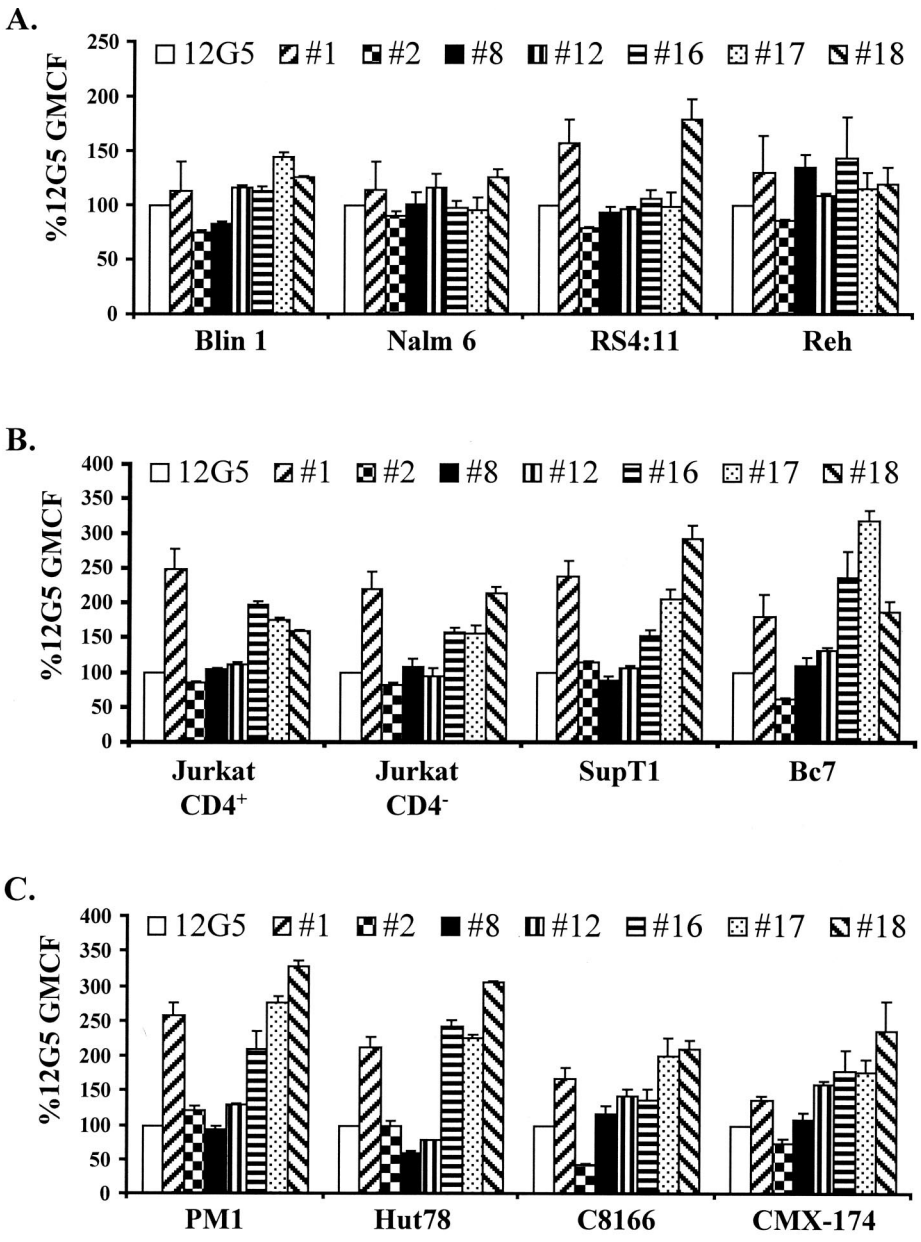


FIG. 3. CXCR4 heterogeneity on lymphoid cell lines. The indicated cell lines were incubated with saturating levels of CXCR4 MAbs 12G5, 1, 2, 8, 12, 16, 17, and 18 for 1 h and then processed for flow cytometry. The cell lines are grouped into three panels (A, B, and C) for convenience. All results have been normalized to the GMCF value obtained for 12G5, which was set at 100%. The results are representative of five different experiments and are reported as the mean and standard error of the mean for each cell line.

(RRHR) but not to the reciprocal chimera (HHRH) containing the rat ECL2 domain in a human CXCR4 background (Table 1). From these results, we conclude that the determinants recognized by the conformation-dependent MAbs are composed largely of residues in ECL2.

To more finely map the epitopes recognized by the CXCR4 MAbs, we tested each for the ability to recognize a panel of previously described CXCR4 constructs in which individual amino acids in ECL2 were mutated (5). MAb 4G10, directed against the N-terminal domain of CXCR4, was used to control for receptor expression. The panel of mutants showed that under the conditions tested, substitutions at residues 176 and 179 abrogated the binding of all MAbs tested, while a substitution at residue 181 reduced but did not abolish recognition of MAb 2 and a substitution at residue 183 reduced but did not abolish recognition of MAb 17 (Table 2). Thus, the epitopes recognized by the panel of conformation-dependent MAbs overlap, involving residues in ECL2.

Finally, we tested the ability of each MAb to recognize feline CXCR4, which differs from human CXCR4 at a number of positions in the ECL domains (Fig. 6A). Interestingly, the MAbs that recognized CXCR4 most efficiently on primary cell types (i.e., 1, 16, 17, and 18) all reacted with feline CXCR4, whereas the MAbs that recognized only a fraction of available cell surface CXCR4 molecules (i.e., 12G5, 2, 8, 12, and 4G10) did not (Fig. 6B). Taken together, these results suggest that

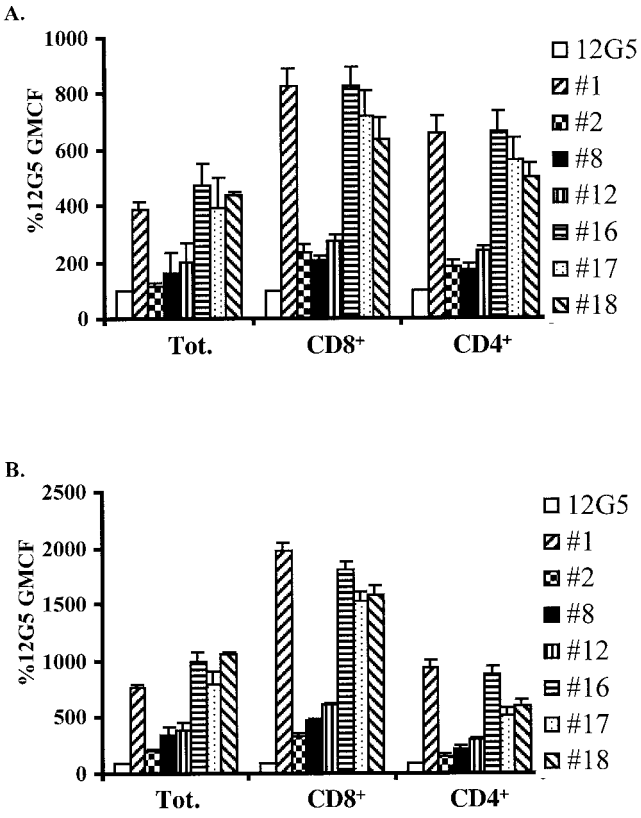


FIG. 4. CXCR4 heterogeneity on PBLs. Human PBLs treated for 4 days with IL-2 alone (A) or with IL-2–PHA (B) were stained with the panel of CXCR4 MAbs and analyzed by flow cytometry as described in Materials and Methods. Antibodies to CD8 and CD4 were also used to examine CXCR4 heterogeneity on these T-cell subsets. Tot., total. The results shown are the mean and standard error of the mean obtained for two or three donors in five different experiments.

residue 180, which is Ala in human and feline CXCR4 and Gly in rat CXCR4, forms part of the epitope recognized by MAbs 1, 16, 17, and 18 (Fig. 6A and Table 2) but not by the other antibodies. In conclusion, the MAbs in our panel can be segregated into two groups based on epitope mapping, with the two groups in turn reacting differently with CXCR4 in primary cell types. Thus, it seems likely that conformational differences

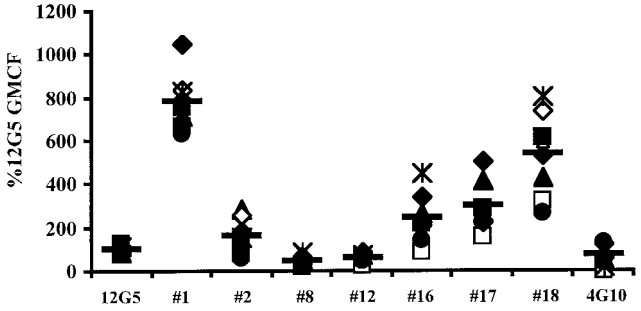


FIG. 5. CXCR4 heterogeneity on B cells. Fresh blood was stained with the panel of CXCR4 MAbs as described in Materials and Methods. All results have been normalized to the GMCF value obtained for 12G5, which was set at 100%. A representative experiment out of three done with eight donors each is shown. The mean value is indicated by a horizontal bar.

TABLE 1. Reactivity of MAbs with receptor chimeras[a]

MAb	Reactivity with the following chimera:						
	4444	4222	2444	4442	RRRR	RRHR	HHRH
12G5	+	−	+	+	−	+	−
1	+	−	+	+	−	+	−
2	+	−	+	+	−	+	−
8	+	−	+	+	−	+	−
12	+	−	+	+	−	+	−
16	+	−	+	+	−	+	−
17	+	−	+	+	−	+	−
18	+	−	+	+	−	+	−
4G10	+	+	−	+	−	−	+

[a] 293T cells were transfected with plasmids expressing CXCR4 (4444) or the indicated receptor chimeras. Chimeras are designated according to the extracellular domains included in each molecule. Thus, 4222 contains the N-terminal domain of CXCR4 in a CXCR2 background. HHRH contains the second extracellular domain of rat CXCR4 in a human CXCR4 background. Cells were stained with the indicated MAbs and analyzed by FACS. +, reactive; −, not reactive.

in the CXCR4 ECL domains, in particular in ECL2, are responsible for CXCR4 antigenic heterogeneity.

Functional consequences of CXCR4 conformations. The existence of at least two distinct CXCR4 conformations in T cells could influence the ability of MAbs and small-molecule inhibitors to block coreceptor or chemokine receptor function. Indeed, it has been noted that 12G5, the most commonly used CXCR4 MAb, inhibits X4 virus infection in some cell lines but not others (38, 50). McKnight and coworkers (38) speculated that this could be due to differences in CXCR4 presentation in some cell types. To test this idea, we examined the ability of a subset of the MAbs (two with low reactivity, 12G5 and 2, and two with high reactivity, 1 and 18) to inhibit virus infection on cell lines displaying conformationally heterogeneous CXCR4 molecules. We used an X4 replication-competent NL4-3 construct (44), bearing a luciferase reporter gene in place of *nef*, for these experiments. As shown in Table 3, we found that the inhibition of virus infection of C8166 cells closely paralleled the ability of the MAbs to recognize CXCR4. MAbs 1 and 18 inhibited virus infection more efficiently than MAbs 12G5 and 2 (Table 3). Neutralization was more efficient when 12G5 was

TABLE 2. Recognition of CXCR4 point mutants[a]

Amino acid	Position	Staining with the following antibody:								
		12G5	1	2	8	12	16	17	18	4G10
N	176	−	−	−	−	−	−	−	−	+
E	179	−	−	−	−	−	−	−	−	+
D	181	+	+	±	+	+	+	+	+	+
D	182	+	+	+	+	+	+	+	+	+
R	183	+	+	+	+	+	+	±	+	+
Y	184	+	+	+	+	+	+	+	+	+
I	185	+	+	+	+	+	+	+	+	+
D	187	+	+	+	+	+	+	+	+	+
R	188	+	+	+	+	+	+	+	+	+
F	189	+	+	+	+	+	+	+	+	+
D	193	+	+	+	+	+	+	+	+	+

[a] 293T cells were transfected with plasmids expressing wild-type CXCR4 or CXCR4 mutants containing the indicated point mutations. Cells were stained with the indicated MAbs and analyzed by FACS. +, positive staining (>30% positive cells); ±, reduced staining (between 20 and 30% positive cells); −, no staining (<10% positive cells).

A.

		1 38
Human	N-term.	MEGISIYTSDNYTEE.MGSGDYDSMKEPCFREENANFNK
Rat	N-term.	--...-------S--.V-------N------D--E---R
Feline	N-term.	-D-FR--P------DDL----------H--R
		97 110
Human	ECL1	DAVANWYFGNFLCK
Rat	ECL1	--M-D----K----
Feline	ECL1	---------K----
		176 202
Human	ECL2	NVSEADDRYICDRFYPNDLWVVVFQFQ
Rat	ECL2	D--QG-G------L--DS--M------
Feline	ECL2	--R---G---------S-S-L------
		262 282
Human	ECL3	DSFILLEIIKQGCEFENTVHK
Rat	ECL3	-------V--------SV---
Feline	ECL3	----------------S----

B.

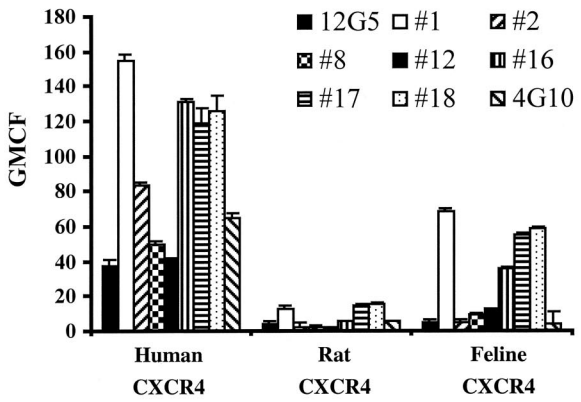


FIG. 6. Reactivity of the CXCR4 MAbs to human, rat, and feline CXCR4. (A) Amino acid sequence comparison of the amino-terminal domain (N-term.) and the first, second, and third extracellular loops (ECL1, ECL2, and ECL3) of human, rat, and feline CXCR4. Human CXCR4 is used as the reference sequence, with different amino acids in the rat or feline sequence indicated in bold. A dash indicates amino acid identity, and a dot indicates a gap created to maximize the alignment. (B) 293T cells were transfected with human, rat, or feline CXCR4, and the reactivities of MAbs 12G5, 1, 2, 8, 12, 16, 17, 18, and 4G10 were analyzed by flow cytometry. The mean and standard error of the mean for one representative experiment out of three done in triplicate are shown.

used in combination with MAb 1 or 18 but not when it was used in combination with MAb 2 (Table 3). This result suggests that MAbs 12G5 and 2 recognize a subset of CXCR4 molecules recognized by MAbs 1 and 18. We conclude that the HIV-1 Env protein studied here could utilize CXCR4 conformations not recognized by 12G5 and some other MAbs on T-cell lines and primary T cells. When antigenic heterogeneity is significant, as on primary T cells, 12G5 binds to only a subset of CXCR4 molecules, enabling HIV-1 to infect cells by using alternative CXCR4 conformations.

To determine if CXCR4 heterogeneity had an impact on SDF-1α-mediated receptor signaling (49), we used a chemotaxis assay with HS-Sultan cells (26), a mature B-cell line which displays heterogeneous CXCR4 molecules (Fig. 7) and which efficiently undergo chemotaxis in response to SDF-1α. The inhibition of chemotaxis by the different MAbs inversely correlated with the amount of CXCR4 recognized by each MAb on this cell line, with one exception: MAb 2 inhibited the chemotaxis of HS-Sultan cells toward SDF-1α as well as did MAb 1, although its reactivity measured by flow cytometry was about half that of MAb 1 (Fig. 7). The reason for this apparent discrepancy is not clear. One possibility is that the epitope of MAb 2 more closely matches the binding site of SDF-1α than does that of MAb 1, enabling it to block chemotaxis more efficiently.

Mechanisms of conformational heterogeneity. The presence of antigenically distinct conformations of CXCR4 could be the result of several factors, including posttranslational modifications (3, 8, 23). CXCR4 contains two N-linked glycosylation sites, only one of which is utilized (8). In addition, it may be sulfated (23) and, like other 7TM receptors, it binds to G proteins (reviewed in reference 2). To determine if any of these factors could account for the antigenic differences in CXCR4 observed in this study, we transiently expressed wild-type and N-terminally truncated forms of CXCR4 in 293T cells. We found that transient expression of CXCR4 in 293T cells gave rise to the now familiar reactivity pattern in which MAbs 1, 16, 17, and 18 recognized CXCR4 more efficiently than MAbs 12G5, 2, 8, and 12 (Fig. 1 and 8). This reactivity pattern was also observed with a CXCR4 construct lacking the first 23 amino acids of the N-terminal domain (Fig. 8) and, as a consequence, both the N-linked glycosylation site and the Tyr residues in this domain that could be potential targets of sulfation. Likewise, a double point mutant (Y7Y12), upon transfection, showed the same reactivity pattern as CXCR4 (data not shown). In addition, MAb reactivity was not obviously affected by treatment of cells with pertussis toxin (Fig. 9) or by expression of forms of CXCR4 bearing mutations in the second intracellular loop that have been implicated in G-protein binding (data not shown). Thus, we conclude that the antigenic heterogeneity of CXCR4 that we have observed is not the result of differential N-linked glycosylation, sulfation of the N-terminal domain, or G-protein coupling.

TABLE 3. Infection inhibition of T cells correlates with CXCR4 reactivity[a]

MAb	GMCF	Result obtained with secondary antibody:				
		None	12G5	#1	#2	#18
None	0	100 ± 13	—	—	—	—
mIg	8.4 ± 0.1	90 ± 5	37 ± 5	16 ± 3	49 ± 3	6 ± 1
12G5	25.7 ± 0.7	40 ± 8	35 ± 7	5 ± 1	35 ± 4	5 ± 1
1	70.2 ± 0.8	8 ± 2	5 ± 1	8 ± 1	4 ± 1	6 ± 1
2	24.7 ± 0.9	57 ± 14	35 ± 4	4 ± 2	56 ± 5	7 ± 1
18	86.4 ± 0.6	6 ± 2	5 ± 1	6 ± 1	7 ± 1	3 ± 1

[a] C8166 T cells were incubated with buffer alone (None), with mIg, or with the indicated CXCR4 MAb. Alternatively, cells were further incubated with an additional CXCR4 MAb (secondary antibody) to determine if there was additive or synergistic inhibition of CXCR4-dependent virus infection. In addition, a fraction of the cells were analyzed by flow cytometry, and the remaining cells were infected with NL4-3 replication-competent luciferase reporter viruses. GMCF, geometrical mean channel fluorescence. Data are mean percentages of infection ± standard errors of the means. The results represent one experiment out of two complete experiments performed.

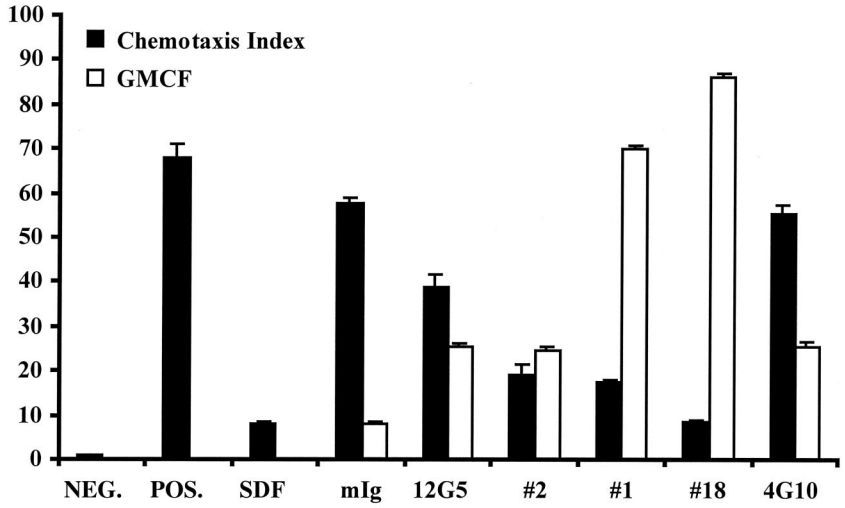


FIG. 7. Chemotaxis inhibition of T cells correlates with differential antibody reactivity. HS-Sultan cells were incubated with CXCR4 MAbs 12G5, 2, 1, 18, and 4G10. A fraction of the cells was analyzed by flow cytometry (white columns), and the rest was used for a chemotaxis assay (black columns). The mean and standard error of the mean for one representative experiment out of three done in triplicate are shown. The chemotaxis index is defined as the ratio of the number of cells that migrate under a given condition to the number of cells that migrate in the absence of the chemoattractant. NEG., chemotaxis index with no chemoattractant in the lower chamber; POS., chemotaxis in response to SDF-1α. The column labeled SDF is the chemotaxis control and shows the chemotaxis index when the concentration of SDF-1α in the upper chamber is equivalent to that in the lower chamber.

DISCUSSION

Interactions between the HIV-1 Env protein and its receptors are conformationally complex (reviewed in reference 25). The structure of gp120 complexed with CD4 shows that multiple residues, often far apart in primary sequence, participate in receptor contacts (31, 45). Likewise, several regions in Env participate in coreceptor interactions (reviewed in reference 25). The V3 loop of gp120 plays a major role in defining which coreceptors are used, with a more subsidiary role being played by the V1-V2 domains. A highly conserved region in gp120, residing largely in the bridging sheet region between the bases of the V1-V2 and V3 loops, also participates in coreceptor binding (31, 45, 58). It is also clear that coreceptor conformation is an important functional determinant. Numerous mutations that have an impact on coreceptor function have been identified for virtually all ECL regions of CCR5 and CXCR4 (5, 17, 19, 24, 46; reviewed in reference 2). In addition, solubilization of CCR5 and CXCR4 with most nonionic detergents prevents gp120 binding, indicating that the lipid environment affects receptor conformation. As a consequence, conditions that alter CXCR4 conformation could have an impact on its coreceptor and chemokine receptor functions.

Seven-transmembrane domain receptors are subject to a number of factors that could alter receptor conformation. The CXCR4 receptor is glycosylated (3, 8) and sulfated (23) and can be phosphorylated as well (41). Heterogeneity in any of these posttranslational events would result in structurally distinct forms of CXCR4. CXCR4 may also associate with other membrane proteins, such as CD4 and cytosolic G proteins (32). Given the diversity of cell types in which CXCR4 is expressed, heterogeneity in CXCR4 interactions with other cellular proteins would not be unexpected either between cell types or within an individual cell. Since the bulk of CXCR4 resides within the lipid bilayer, changes in lipid composition could influence receptor structure. Glycosphingolipid-rich, detergent-insoluble rafts represent one such membrane domain into which some cell surface receptors cluster, although often not quantitatively (7). Since CXCR4 transduces a signal across the membrane upon ligand binding (36), it is clear that its structure is compatible with assuming different conformations under the appropriate conditions (27). In this regard, CXCR4 most likely is not different from other seven-transmembrane domain receptors.

Differences in receptor conformation can be inferred from ligand binding studies, particularly with MAbs that bind to a receptor with similar affinities and that recognize defined epitopes. We have previously used a panel of well-defined MAbs to CCR5 to show that this receptor exists in antigenically distinct states (33). Using the same approach, we found that

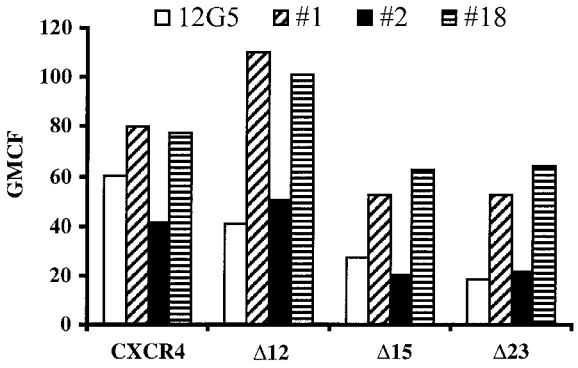


FIG. 8. Sulfation and glycosylation do not influence antibody reactivity. 293T cells were transfected with CXCR4 or a CXCR4 construct lacking the first 12 (Δ12-CXCR4), 15 (Δ15-CXCR4), or 23 (Δ23-CXCR4) N-terminal amino acids. At 48 h later, the cells were stained with MAbs 12G5, 1, 2, and 18 and analyzed by flow cytometry. A representative experiment out of three done is shown.

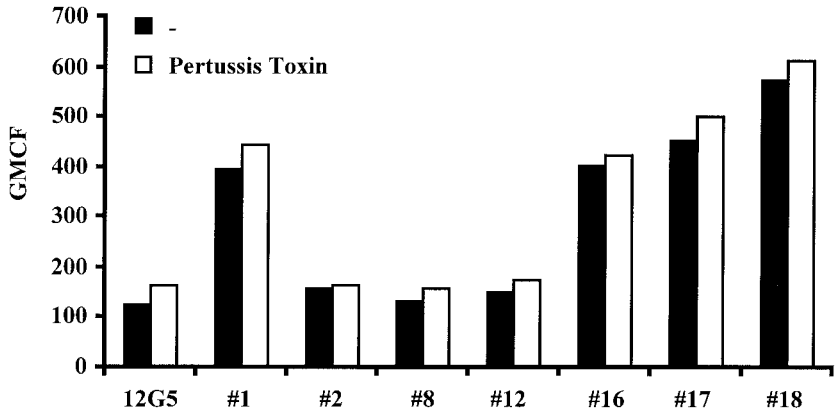


FIG. 9. Pertussis toxin treatment does not influence antibody reactivity. Jurkat cells were incubated overnight in the presence or absence (−) of 100 ng of pertussis toxin/ml. The cells were then stained with MAbs 12G5, 1, 2, 8, 12, 16, 17, and 18 and analyzed by flow cytometry. One experiment out of two done is shown.

CXCR4 also exhibits antigenic heterogeneity, since for most cell types stained with our CXCR4 MAbs under saturating conditions, we found two reactivity patterns. Approximately one-half of the MAbs recognized CXCR4 more efficiently than the second group of MAbs. For the most part, antibodies within each group recognized CXCR4 equally well. Since the MAbs exhibited similar binding constants for CXCR4 and we took care to stain cells under saturating conditions, we conclude that CXCR4 exhibits antigenic heterogeneity. The fact that some lymphoid cell lines did not exhibit this heterogeneity served as a useful control, indicating that the entire panel of MAbs could bind to CXCR4 equally well under some circumstances. Thus, the antigenic heterogeneity that we observed was in part cell type dependent.

We have demonstrated that CXCR4 exists in antigenically distinct states in most cell types, including primary $CD4^+$ and $CD8^+$ T cells. We believe that this is due to conformational heterogeneity for several reasons. First, the entire panel of MAbs bound to conformation-dependent epitopes, since solubilization of CXCR4 with a variety of detergents prevented immunoprecipitation of this receptor by all of the MAbs, except for 4G10, which recognizes a linear determinant in the N-terminal domain (57). Second, the known posttranslational modifications of CXCR4 (N-linked glycosylation and sulfation of the N-terminal domain) had little or no effect on antibody reactivity (3, 8, 23). Third, the two reactivity groups correlated perfectly with our epitope mapping studies. Both the poorly reactive and strongly reactive groups of antibodies bound to distinct but overlapping conformation-dependent epitopes that were largely dependent upon ECL2. As ECL2 is the largest extracellular loop in CXCR4 and this region plays an important role in SDF-1α-mediated signal transduction (53), we suspect that conformational differences in this region are responsible for differential antibody binding in most cell types. We would anticipate that the epitope recognized by the poorly reactive MAbs is occluded on a variable portion of cell surface CXCR4 receptors, being influenced by donor variability, cell type, and potentially growth conditions, receptor oligomerization, or interactions with as-yet-unidentified molecules.

Our panel of MAbs necessarily gives an imperfect view of CXCR4 antigenic structure given the limited antigenic repertoire of the panel. Specifically, these MAbs cannot directly measure conformational differences in the N-terminal domain of CXCR4 or ECL3. Therefore, while we can determine that there must be at least two CXCR4 conformations present in T-cell lines and primary T and B cells, there could be more. We have found that antibodies in the strongly reactive group effectively compete with 12G5 for CXCR4 binding. Thus, these MAbs recognize a conformation of CXCR4 that is recognized by 12G5 and other members of the poorly reactive antibody group. However, they must also recognize a second conformation that does not support the binding of MAbs such as 12G5. Thus, antibodies such as 12G5 recognize a subset of CXCR4 conformations in most cell types.

Does the conformational heterogeneity revealed by this panel of MAbs have an impact on CXCR4 coreceptor or chemokine receptor functions? Our experiments indicate that HIV-1 recognizes both CXCR4 conformations. Indeed, with cells (such as primary T cells or T-cell lines) that exhibit both CXCR4 conformations, HIV-1 was not inhibited by antibodies such as 12G5. Cell-type-dependent inhibition of X4 HIV-1 strains by 12G5 has been observed previously by McKnight and coworkers, who suggested that CXCR4 might exist in different conformations on some cell types (38). Our data show this hypothesis to be correct. The use of the MAbs to inhibit SDF-1α-induced chemotaxis gave similar results. Thus, HIV-1 Env and SDF-1α can bind to multiple CXCR4 conformations. We cannot determine, however, if they bind to different CXCR4 conformations equally well. To study this idea, we need to identify the factors that control CXCR4 structure so that they can be varied in a systematic fashion and then correlated with coreceptor and chemokine receptor functions.

Our results argue that differential N-linked glycosylation, sulfation, and pertussis toxin-sensitive G-protein coupling do not account for CXCR4 antigenic heterogeneity. There are a multitude of other factors that could have an impact on CXCR4 structure and that we have not examined. There is evidence, for example, that CXCR4 can, in some cell types, associate with CD4 (32). However, we found that CD4 coexpression in 293T cells did not obviously affect CXCR4 conformation and that CXCR4 did exhibit antigenic variation in some $CD4^-$ lymphoid cell lines (Fig. 3; Jurkat $CD4^-$ and Bc7

cells). CXCR4 could associate with other cellular proteins as well as pertussis toxin-insensitive G proteins, although we have no evidence for this notion. CXCR4 could potentially form oligomers or partition into detergent-insoluble lipid rafts. Unfortunately, we have been unable to establish conditions that make it possible to quantitatively immunoprecipitate CXCR4, a situation which would enable us to address these possibilities. Therefore, while we can identify some factors that are unlikely to account for CXCR4 heterogeneity, there are others whose contributions to receptor conformation cannot be determined with the assays presently available.

The most obvious implications associated with this work concern the development and use of CXCR4 inhibitors and CXCR4 expression patterns. Individuals who lack CCR5 are highly resistant to virus infection and do not exhibit any obvious immunological deficits, making CCR5 an attractive drug target (37, 47). However, the use of CCR5 antagonists could potentially exert selective pressure for the evolution of X4 viruses, and many individuals with advanced HIV-1 infection or AIDS possess X4 virus types that would not be directly affected by CCR5 antagonists (51, 52). Thus, the development of CXCR4 antagonists as antiviral agents would be desirable. Several CXCR4 antagonists have been described thus far, and at least one has been used in a clinical trial (15). Our study indicates that it will be important to determine if CXCR4 antagonists recognize all CXCR4 conformations or only a subset of the available structures. If cell lines expressing CXCR4 are used to screen for inhibitors, then it will be important to use cells that express different CXCR4 conformations, like the T-cell lines used in this study. Otherwise, there is a risk that inhibitors will be selected that bind to only a fraction of CXCR4 molecules, making them much less effective antiviral agents.

Finally, coreceptor expression can be limiting for HIV-1 infection (29). The membrane fusion process requires the participation of multiple Env proteins and multiple virus receptors (reviewed in reference 13). Generally, CD4 is expressed in excess of 50,000 copies per T cell, while coreceptor expression on freshly isolated cells is typically less than 10,000 copies per cell (34). Therefore, it comes as little surprise that differences in coreceptor expression can influence virus infectivity. That this situation can be relevant in vivo comes from the finding that individuals who have a single copy of the mutant Δ32-ccr5 allele exhibit somewhat reduced virus loads and prolonged survival, likely as a consequence of a modest reduction in CCR5 expression levels (10, 42). Thus, it is important to be able to accurately measure CXCR4 levels and ascertain the factors that regulate its expression. By far the MAb most commonly used to measure CXCR4 expression is 12G5. However, our study shows that 12G5 recognizes only a subset of receptor conformations and, for some donors, leads to a gross underestimation of receptor density. We suggest that to accurately measure CXCR4 expression levels, antibodies competent to bind to multiple receptor conformations should be used. MAbs such as 1, 16, 17, and 18 appear to meet this requirement.

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