Biochemical Pharmacology, Vol. 57, pp. 451–463, 1999.

ISSN 0006-2952/99/$–see front matter
PII S006-2952(98)00339-6

COMMENTARY

Chemokine Receptors—Future Therapeutic Targets for HIV?

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ABSTRACT. To date, triple drug therapies for HIV have resulted in spectacular reductions in the number of virus particles and often remarkable recovery from disease in infected people. There is still, however, a great need for improved therapies. A battery of drugs aimed at different stages in the life cycle of HIV will enable switching of treatments if resistant viruses emerge or if patients are unable to tolerate particular therapies. Intense efforts are now underway to produce drugs that target chemokine receptors used by HIV to gain entry into cells. HIV needs two receptors on the host cell surface for efficient attachment and infection. HIV first interacts with CD4 but requires a coreceptor to penetrate the cell membrane. The first coreceptor, identified in 1996, is a member of the family of chemokine receptors, members of the G-protein coupled 7TM superfamily, which are involved in the trafficking of leukocytes in immune surveillance and inflammation. Such a therapeutic approach would differ from those used successfully to date, which focus largely on proteins coded by the HIV virus itself, and which are required for the replicative cycle of the virus. Many small, orally bioavailable molecules that block various 7TM receptors are used to treat a panoply of diseases including ulcers, allergies, migraines, and schizophrenia. These molecules are the cornerstone of the pharmaceutical industry's contribution to the fight against so many diseases, and it is hoped that a small molecule inhibitor of coreceptors can be developed that will become an invaluable drug in the fight against AIDS. BIOCHEM PHARMACOL **57**;5:451–463, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. chemokines; chemokine receptors; HIV; chemokine receptor antagonist; inflammation; therapeutic target

THE CHEMOKINE FAMILY

The chemokine family is a rapidly expanding group of immunoregulatory proteins [1–4]. The first pro-inflammatory chemokine to be discovered, due to its capacity to attract neutrophils, was IL-8§ [5]. IL-8 differs structurally from all the other ILs and also is the only IL that binds to a 7TM receptor. Following the discovery of IL-8 as a modulator of neutrophil migration, proteins with a high level of sequence identity to IL-8 that attracted monocytes were identified: MIP-1α and MIP-1β [6] and MCP-1 [7]. The family of chemokines, their name being derived from ***Chemo***attractant Cyto***kines,*** was therefore discovered a few years after the identification of HIV as the virus that causes AIDS.

The amino acid sequences of the chemokines showed that they possessed a conserved motif of four Cys residues that made it easy to rapidly identify numerous members of this family. Chemokines such as IL-8 have a single amino acid between the first two cysteines, whereas others such as MCP-1, MIP-1α, and MIP-1β have the first two cysteines adjacent. This different cysteine spacing allowed the chemokines to be divided into two subclasses: the CXC or α-subclass and the CC or β-subclass. This division into two subclasses, based on their primary sequence, was further substantiated by differences in their biologic activities. Thus, members of the α-subclass are found to principally activate neutrophils, while members of the β-subclass activate other types of leukocytes such as monocytes/macrophages, T lymphocytes, NK cells, eosinophils, basophils, and dendritic cells, although it should be noted that this rule is not absolute.

During the last few years, exceptions to this classification have been identified. Chemokines have been found with modifications to the disulfide bonding pattern, such as lymphotactin [8], which has only two Cys residues of the conserved four-cysteine motif, and I-309, which has six cysteines [9]. In addition, a molecule that has a CX_3C motif and is also joined to the cell membrane by a large mucin homology domain has been identified. This receptor has been called either Fractalkine [10] or Neurotactin [11].

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§*Abbreviations*: IL, interleukin; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; 7TM, seven transmembrane; MIP-1α and β, macrophage inflammatory protein 1α and β; MCP-1, monocyte chemoattracting protein 1; RANTES, Regulated And Normal T-cell Expressed and Secreted; Cys, cysteine; EST, Expressed Sequence Tag; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; SI, syncytium inducing; and NSI, non-syncytium inducing.

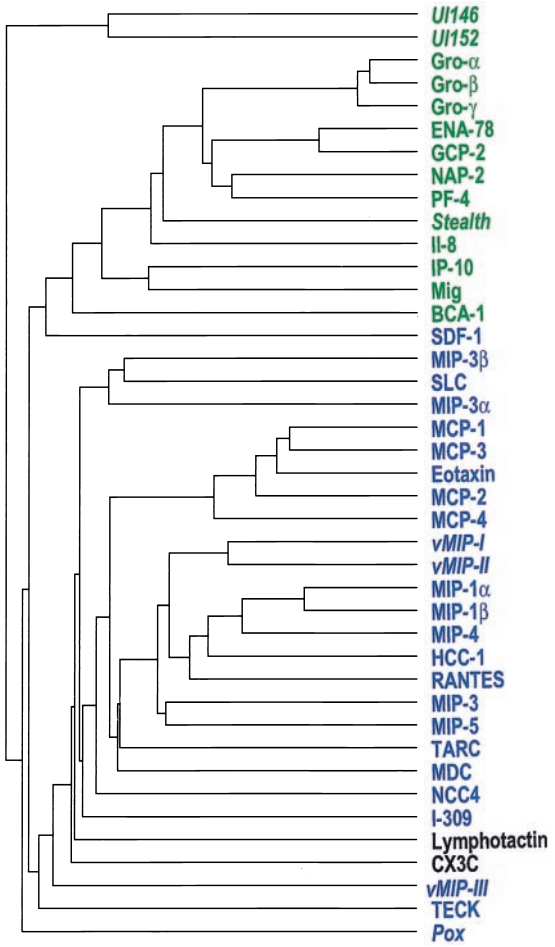

FIG. 1. Dendrogram showing the relative similarities between the human CXC and CC chemokines and sequences coded by certain viruses. The chemokine sequences with the CXC motif are shown in green and the CC sequences in blue. Viral sequences are in italics. The sequences *Ul146* and *Ul152* are coded by the human cytomegalovirus, *Stealth* by the Stealth virus, *vMIP-I*, *vMIP-II*, and *vMIP-III* by human herpes virus-8 (Kaposi's sarcoma-associated herpes-like virus), and *Pox* by the poxvirus, molluscum contagiosum.

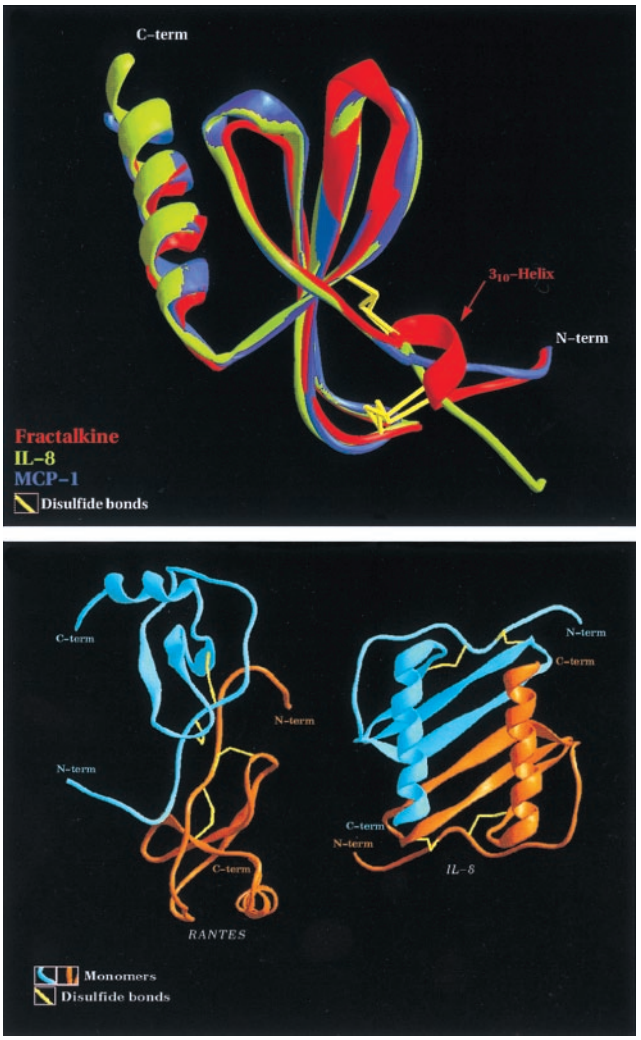

FIG. 2. (Top panel) Superposition of molecular models of monomers of a CC chemokine, MCP-1 [17], shown in purple, a CXC chemokine, IL-8 [13], shown in green, and the CX_3C chemokine, Fractalkine or Neurotactin, shown in red. The model of the CX_3C chemokine was built and validated using techniques described in Ref. 12. The 3_{10} helix formed by the three amino acids between the first two Cys residues is indicated. (Bottom panel) Comparison of the dimeric structures of a CC chemokine, RANTES, and a CXC chemokine, IL-8. The three-dimensional structure of RANTES [15] shows that dimerization occurs though interactions of the amino terminal regions forming an elongated cylindrical dimer. CXC chemokines, as shown by the structure of IL-8 [13, 18], form compact dimers with the interphase formed by interactions along the first beta strand, and are stabilized by the alpha helices.

Several Herpes and pox viruses also encode chemokines (see below), which have conserved the amino terminal motif as well as the position of the fourth cysteine, but show variations in the positioning of the third.

Initially, protein sequencing and polymerase chain reaction (PCR) techniques were exploited to identify new chemokine sequences, but more recently bioinformatic approaches searching databases containing the EST have identified new members [12]. Thus, the family currently comprises between 40 and 50 members that are evolutionarily related, as shown in Fig. 1. Despite the weak identity at the primary sequence level, which in some instances is as low as 20%, the chemokine monomers fold into highly conserved three-dimensional structures. Molecular modelling of the C and the CX_3C chemokines show that they, too, conserve the three-dimensional structure determined for CC and CXC chemokines (Fig. 2 top panel). However, the chemokine structures solved to date, whether by NMR spectroscopy in solution [13–15] or by X-ray crystallography [16, 17], show a large variation in the quaternary structures between the α-subclass and the β-subclass (Fig. 2, bottom panel). The α-chemokines form compact dimers with a groove, while the β-chemokines form elongated cylindrical dimers. The physiological significance of these quaternary structures, however, is controversial, since the dissociation constant for dimers in solution is several orders of magnitude higher than the concentration required for activity in

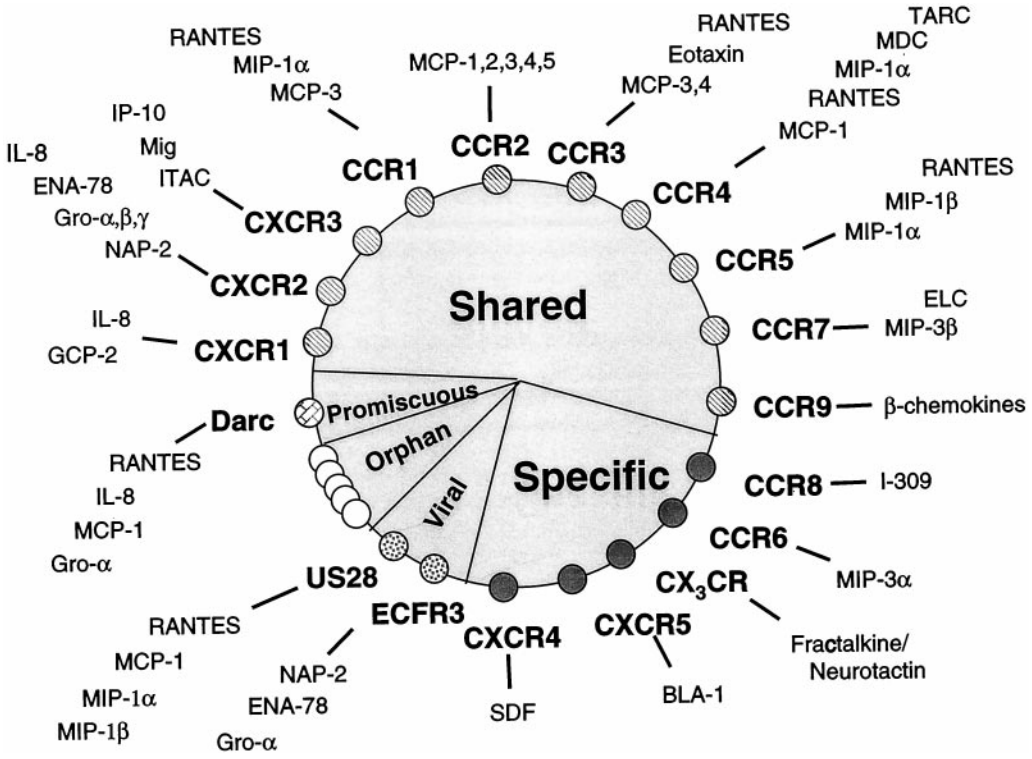

FIG. 3. Chemokine receptor and ligand specificities. Chemokine receptors are either shared, where they bind more than one ligand, or are specific, where they have been shown to bind only one ligand. The Duffy antigen, or DARC receptor, is termed promiscuous in that it binds members of both the CC and CXC subclasses. Orphan receptors are so defined since their ligand(s) has not been identified. Two sequences coded by viruses have been shown to bind and signal in response to certain β-chemokines. US28 is coded by cytomegalovirus and ECFR3 by *Herpes saimiri* virus. It should also be noted that certain ligands, such as RANTES, MIP-1α, and MCP-3, bind to more than one receptor.

bioassays or the receptor binding constant [9, 19]. Glycosaminoglycans on cell surfaces may act to increase local chemokine concentrations by enhancing oligomerization [20]. Such complexes have been observed to be secreted from activated T-cells [21].

CLASSIFICATION OF CHEMOKINE RECEPTORS

The chemokines act through receptors belonging to the superfamily that span the membrane seven times. These receptors are coupled to heterotrimeric G-proteins, mainly of the $G_{\alpha i}$ class, and receptor activation often triggers intracellular calcium mobilization, reduction in cyclic AMP levels, and increased kinase activity [22]. For a long time, the number of chemokine receptors was small, but it recently has increased significantly [1–4]. Currently, there are five CXC receptors. The first two to be identified were the neutrophil IL-8 receptors, subsequently called CXCR1 and 2. Initially, it was thought that the CXC chemokine effects would be involved in acute, mainly neutrophil-mediated pathologies. However, this pattern was broken with the identification of the receptors for IP-10 (CXCR3) and SDF-1 (CXCR4) on T cells. CXCR5 had been known for some time as a B cell-expressed orphan, but its ligand, BCA-1, was identified recently [23]. To date, nine CC chemokine receptors have been identified: CCR1 from pro-monocytes; CCR2 from monocytes; CCR3 from eosinophils; CCR4 from basophils, platelets, and T cells; and CCR5 from macrophages. These receptors all bind multiple ligands. Two of the receptors identified most recently, CCR6 and CCR8, appear to be more specific in that they only have one ligand, while CCR7, initially thought to bind only MIP-3β, has been shown recently to be the receptor for SLC (secondary lymphoid-tissue chemokine) as well [24]. On the contrary, CCR9 is the least specific of the receptors known to date, in that it binds all of the CC chemokines tested, and is widely distributed on cell types throughout the body [25]. The receptor for the CX_3C chemokine has been shown to be a previously identified orphan receptor, while the C chemokine receptor remains unidentified. Finally, the Duffy antigen on red blood cells (DARC) is unique in that it binds both CC and CXC chemokines, which are basic, but does not bind those that are acidic. Furthermore, this receptor has not been shown to be capable of transmitting a signal.

The ligand selectivity profile is complicated in the chemokine system. As shown in Fig. 3, few receptors bind only one ligand, and these are often the more recently identified receptors; whether this is because the specific receptors are more difficult to identify, or because all receptors will turn out to be promiscuous as more ligands are identified is a matter of speculation for the moment. An

added complexity is found in that certain chemokines bind to more than one receptor. For instance, RANTES binds to CCR1, 3, 4, and 5 as well as to DARC, and MIP-1α binds to CCR1, 4, and 5, but not to CCR3 and DARC. However, chemokines do exist that at low nanomolar concentrations bind to only one of the shared receptors, such as eotaxin to CCR3, and MIP-1β to CCR5.

It is now becoming apparent that the chemokine family may also be grouped into two classes based on their activities: (a) inducible chemokines involved in inflammation, and (b) constitutively expressed chemokines involved in homing. The first group contains the MIP and MCP families, as well as the closely related eotaxin and RANTES proteins. Evidence for their involvement in inflammation has come from studies of both increased levels of their mRNA and protein in both human pathology and animal models. The second group has emerged later and includes recently discovered members such as SDF-1, BCA-1, SLC, and MIP-3β. Evidence of their specific role in leukocyte homing *in vivo* comes from the deletion of the SDF-1 gene in mice where SDF-1 is shown to be essential for B cell lymphopoiesis and bone marrow myelopoiesis during fetal development [26].

THE DISCOVERY OF THE FIRST HIV CORECEPTOR

For more than a decade the immunoglobulin-like molecule CD4 was known to be the major cell surface receptor for all HIV and SIV strains. However, HIV infection required a cofactor, since murine cells transfected with human CD4 resisted HIV-induced fusion and entry [27]. In contrast, many (but not all) human cells transfected with CD4 were fully permissive to HIV infection [27–30]. Since $CD4^+$ murine cells can efficiently bind HIV virus particles [31], the block to entry was prior to fusion of cell and virus membranes, implying an additional cofactor.

This was eventually identified by expression cloning techniques. A human cDNA library was expressed in non-permissive murine NIH 3T3 cells expressing T7 polymerase. These cells were mixed with cells expressing an HIV-1 envelope gene as well as a *lacZ* reporter gene with an upstream T7 promoter. When fusion occurred between the two types of cells, the *lacZ* reporter was switched on, thus allowing detection of fused cells. Eventually by subdividing the cDNA library and repeated cycles of assays, a single cDNA clone was isolated that allowed fusion of the 3T3 cells with HIV. This cDNA encoded for an orphan seven-transmembrane spanning protein having high similarity to the chemokine receptor family [32]. The gene already had five different names (LESTR, HUMSTR or HM89, L5, D2S201E, and hFB22), and was renamed Fusin since it allowed fusion of the HIV virus with the host cell. It has since been renamed CXCR4.

HIV TROPISM AND MORE CORECEPTORS

The HIV-1 strain used to identify CXCR4 was a T-cell line tropic, SI strain. Tropism is a phenomenon used to describe the "specificity" of HIV viruses in their capacity to infect different cell types. This specificity, and therefore the ability of the virus to infect one cell type rather than another, resides in the viral surface glycoprotein gp120 that is encoded by the *env* gene of HIV. Different HIV and SIV strains were shown to be tropic for different cell types [28, 30]. These tropism differences act during virus entry and are controlled by cell surface determinants, suggesting that different coreceptors could be expressed on distinct cell types and be exploited by HIV/SIV strains with different cell tropisms. Moreover, primary isolates of HIV-1 can be divided into two categories depending on their properties *in vitro* [33, 34]. The first group of viruses have been called SI (as well as rapid/high, T-cell line tropic, or T-tropic) since they could infect and induce syncytia in $CD4^+$ T-cell lines. The second group were termed NSI (as well as slow/low, and macrophage-tropic/M-tropic) since they could not infect such $CD4^+$ T-cell lines and, therefore, did not induce syncytia. This nomenclature, however, is misleading, since NSI strains efficiently induce syncytia in infected cultures of macrophages [35]. *In vivo*, NSI strains are predominantly transmitted and are usually present throughout the course of infection, while SI viruses frequently emerge late in infection and are associated with the decline of $CD4^+$ cells that accompanies the onset of AIDS [33, 34, 36]. Mutations in the V3 loop of the viral gp120 often confer the change from an NSI phenotype in the early stages of HIV infection to CXCR4-using SI late on [37].

The identification of CCR5 as the coreceptor for the NSI strains followed a few weeks after the discovery of CXCR4 as the SI coreceptor. This discovery was aided by the identification of three β-chemokines (RANTES, MIP-1α, and MIP-1β) contained in $CD8^+$ T-cell secretions that inhibited HIV infection of lymphocytes [38]. The only receptor that binds all three chemokines is CCR5, which was soon shown to be the coreceptor for NSI HIV-1 strains [39, 40]. CCR5 is the principal receptor for primary infection, since individuals carrying a 32 nucleotide deletion in the CCR5 gene (Δ32 CCR5), and thus lacking a functional surface expressed CCR5 receptor, are substantially protected from infection [41–44].

All HIV strains tested thus far use CCR5 and/or CXCR4. Other members of the chemokine receptor family are also capable of supporting viral fusion and entry, and the list of coreceptors is gradually increasing. The β-chemokine receptors CCR2b, CCR3 [45, 46], and CCR8 [47–49] have been shown to allow infection by certain strains, as well as the virally encoded US28 [50] and several orphan 7TMs that have similarity to the chemokine receptor family. These include BOB/gpr15 [51, 52], BONZO/STRL-33 [53], and gpr1, so far shown to be used by SIV strains [51] (Fig. 4). As disease progresses, so coreceptor-use broadens, and viruses that use multiple coreceptors can often be isolated

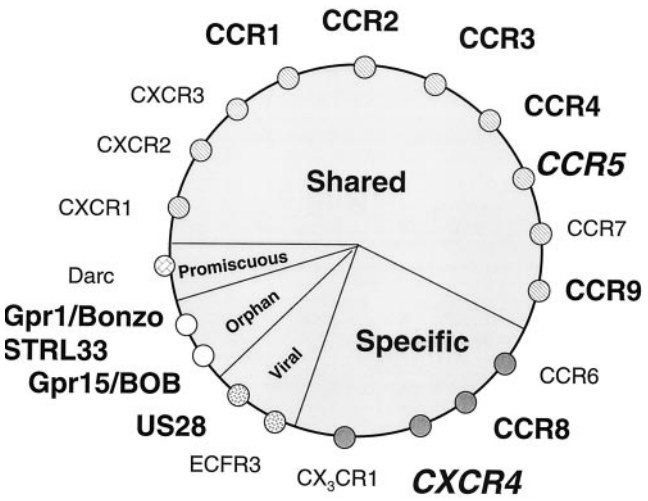

FIG. 4. Chemokine HIV coreceptors. Chemokine receptors and chemokine receptor-like sequences that can act as HIV and/or SIV coreceptors are shown in bold. The principal receptors believed to be responsible for infection *in vivo*, CCR5 and CXCR4, are shown in italics.

from AIDS patients. Yet, SI strains that apparently only use CXCR4 are also isolated from AIDS patients [54], and it is currently not clear if they evolve from multi-coreceptor strains or switch directly from CCR5.

Recently, a new terminology for HIV strains has been proposed that depends on coreceptor use. CCR5-using viruses will be called R5, CXCR4 viruses X4, and strains using both coreceptors, R5X4. This system can be extended for viruses that can use a range of coreceptors, e.g. R2bR3R5X4 for the 89.6 strain [55]. We will therefore adopt this nomenclature for the remainder of this review.

CELL TYPES INFECTED, AND THEIR RECEPTOR EXPRESSION

The main targets for HIV-1 infection *in vivo* are $CD4^+$ T-lymphocytes, macrophages, and dendritic cells. In the blood and lymph, $CD4^+$ T-lymphocytes are the predominant infected cell type [56]. On T-lymphocytes, CCR5 is expressed principally on memory T-cells, while CXCR4 is more widespread but predominates on naive T-cells [57]. It is therefore easy to envisage that emerging X4 variants will be able to infect and colonize new T-cell populations, perhaps critical for the homeostasis of the $CD4^+$ T-cell numbers. Their infection could thus precipitate the sharp decline of $CD4^+$ T-cells that follows X4 emergence [33, 34, 36]. CCR3 is expressed on $CD4^+$ TH2 cells [58]; however, its role in HIV infection of these cells has not been described.

HIV can be detected in blood monocytes only in a minority of HIV^+ individuals [56, 59]. *In vitro*, as monocytes differentiate into macrophages, they become susceptible to HIV replication [60, 61], and it is the more mature macrophages in lymph nodes and tissues that harbour virus. CCR5 expression is very low or undetectable on freshly isolated monocytes by flow cytometry, but is up-regulated significantly as they differentiate into macrophages [62–64].

PBMCs or macrophages derived from individuals homozygous for Δ32 CCR5 are resistant to infection by R5 strains [36, 42], confirming CCR5 as a crucial coreceptor for infection of these cells. Whether X4 viruses infect macrophages is controversial. The first X4 strains analyzed had been cultured in T-cell lines and were not competent for macrophage infection even though cultured macrophages express low levels of CXCR4 [65, 66]. These T-cell line adapted (TCLA) viruses have often acquired mutations in accessory genes, such as *vpr*, that are essential for efficient replication in macrophages. Regardless, some TCLA strains have envelope glycoproteins that are unable to trigger entry of virus into macrophages [29]. In contrast to TCLA X4 strains, primary X4 viruses infect cultured blood-derived macrophages proficiently [54, 67] using CXCR4 for entry [66]. These observations show that there is no specific restriction for X4 virus replication in macrophages *per se*. However, the cell surface expression of CXCR4 by macrophages may depend on the isolation procedure used, and other groups have also noted that their preparations of macrophages resist primary X4 virus infection [60, 68, 69]. Whether all these macrophages express CXCR4 is not known.

Langerhans cells and blood dendritic cells also contain HIV DNA *in vivo* [70–72]. Dendritic Langerhans cells are likely to be at or near mucosal surfaces and could be the first cell type infected following transmission. Freshly prepared, skin-derived Langerhans cells express cell surface CCR5, while CXCR4 is contained inside the cell in vesicles and requires priming by granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce surface expression [73]. Cultured dendritic or Langerhans cells are susceptible to both R5 and X4 viruses. Infection can be blocked by the addition of appropriate CCR5 and CXCR4 receptor ligands, thus implicating these 7TMs as the coreceptors used [74]. HIV replication in pure dendritic cells is inefficient, but is enhanced significantly by interaction with T-cells [75], as well as being dependent on the stage of dendritic cell maturation [76].

The brain is colonized by HIV-1 early after infection [77]. Microglial cells are of monocyte/macrophage lineage, and are the main infected cell type in brain sections from autopsy material. CCR3, CCR5, and other coreceptors have been implicated for infection of adult microglial cells [78, 79]; however, a consensus is yet to emerge. CD4-negative astrocytes may sometimes become infected, particularly in pediatric cases [80], although this infection may not be productive [81]. Coreceptors used for infection of astrocytes have not been described.

Nine different 7TM receptors that act as coreceptors for HIV-1 have been identified. Thus far, convincing evidence for only CCR5- and CXCR4-use has been forthcoming for infection of primary cell cultures *in vitro*. The role of coreceptors (other than CCR5 or CXCR4) in HIV replication and pathology is, therefore, a key issue. If drugs

targeted to CCR5 and/or CXCR4 simply leave space for strains using other coreceptors to expand and predominate, this therapeutic approach will fail.

CORECEPTORS, HIV TRANSMISSION, AND PATHOGENESIS

Following sexual transmission of the virus, early isolates are usually R5 strains. One possible explanation is that the first cells to be infected at transmission are $CCR5^+$ but do not express CXCR4. Mucosal Langerhans cells or submucosal macrophages are such candidate cell types. Cultured macrophages and Langerhans cells do express CXCR4, but both have the potential to express cell surface CCR5 without CXCR4 [69, 73]. Yet, many X4 viruses can use CCR5 as a coreceptor, and so their mechanism of exclusion is unknown. R5 viruses may be preferentially transmitted because all infected individuals carry them, while SI viruses (both X4 and R5X4) are found only at later stages of disease and can only usually be isolated from about 50% of AIDS patients. Therefore most HIV^+ individuals do not carry an SI virus to transmit. When an SI virus is transmitted, however, then the disease course of the infected individual is faster [82].

The role of chemokine receptors in the progression of infection to AIDS has been studied in long-term nonprogressors (LTNPs). Individuals heterozygous for Δ32 CCR5 are likely to survive longer [44]. A polymorphism in the CCR5 promoter region linked to a mutation in the CCR2b gene also predisposes for longer survival [83]. These observations emphasize the importance of CCR5 as a coreceptor in pathogenesis as well as transmission.

Recently, a polymorphism in the SDF-1 gene was also shown to slow disease progression [84]. This mutation lies in the 3′ untranslated region of the SDF-1 gene that may influence mRNA stability, resulting in more stable SDF-1 mRNA and, therefore, higher levels of SDF-1. It is possible that infection of X4 viruses via CXCR4 is therefore inhibited, suggesting that therapeutics aimed at CXCR4 could be useful in slowing disease progression.

MOLECULAR MECHANISM OF THE HIV/CD4/ CHEMOKINE RECEPTOR INTERACTION

The HIV viral membrane contains glycoprotein spikes essential for fusion with the host cell plasma membrane. Each spike contains three molecules of the glycoproteins gp120 and gp41, products on the viral *env* gene. This complex mediates its binding to the host cell surface CD4, driving a conformational change in gp120 [85, 86], which induces or exposes a binding site for the chemokine receptor [87, 88]. Physical evidence for a tight association between gp120, CD4, and the chemokine receptor has been provided by immunoprecipitation of a molecular complex of soluble gp120, membrane-associated CD4, and CXCR4 [89]. Direct binding studies using radiolabelled gp120 and CCR5 expressing cells showed a nanomolar interaction in the presence of soluble or cell surface CD4 [87, 88]. The exact regions of gp120 interacting with coreceptors are currently unclear but are likely to involve the V3 loop and relatively conserved epitopes that become more exposed on CD4 binding. Antibodies to these regions block the association of the HIV envelope with coreceptors.

The gp120/coreceptor interaction is thought to trigger conformational rearrangements in gp41, the transmembrane glycoprotein. These changes may release the hydrophobic amino terminal region of gp41, which must insert into the host cell membrane to initiate the fusion of the cell and virus membranes. This scenario is based on the model for influenza virus fusion, where the conformational change in the envelope protein has been shown to be induced by low pH, rather than receptor interaction [90]. Once the virus has fused with the host cell membrane, the virus core must detach from the membrane to form a preintegration complex. The virion RNA is reverse transcribed into DNA and is carried by the preintegration complex to the nucleus where the DNA genome becomes integrated into a host cell chromosome. The integrated provirus directs the new replicative cycle.

MOLECULAR BASIS FOR CORECEPTOR/ gp120 SELECTIVITY

The molecular basis of the interaction of the viral envelope protein with the chemokine coreceptors is an area of active research. For NSI/R5 viruses, the N-terminus of CCR5 is crucial for coreceptor recognition. Chimeric CCR2b/CCR5 receptors containing only the CCR5 N-terminus are active for R5/NSI virus infection [91, 92]. CCR5 has three tyrosine residues in this N-terminus, which have been suggested to be important since they are conserved in the orphan coreceptors BONZO and BOB [51]. Yet, the negatively charged residues at the CCR5 N-terminus are crucial for coreceptor activity; mutation of aspartic acid at positions 2 and 11, as well as a glutamic acid at position 18, impair or abolish CCR5 coreceptor activity [40]. In addition, mAbs recognizing the CCR5 second extracellular loop (E2) efficiently blocked HIV-1 infection, while mAbs to N-terminal epitopes inhibited only weakly [88]. Together, these observations suggest that both regions (E2 and the N-terminus) play a role in coreceptor activity. HIV-1 strains that can exploit CXCR4 and other coreceptors as well as CCR5 have a weaker CCR5 interaction than pure R5 viruses. These R5/X4-using viruses are particularly sensitive to mutations throughout the CCR5 sequence [93] as well as extremely sensitive to inhibition of infection by CCR5 ligands [94].

In contrast to this, the X4 viruses recognize the second extracellular loop of CXCR4. Thus, a chimeric receptor containing the N-terminus of CCR5 and E2 of CXCR4 functions for a range of X4 and R5 viruses [92]. Truncation of N-terminal sequences of CXCR4 showed that not all X4 viruses required a full-length N-terminus. This dependency

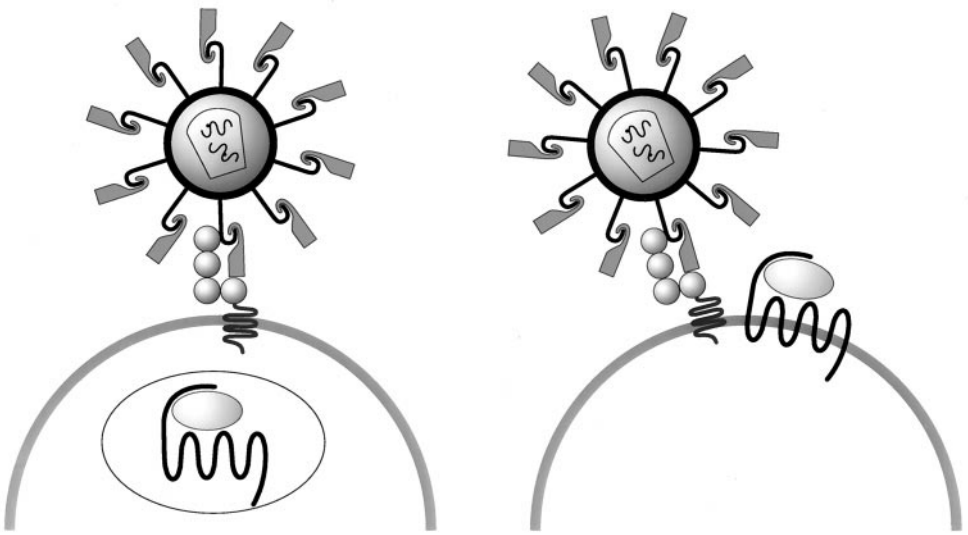

FIG. 5. Two hypothetical mechanisms whereby chemokines or chemokine receptor antagonists can prevent the chemokine receptor from acting as a coreceptor. The cartoon on the left indicates that activation of the receptor after ligand binding results in the internalization of the ligand/receptor complex into endosomes, thereby removing the receptor from the surface which prevents it from functioning as a coreceptor. The alternative mechanism shown on the right indicates that ligand binding to the receptor results in sterically hindering the CD4/viral complex from interacting with the chemokine receptor in what can be regarded as classic competitive inhibition.

did not correlate with strains that used CXCR4 only or were able to use multiple coreceptors [95].

FUNCTIONAL CHEMOKINE RECEPTOR ANTAGONISTS

Minor mutations of cytokines can sometimes abolish their ability to activate receptors while retaining their ability to bind, resulting in potent receptor antagonists. Chemokine antagonists and partial agonists can be produced by modifications of the amino terminus. Amino terminal truncation of IL-8 up to the arginine residue results in a receptor antagonist [96]. RANTES can also be inactivated by additions to the amino terminal. When recombinant RANTES is produced in the bacterial host *Escherichia coli*, the initiating methionine is retained at the amino terminus. This extra amino acid makes Met-RANTES a potent antagonist of chemotaxis *in vitro* [97], and *in vivo* where Met-RANTES has profound effects in reducing inflammation [98, 99]. An even more potent RANTES version was created by the chemical coupling of an alkyl chain to the amino terminal serine (AOP-RANTES) [100].

The modified RANTES proteins were initially thought to be pure receptor antagonists. However, both Met-RANTES and AOP-RANTES are able to mobilize calcium through CCR5, although neither achieves the potency and efficacy of RANTES itself [101]. Moreover, two RANTES variants, (9–68) RANTES and AOP-RANTES (but not Met-RANTES), are efficient in mediating receptor internalization [101–103], which is consistent with their ability to activate receptors. It is believed that internalization occurs following activation of all 7TM receptors [104, 105] and is mediated by phosphorylation of the carboxy terminal region, and this process is independent of G-protein signalling.

ANTAGONISM OF VIRAL ENTRY

β-Chemokines were shown to have inhibitory properties for HIV-1 infection before the identification of the chemokine receptors as HIV coreceptors [38]. Chemokines that interact with specific coreceptors are able to block infectivity: RANTES, MIP-1α, and MIP-1β inhibit CCR5-mediated entry; SDF-1 blocks infection via CXCR4 [106, 107], eotaxin inhibits CCR3 infection [45] and I-309, infection mediated by CCR8 [47].

There are two ways that chemokines can block HIV infection, as shown schematically in Fig. 5. The first is simply by steric hindrance, where chemokine binding prevents HIV from interacting with a chemokine receptor. The second is based on the capacity of chemokines to induce endocytosis of their receptors [103, 108]. Removal of a chemokine receptor from the cell surface obviously prevents the receptor from acting as an HIV coreceptor. These mechanisms, in theory, could be distinguished using receptor agonists and antagonists. The three RANTES functional antagonists described to date have all been shown to be effective in preventing the infection of cells by HIV-1 strains.

Internalization of seven-transmembrane receptors, an agonist-triggered event, is mediated by phosphorylation of the carboxy terminal region by the GRK kinase (G protein coupled receptor kinase) family, followed by interaction with the arrestin proteins and sequestration into clathrin coated pits. However, both (9–68) RANTES [106] and

AOP-RANTES [101], antagonists of chemotaxis, induced receptor down-regulation. They are thus agonists for the signal transduction mechanisms required for endocytosis. But chemokine receptors act as HIV coreceptors independently of functional responses, at least those mediated by G-proteins. Both X4 and R5 viruses are able to infect cells in the presence of pertussis toxin [38, 109], which inhibits seven-transmembrane receptors coupling to the G_i class of G-proteins, and thus abolishes chemokine-induced responses such as calcium mobilization and chemotaxis. These data are confirmed by mutagenesis studies, where receptors deleted of the C-terminal region (essential for Ca^{2+} flux) still function as coreceptors for HIV [102].

Chemokine inhibition of HIV entry by steric hindrance could be achieved by an agonist or an antagonist, since both types of proteins bind to the receptors. A truncated RANTES protein, (9–68) RANTES [110], as well as Met-RANTES, fails to elicit calcium mobilization and chemotactic responses in primary cells, and these proteins are thus functional antagonists. Both are capable of inhibiting HIV-1 infectivity, albeit less potently than RANTES itself. Very potent inhibition was achieved by the chemically modified AOP-RANTES [100]. This protein was able to efficiently inhibit infection of primary macrophages by R5 strains of HIV-1, whereas RANTES and Met-RANTES showed little or no inhibition at all.

When 7TM receptors are internalized into endosomes, they can follow one of two pathways: transport to the lysosomal compartment where they are degraded, or recycling back to the cell surface. These alternative pathways have not been studied extensively to date for the chemokine receptors, but evidence exists for both. CXCR2 has been shown to undergo lysosomal degradation [111], whereas CXCR4 [108] and CCR5 [101] have been shown to recycle to the cell surface. When RANTES is removed from the medium following down-regulation in PBMCs, all of the CCR5 receptors are shown to recycle, even in the presence of cycloheximide, which prevents *de novo* protein synthesis [101]. Yet, in the same study, it was shown that removal of AOP-RANTES from the medium did not result in receptor recycling. Furthermore, culturing PBMCs for up to 12 days in the presence of RANTES eventually results in re-expression of surface CCR5, while culture with AOP-RANTES does not. The capacity of AOP-RANTES to prevent CCR5 recycling correlates with its higher affinity for CCR5 and its greater potency in inhibiting HIV infectivity than RANTES, suggesting an efficient mechanism to prevent the infection of cells by HIV. The absence of surface coreceptor in preventing HIV infection has also been validated by an approach based on gene targeting. The CCR5 ligands RANTES and MIP-1α were modified such that a KDEL sequence added to their carboxy terminus prevents newly synthesized receptor from trafficking from the endoplasmic reticulum to the cell surface, rendering these cells resistant to HIV infection [112]. The challenge, therefore, is to design similar agents that simply strip CCR5 from the cell surface and prevent its re-expression.

CHEMOKINES ENCODED BY VIRUSES

One interesting complexity to the chemokine story built up last year as a result of chemokine open reading frames being identified in herpes viruses (see Ref. 113). Human herpes virus-8 (also known as Kaposi's sarcoma herpes virus) has long been found associated with the Kaposi sarcoma, a known complication of AIDS. The sequencing of its genome yielded three putative chemokines, called vMIP-I, vMIP-II, and BCK or vMIP-III. Of these, vMIP-II has been studied most intensively [94, 114]. Receptor binding studies showed that although vMIP-II had the highest sequence identity to human MIP-1α and MIP-1β, its receptor binding profile was completely different. vMIP-II is able to compete for ligand binding to CCR5 as well as CCR3, CXCR4, and cytomegalovirus US28. This is the first natural chemokine to be found that can bind to both CC and CXC chemokine receptors. However, some selectivity is maintained, since vMIP-II does not compete significantly with IL-8 binding to CXCRI, nor with MIP-Iα binding to CCR1. The role of this and other vMIPs in the HHV-8 life cycle is not clear, but vMIP-II does have angiogenic activity [114], and it acts as a partial agonist in cell recruitment. In HIV infection assays, vMIP-II was able to block all strains, and although less potent than AOP-RANTES and SDF-1 on CCR-5 and CXCR4, it is the most potent chemokine inhibitor of HIV infection via CCR3. However, the identification of a single molecule that can bind selectively to the key HIV coreceptors suggests that it might eventually be possible to produce small molecules with a desired receptor profile for HIV inhibition and therapy.

SMALL MOLECULAR WEIGHT CHEMOKINE RECEPTOR ANTAGONISTS: A PHARMACEUTICAL REALITY?

Many low molecular weight molecules have been described that interact with, and are antagonistic for, seven-transmembrane receptors. Examples include histamine, adrenalin, serotonin, and dopamine. Many have been developed into therapeutics that can be administered orally. Chemokines are much larger ligands, about 8 kDa. The chemokine/chemokine receptor interaction has been proposed to interact in a two-site mode [115, 116] similar to that suggested for C5a [117], where the body of the protein ligand would interact with an extracellular portion of the receptor, and signalling would be triggered by the interaction of the amino terminal region in a smaller site, probably in the seven-transmembrane regions. Evidence supporting this model is found from the susceptibility of the chemokines to modifications at the amino terminus, which appear to greatly affect their signalling capacities while not significantly changing their receptor binding properties. RANTES binding to CCR5 is blocked by mAbs to the second extracellular loop, E2, but not by N-terminal mAbs, indicating that site 1 involves E2 but not N-terminal sequences [118].

Development of small molecular weight ligands for chemokine receptors has not been a trivial task. Intense efforts from the major pharmaceutical companies have resulted in very few reports of coreceptor-specific compounds. Recently, however, several compounds have been noted in the patent and scientific literature [119]. No compounds have been described to date for CCR5. However, three classes of compounds were identified by their ability to block infection of PBMCs by X4 HIV-1 strains. These compounds have all been shown to be CXCR4 receptor antagonists that block binding of its ligand, SDF-1, as well as inhibiting SDF-1-induced receptor activation. The most potent class of compounds is the bicyclam series, where the prototype AMD3100 is 10- to 50-fold more potent in inhibiting HIV infection than SDF-1 itself [120, 121]. The second type of antagonist is a cyclic 18-mer peptide containing two disulfides that was isolated from *Limulus polyphemus* [122], while the third is a pseudo 9-mer peptide [123].

Current HIV treatments are all directed at proteins produced by the virus itself, whereas targeting the chemokine receptors would be a therapy directed to a host protein. The question is often raised as to whether antagonizing a receptor involved in general immune surveillance would not be deleterious to general physiology. Excellent support for this therapy, however, is provided by 0.1 to 1% of the Caucasian population, who are homozygous for a 32 bp deletion in the CCR5 gene. These individuals are effectively human equivalents to experimental "knock-out" animals, since they do not have a functional CCR5. No obvious health problems occur in these individuals, who are highly resistant to HIV infection. CCR5 antagonists that block or reduce its effectiveness as a coreceptor are exciting. Such agents have potential for use as prophylactics in condoms and for post-exposure treatments. Moreover, since polymorphisms that reduce CCR5 expression levels also slow disease progression, there is a clear potential to target CCR5 with therapeutics during the asymptomatic phase and before X4 viruses have emerged. Antagonists of CXCR4 may also have therapeutic benefit to patients who carry X4 strains of HIV-1 and are in an advanced stage of disease. To date, the only available potential therapeutic agents are the chemokines themselves, or modified chemokine proteins, but new synthetic molecules will soon be identified by the intensive screening programs currently undertaken by many laboratories and pharmaceutical companies.

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