

# Cloning of a Novel Chemoattractant Receptor Activated by Leukotriene B<sub>4</sub> and Used by Human Immunodeficiency Virus Type 1 to Infect CD4-positive Immune Cells A Therapeutic Connection to Asthma?

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The superfamily of G protein-coupled receptors (GPCRs) represents the most widely distributed group of membrane receptors in eukaryotic cells (1). These receptors receive chemical signals that consist of molecules of considerable diversity that are released from a great variety of cellular sources. The receptor protein usually consists of some 350–450 amino acid residues, which are arranged as a serpentine-like structure, which runs in and out through the cell membrane seven times—hence the name seven-transmembrane (7TM) or heptahelix receptor. The ligands bind in a varying manner to the N-terminal tail and the extracellular loops, with important contributions also from the deeper helix regions within the cell membrane. Further signaling within the cell is mediated through binding of intracellular receptor domains to the heterotrimeric G proteins.

The exceedingly rapid development during the last decade in our understanding of the molecular mechanisms underlying the functions of GPCRs has to a large extent been based on the cloning and characterization of cDNA and genes encoding these receptors. In this way, entirely unexpected types of receptors have been detected, for example, the cannabinoid receptor (2). It has also been found that the whole odorant system is based on hundreds of genes encoding GPCRs that are expressed in the olfactory epithelium (3). Despite knowledge about the existence of the leukotrienes for more than two decades (4), the cDNA encoding the first leukotriene receptor was not cloned until recently (5) and, at about the same time, it was sensationally shown that two GPCRs serve as cofactors necessary for the entry of human immunodeficiency virus type 1 (HIV-1) into the CD4-positive cells of the immune system (6).

## CLONING OF A NOVEL HEPTAHELIX RECEPTOR

A frequently and successfully used strategy for discovering entirely new GPCRs is to apply homology screening. Using sequence information from highly conserved motifs within previously known receptor molecules, degenerate oligonucleotides are synthesized and applied at low stringency as polymerase chain reaction (PCR) primers or hybridization probes when screening cDNA or genomic libraries. In our own work we based the oligonucleotide synthesis on sequence information

from some 10 previously cloned peptide receptors and screened, using PCR and Southern blot hybridization, a human B cell lymphoblast cDNA library constructed according to the principles of Okayama and coworkers (7).

Thus, PCR experiments with degenerate primers corresponding to the TMII and TMVI regions of the selected receptors resulted in the amplification of a 430-bp sequence, whose deduced amino acid sequence showed homology with, and a predicted hydrophobicity pattern corresponding to, a new GPCR. Oligonucleotide probes derived from this sequence were synthesized and used to screen the human lymphoblast library in an attempt to obtain the full-length sequence. A 1.7-kb-long cDNA clone was isolated, and found to contain an open reading frame, with a putative initiator methionine conforming to the optimal sequence for translation (10) and an in-frame downstream stop signal. This DNA sequence encoded a 352-amino acid protein whose general sequence structure conformed with that of GPCRs.

## PROTEIN SEQUENCE SUGGESTED A CHEMOATTRACTANT RECEPTOR

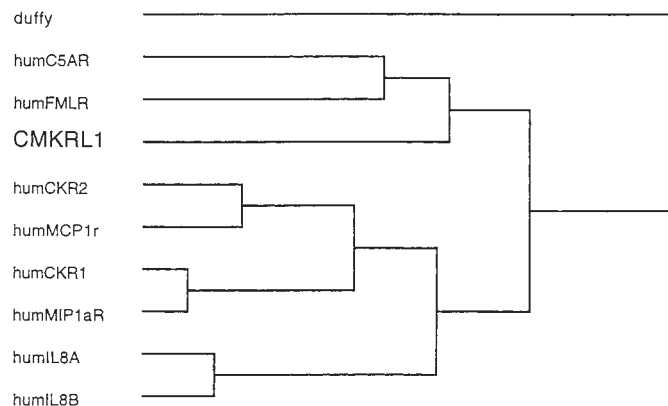
In sequence alignments from databases, the predicted amino acid sequence was found to possess many features common to the superfamily of GPCRs: (1) consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr, where X is any amino acid) near the amino terminus (Asn-2) and in the second extracellular loop (Asn-164), (2) a conserved cysteine residue in each of the first two extracellular loops (Cys-90 and Cys-168), providing possibilities to form a disulfide bond that stabilizes the functional protein structure, (3) proline residues in all transmembrane regions (except TMIII), which are thought to induce flexibility within the helix formations, and (4) a carboxyl terminus with several serine and threonine residues (10/4), which could serve as substrate for serine/threonine protein kinases. The predicted third intracellular loop, thought to be particularly important in the coupling to G proteins, and varying most in length among the different GPCRs, consisted of only 21 amino acid residues.

This previously unknown receptor showed highest structural similarity with the family of leukocyte chemoattractant receptors (Figure 1), particularly the so-called “classic” chemoattractants C5a (30% overall sequence identity with the new receptor) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 28% sequence identity). In view of the preceding considerations, the newly cloned receptor was named CMKRL1 (“chemoattractant receptor-like 1”) according to genome database nomenclature (5). The sequence has been deposited with the EMBL/GenBank/DBJ databases under accession no. X98356. Further work was focused on the identification of the parent ligand for this “orphan” receptor.

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**Figure 1.** Dendrogram (the horizontal distances to the branching points corresponding to the relative degree of sequence identities) based on eight human chemoattractant receptors as well as the Duffy antigen, together with the presently cloned CMKRL1, illustrating the degree of similarity in the evolutionary pattern. Full amino acid sequences were used in the multiple alignments and the dendrogram, and were done using the PileUp program in the GCG software package.

### CELLULAR AND TISSUE DISTRIBUTION OF CMKRL1

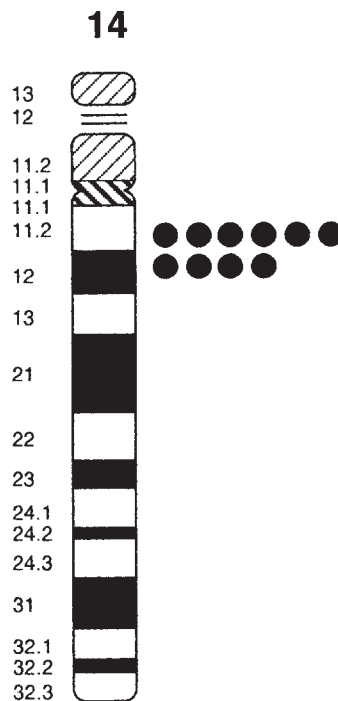
Knowledge about the cellular expression and tissue distribution of an unknown receptor may be of key importance in trying to establish the functional identity of the receptor. Thus, Northern blot hybridization of the receptor message at high stringency (5) revealed two primary transcripts, slightly more than 5 and 7.5 kb in size, in the spleen, thymus, lymph nodes, bone marrow (bigger transcript predominating), and peripheral blood leukocytes (smaller transcript predominating), in order of autoradiographic signal intensity. Some tissues—skeletal muscle, pancreas, and heart—showed only one, relatively weak hybridizing band that was smaller, about 3 kb. Two small bands appeared in the bone marrow, and they were seen at a lower signal intensity also in peripheral leukocytes.

There may be several reasons for the identification of several band sizes, in spite of the stringent hybridization conditions used: it could indicate the presence of several splice variants of the message; and/or there might be a varying degree of expression of different RNA entities in different types of cells, in various stages of cell maturation, or in different functional stages of a particular cell.

Analysis by *in situ* hybridization histochemistry corroborated the ubiquitous distribution of CMKRL1 message in cells within the immune system. In immunocytochemical studies with a monoclonal antibody applied to transfected cells stably expressing CMKRL1 we could show (8) that a positive signal was located in a dotlike fashion within the cell membrane where the receptor should normally be expressed. With fluorescence *in situ* hybridization (FISH) we could, in addition, demonstrate that the receptor gene is located in the long arm of chromosome 14, within region q11.2-q12 (Figure 2).

### IDENTIFICATION OF THE PARENT LIGAND FOR THE ORPHAN RECEPTOR

To allow for functional studies of the novel chemoattractant receptor, the cloned cDNA was transfected into various standard cell lines. The level of expression, and hence the selection of suitable cell clones, was monitored in Northern blot hybridizations. In attempts to identify the ligand for CMKRL1 we screened, at first not successfully, large numbers of more or

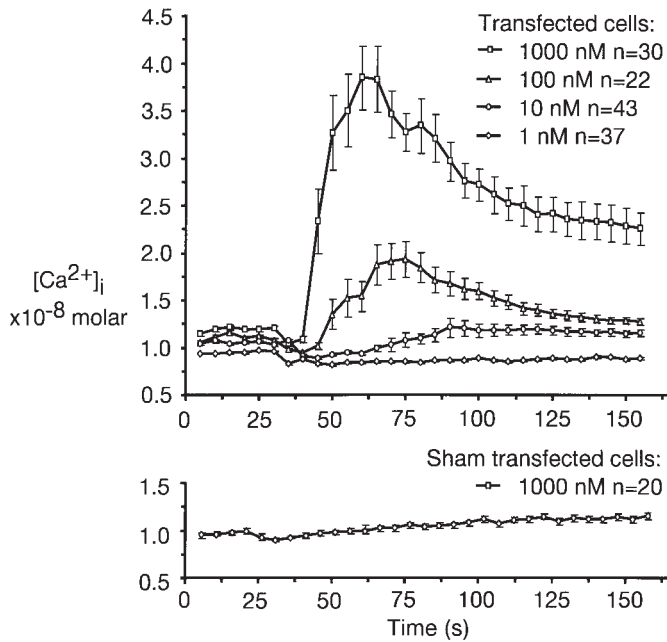


**Figure 2.** Line drawing based on the FISH mapping of the gene encoding CMKRL1. Each dot represents the double fluorescence signals detected on chromosome 14 (images from 10 photographs). Reprinted by permission from Reference 5.

less likely ligand candidates by functional measurements of intracellular calcium fluxes in the transfected cells, thus reflecting activation of certain intracellular transduction mechanisms (the chemokine receptors, e.g., couple to the  $G_q$  protein) following any induced stimulation of the receptor. Subsequent to numerous negative recordings, reproducible calcium signals were eventually obtained with leukotriene  $B_4$  ( $LTB_4$ ). To corroborate the findings, which thus provided the identity of the hitherto unknown receptor, functional blocking experiments were performed with the monoclonal antibodies, and binding studies were performed with radiolabeled  $LTB_4$  and isolated membranes of transfected COS-7 cells (8).

Figure 3 illustrates that the addition of  $LTB_4$  to CHO cells stably expressing the CMKRL1 receptor and preloaded with Fura-2 results in a rapid increase in the fluorescence ratio, reflecting increased intracellular levels of calcium. The effect was concentration dependent between 1 and 1000 nM final concentrations, the onset being more rapid and the elevation steeper at the higher concentrations. After the maximum response had been attained the cellular calcium concentration slowly returned to baseline levels.  $LTB_4$  was completely without effect at any dose level when added to sham-transfected control cells (Figure 3). When the regular cell medium was exchanged for calcium-free medium,  $LTB_4$  no longer induced any significant increase in intracellular calcium levels. Preincubation of CMKRL1-expressing cells with one of the monoclonal antibodies (directed against the amino terminal of the receptor) almost completely blocked the  $LTB_4$ -induced calcium response (8).

Binding of [ $^3H$ ] $LTB_4$  to isolated membrane preparations from transfected COS-7 cells expressing CMKRL1 showed a concentration-dependent increase in both total and specific binding at 4° C until saturation was reached (Figure 4). Non-specific binding represented less than 15% of the total binding, and did not vary significantly with radioligand concentration. There was a near-linear increment in the specific binding of [ $^3H$ ] $LTB_4$  with increasing concentrations of membrane protein, whereas membranes from sham-transfected cells exhibited no specific binding. The saturation binding studies yielded

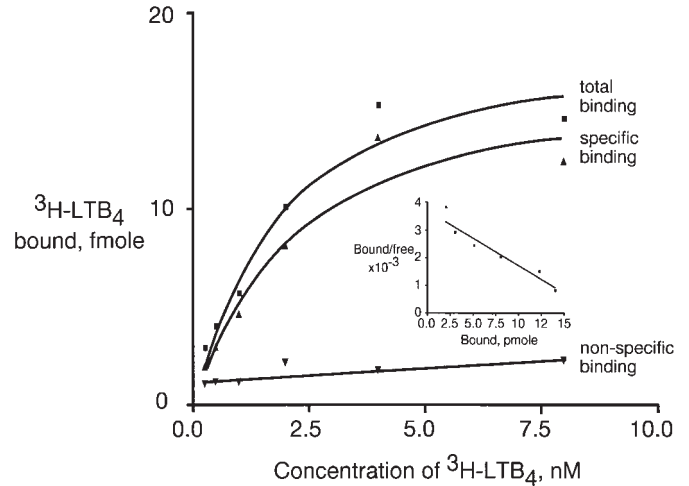


**Figure 3.** Action of four different concentrations of  $LTB_4$  (figures given are final concentrations in the test well) on intracellular calcium levels in CHO cells stably transfected to express CMKRL1 and preloaded with Fura-2 for monitoring of intracellular calcium concentration (fluorescence ratios). The lower curve illustrates the effect of the highest  $LTB_4$  concentration tested on sham-transfected control cells. Values represent means  $\pm$  SEM (n = 20). Reprinted by permission from Reference 7.

a linear Scatchard plot (Figure 4, inset). Thus, the radioligand identified a single class of high-affinity binding sites with an affinity constant ( $K_d$ ) of  $2.1 \pm 0.5$  nM (mean  $\pm$  SEM) and a  $B_{max}$  of  $17.0 \pm 2.0$  pmol/mg of protein (8). This affinity constant is close to the figure produced by guinea pig eosinophil cell membranes, in which the receptor is endogenously present.

Our results show that tritiated  $LTB_4$  binds with high affinity to a single receptor population in transfected (but not sham-transfected) cells transiently expressing CMKRL1. Moreover,  $LTB_4$  in a concentration-dependent manner functionally activates cells stably expressing the previously "orphan" receptor. The effect is blocked by monoclonal antibodies directed against a receptor epitope known to be of key importance during ligand binding to the GPCR. The results provide strong evidence that  $LTB_4$  is a natural ligand for CMKRL1, which is, thus, the first cloned leukotriene receptor and should hence be named BLTR according to the established pharmacological (NC-IUPHAR) nomenclature.

Shortly after CMKRL1 was published (5), an identical cDNA sequence was obtained by another group working with human erythroleukemia cells and, mainly on the basis of radioligand-binding experiments with  $[^{35}S]dATP\alpha S$ , the corresponding receptor was proposed to be a new member of the P2Y group of purinoceptors (9). The interpretation of the binding data and of functional assays performed with transfected cells, as well as the comparatively low relatedness of the deduced amino acid sequence to hitherto cloned P2Y receptors, warranted a reevaluation of this conclusion. Thus, in more extensive studies of the binding and function of the receptor stably expressed in a human astrocytoma cell line, others were able to assess that the purported P2Y<sub>7</sub> receptor is not a member of the P2Y family of signaling molecules (10). By



**Figure 4.** Saturation and Scatchard (inset) analyses of  $[^3H]LTB_4$  binding to membranes prepared from COS-7 cells transfected with cDNA to express CMKRL1. Results shown are the mean values of three experiments performed in triplicate. Reprinted by permission from Reference 7.

applying a subtraction strategy to retinoic acid-differentiated HL-60 cells, a cDNA sequence identical to the coding sequence of CMKRL1 was again reported, and the corresponding receptor for the first time was identified as BLTR (11). The receptor clone R2 obtained from a human genomic library (12), on the other hand, deviates from the above-mentioned three (5, 9, 11) DNA sequences in its 5' end, which led to the identification of a different methionine initiation site in the proposed coding sequence.

#### CMKRL1/BLTR FACILITATES CELLULAR ENTRY OF SELECT PRIMARY ISOLATES OF HIV-1

It is now well understood that HIV-1 during infection fuses with and enters the target cell through a series of events involving two classes of cell membrane receptors (6). First, the virus associates with the tyrosine kinase-activating receptor, CD4, which induces conformational changes in the glycoprotein envelope, allowing the virus subsequently to bind to a GPCR that, in turn, facilitates the necessary fusion of the envelope with the cell membrane and thus viral entry. Two major coreceptors have been identified, both belonging to the chemokine family: CCR5, which is primarily involved in the infection of M-tropic viral strains, and CXCR4 (also called fusin), which is required for the fusion of primarily T-tropic strains (6).

With progression of the infection there appears to be a shift in chemokine receptor usage, from CCR5 to CXCR4, reflected by a loss of sensitivity to  $\beta$ -chemokines (13). The  $\beta$ -chemokines are the natural ligands for CCR5 and they are able to compete with M-tropic viruses for that particular receptor (14). The usage of the two major cofactors may, thus, be viewed as extremes in an adaptation process, along which the virus expands its coreceptor usage to include several different receptors that bind different variants within the V3 loop of the envelope glycoprotein 120 (gp120).

As already mentioned, mapping of the distribution of CMKRL1—now identified as the  $LTB_4$  receptor, BLTR—revealed a widespread expression in CD4-positive cells of the immune system, including thymus, spleen, lymph nodes, and peripheral blood mononuclear cells (PBMCs). Further, the re-

TABLE 1  
CLINICAL AND LABORATORY ISOLATES OF HIV-1 USED  
IN THE INFECTION EXPERIMENTS: DATA FROM THE  
PATIENTS AND CHARACTERISTICS OF THE ISOLATES\*

Patient	CD4 <sup>+</sup> Cell Count <sup>†</sup>	Isolate	Virus Phenotype <sup>‡</sup>	p24 Core Antigen <sup>§</sup>
A	400	DS pbmc CD8-	SI	150
B	354	OR pbmc CD8-	SI	262
C	79	5775 PBMC	SI	68
D	—	G3	NSI	120
E	923	OT pbmc CD8-	SI	110
F	411	P001 pbmc CD8-	SI	24
G	386	L002 PBMC	SI	36
H	—	22069-65	SI	356
J	—	JV 1083	NSI	185
K	—	571	SI	170
—	—	IIIB	SI	237
—	—	BaL	NSI	688

\* Clinical virus isolates from 10 patients, as well as 2 laboratory-adapted model isolates were tested on CD4-positive mouse fibroblasts (NIH 3T3) expressing various types of human chemotactic receptors. Primary isolates were grown by coculturing with stimulated human PBMCs (in the four isolates designated "pbmc CD8-," PBMCs were depleted of CD4-positive cells). G3 and JV 1083 are primary isolates from Nigerian clade G/A.

<sup>†</sup> Absolute counts of CD4-positive lymphocytes (cells/mm<sup>3</sup>) in patients were measured at the time of initial blood draw.

<sup>‡</sup> Phenotype was subsequently classified in MT-2 cell assays performed after the infection experiments.

<sup>§</sup> Viral titers (ng/ml) were evaluated *in vitro* by p24 core antigen ELISA.

ceptor shows approximately 30% identity with both CCR5 and CXCR4, which, in turn, exhibit the same degree of homology when compared with each other. This would make our receptor an excellent target for HIV-1. Moreover, the gene is located close to the gene encoding the inhibitor of NF- $\kappa$ B, a transcription factor important in immune function, inflammation, and in the control of cell growth; and also for the expression of viruses such as HIV-1.

We therefore set out to perform infection experiments with primary HIV-1 isolates (Table 1), using a murine host cell model consisting of NIH 3T3 cells stably expressing exogenous human CD4 receptors (15). These cells were then cotransfected with either of the two major HIV-1 coreceptors, CCR5 or CXCR4, or, alternatively, with the receptor under study, CMKRL1 (the infection experiments were, in fact, carried out when our receptor clone was still an "orphan," before LTB<sub>4</sub> had been identified as the natural ligand). Entry of HIV-1 into the murine host cell model was estimated semiquantitatively in terms of the amount of PCR-amplified virus DNA, as measured by computer-assisted densitometry in Southern blots after cellular entry and reverse transcription of viral RNA.

The infection experiments showed (15) that there was more than a 50-fold increase in the cellular entry of patient isolates A-G (Table 1) when testing cells expressing CMKRL1 compared with the CD4-positive controls (Figure 5). All isolates (except DS CD4) also entered into cells that instead expressed CXCR4. In the case of isolates A-D, the entry into cells expressing CMKRL1 was even higher than the entry mediated by CXCR4. A particular high level of viral entry into cells expressing CMKRL1 was observed when the isolate L002 had been enriched in PBMCs. Preliminary data using a different infection model of human astrocytes coexpressing CD4 and CMKRL1 have shown that the receptor also supports the infection of yet another syncytium-inducing (SI) virus isolate (designated LW), as reflected by p24 enzyme-linked immunosorbent assay (ELISA) measurements.

In accordance with the SI characteristics of the isolates tested, the usage of CCR5 was in most instances (except for isolate LR

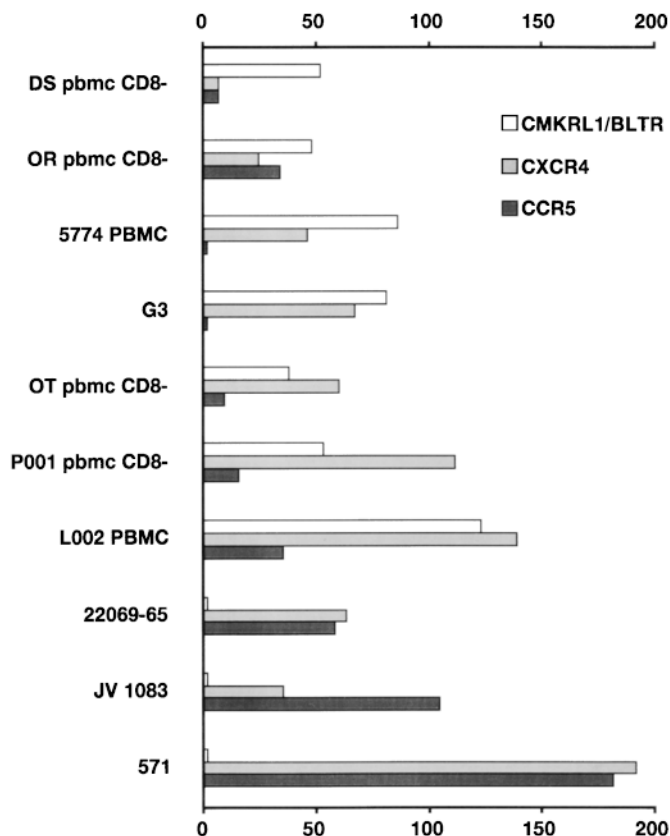


Figure 5. Infection experiments in which CD4-positive NIH 3T3 cells (murine fibroblasts), coexpressing one of three different human chemotactic receptors (BLTR, CXCR4, or CCR5), were infected with 10 clinical isolates of HIV-1 (see Table 1). The LTB<sub>4</sub> receptor (BLTR) clone used in the experiments is that designated CMKRL1. After a 4-h exposure to the various isolates, followed by washing and an additional 16-h incubation of the infected cells, viral cDNA was amplified in semiquantitative PCR followed by hybridization of Southern blots with a <sup>32</sup>P-labeled oligonucleotide probe corresponding to an internal HIV-1 DNA sequence. The graph illustrates computer-assisted densitometry of the autoradiograms, and is expressed as relative density (pixel saturation level) of the hybridizing bands after subtraction of the film background and of any positive signal from control cells expressing CD4 alone. Bars show averages of two to four experiments, the standard deviation being less than 20% of the mean absolute pixel values. Reprinted by permission from Reference 15.

CD4) lower than that of CXCR4. This is in contrast to the situation with isolates H-K, which entered to a high degree into both CD4-positive cells expressing CXCR4 and into those expressing CCR5. None of these isolates utilized CMKRL1 for entry (15).

Consistent with the preceding findings with the clinical isolates, the SI type of laboratory isolate, IIIB, also showed significant entry into cells expressing CMKRL1, in addition to the entry into cells transfected with CXCR4. The other prototypic isolate, BaL—which is an NSI-type of laboratory-adapted virus that, as expected, is efficiently taken up into CD4-positive cells equipped with CCR5—did not enter CMKRL1-expressing cells to any significant degree (15).

#### CONCLUDING REMARKS

Our experiments introduce the LTB<sub>4</sub> receptor, BLTR, as a novel type of HIV-1 coreceptor for certain primary virus iso-

lates. These differ from the viruses frequently used in other studies of coreceptor function in that they are clinical isolates, which have not been passaged and adapted in immortalized cell lines. They may therefore more closely reflect the actual mode of virus usage at a given stage of disease progression. Indeed, the presently described coreceptor function, like that of several other proposed coreceptors, has been identified in artificial target cell models, and it therefore remains to be established what relevance this—and some other—coreceptors have for the HIV infection mechanism in a natural milieu, for example, in PBMCs endogenously expressing the proposed coreceptor. CMKRL1/BLTR shows the same general distribution in CD4-positive human tissues and cells belonging to the immune system as previously identified coreceptors, but differs in that the major coreceptor “partner” is CXCR4 rather than CCR5. This agrees with the finding that the virus isolates using BLTR turned out to be primarily of the SI phenotype. This is a more highly adapted and virulent virus subtype, suggesting that the coreceptor function of BLTR might be important in late stages of infection. Blockade or inactivation of this receptor might therefore be able to slow disease progression in a manner reminiscent of the V641I mutant of another coreceptor, CCR2b (16).

HIV-1 entry after infection of the CD4-positive mouse fibroblasts expressing CMKRL1/BLTR was monitored by PCR amplification of viral DNA, which is a sensitive method. Since entry of the primary isolates in this model, whether expressing BLTR or CXCR4 (or CCR5), was relatively low compared with the “enormous” entry of the laboratory-adapted isolates (IIIB or BaL) into cells expressing their respective prototypic coreceptors (CXCR4 or CCR5), it is possible that other target cell models are not sufficiently sensitive to allow for the detection of certain HIV-1 test isolates using the BLTR (17). Moreover, the cell type used for BLTR expression (and even the cDNA clone used) may be of importance, as may the number of receptors expressed on the cell surface. Our experience is that BLTR is easily desensitized (internalized?) in some types of stably transfected host cells as evidenced by the capricious response to the natural ligand. The virus phenotype used by BLTR in the course of a complex adaptation process may be select, and a given isolate may even have changed phenotype when tested in various experimental settings.

## PERSPECTIVES

The identification of the chemokine receptors as entry factors of key importance in the cellular infection process of HIV-1 has meant a major breakthrough for many reasons.

1. Our understanding of the interaction between the virus and the target cells has taken a major step forward. It now seems evident that these heptahelix-type receptors are not “only” secondary receptors or coreceptors, but instead constitute the primary mechanism for viral entry, which in some instances may be mediated by these receptors even in the absence of CD4. The role of the latter receptor may rather be to provide enrichment of viral particles at the target cell surface, before membrane fusion takes place.
2. It can be assumed that the involvement of heptahelix receptors in viral infection mechanisms will introduce an entirely new pathophysiologic principle that may not be restricted only to HIV (and SIV). The presently observed role of BLTR may widen the heptahelix receptor usage, from chemokine receptors to their structurally related “cousins” within the family of “leukocyte chemoattractant receptors.” In view of the considerable degree of homology between heptahelix receptors also outside this subfamily of inflammatory receptors, the decisive criterion for viral coreceptor function might be in which cell type, or how widely, the receptor is expressed in order to serve as a suitable target for the “viral cadger.”
3. Quite a number of coreceptors have now been identified (18). They seem to be utilized in a complex viral adaptation process in the course of disease progression, where multiple receptor usage spans between the two major coreceptors, CCR5 and CXCR4, as HIV-1 changes its phenotype. Little is known about any peculiar role of individual coreceptors within specific populations of viral genotypes, in a more global perspective.
4. The important role of the chemokine receptors, particularly CCR5, and the resistance to infection linked to the  $\Delta 32$  mutation (6), have initiated important new directions in the development of useful drugs that do not target the virus itself, but rather its entry routes by way of the chemokine receptors (18). In this way drugs can be foreseen that not only prevent the primary infection but also, in an already infected patient, inhibit the further spread of the virus among CD4-positive cells. In this context it is notable that a whole new generation of antiasthmatic drugs is presently being developed and applied to patients (19), among which the leukotriene receptor antagonists may now prove to be useful in the treatment also of patients with AIDS.

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