Identification of CCR8, the Receptor for the Human CC Chemokine I-309*

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The nucleotide sequence for a putative chemokine receptor, termed TER1, ChemR1, or CKR-L1, was recently obtained by a polymerase chain reaction-based cloning technique. It encodes a protein of 355 amino acids that shows 32–45% sequence identity with human chemokine receptors. The gene was localized on human chromosome 3p21–24, the site for the genes for the five known CC chemokine receptors, suggesting that the natural ligand may be a CC chemokine. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines. We have stably expressed this receptor in murine pre-B cells 300-19 and have tested their responsiveness to 20 human chemokines.

Several reports have described cDNAs for so-called orphan chemokine receptors for which no ligand has been identified. Burkitt's lymphoma-derived receptor 1 and monocyte-derived receptor 15 are amino-terminal splicing variants of a gene expressed in monocytes, lymphocytes, and tissue cells (6, 7). The Epstein-Barr virus-infected Burkitt's lymphoma receptor EBI1 shows 40% sequence identity with the IL-8 receptors and is expressed exclusively in B and T cell lines (8). The chemokine receptor-like protein CMKBRL1 (9), also known as V28 (10), shows higher similarity to known CCRs than CXCRs. The most recent orphan receptor, referred to as TER1 (11), ChemR1 (12), or CKR-L1 (13), shares 32–45% sequence identity with chemokine receptors. Its gene is co-localized with the genes for all five known CC chemokine receptors on human chromosome 3 band p21–24 (14).

Here, we demonstrate that TER1 (ChemR1 or CKR-L1) is the receptor for I-309. These data demonstrate that TER1 (ChemR1 or CKR-L1) is the receptor for I-309, and we propose to call this receptor CCR8 in agreement with the current nomenclature for chemokine receptors. The expression of CCR8 in blood leukocytes and lymphocytes was analyzed by Northern blot. No transcripts were found in DNA from freshly isolated blood neutrophils, monocytes, cultured macrophages, and phytohemagglutinin-stimulated T lymphocytes, and a faint hybridization signal corresponding to the RNA species of 4 kb was obtained only with RNA from interleukin-2-treated T lymphocytes. CCR8 is unusual for its selectivity for a single chemokine, previously shown only for CXCR1 and CXCR4, which bind interleukin-8 and stromal cell-derived factor 1, respectively. Identification of the receptor for I-309 represents a significant progress in determining the function of I-309 in inflammation and disease.

Chemokines are produced locally at sites of inflammation and infection and regulate the recruitment of leukocytes and lymphocytes (1, 2). All chemokines contain four conserved cysteines linked by disulfide bonds, and two subfamilies, CXC and CC chemokines, are defined on the basis of the first two cysteines, which are separated by one amino acid or are adjacent. Generally, chemokines act on more than one type of leukocyte and in vitro responses include chemotaxis, enzyme release from intracellular stores, oxygen radical formation, shape change through cytoskeletal rearrangement, generation of lipid mediators, and induction of adhesion to endothelium or extracellular matrix proteins (1–3). The overlap in target cell selectivity for many of the currently 34 known human chemokines can be taken as a measure of their importance in the regulation of immunity.

Chemokines act via seven transmembrane domain receptors that couple to Bordetella pertussis toxin-sensitive G-proteins for signal transduction (1, 4, 5). Similar to chemokines, the nine human chemokine receptors are divided into two subfamilies: the CXC chemokine receptors (CXCR) and the CC chemokine receptors (CCR). Based on their selectivity for either CXC or CC chemokines, Ligand cross-selectivity, i.e. CXCRs that bind CC chemokines or vice versa, is not observed. Chemokine receptors consist of 350–368 amino acids and sequence identity among members of the two receptor subfamilies are 36–77 and 47–74%, respectively. Most chemokine receptors recognize more than one chemokine and many chemokines, including IL-8, RANTES, MIP-1α, and the MCPs, bind to more than one receptor.

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1 The abbreviations used are: IL, interleukin; Mig, monokine induced by interferon-gamma; IP10, interferon-gamma inducible 10 kDa protein; NAP-2, neutrophil-activating peptide-2; ENA78, epithelial-derived neutrophil-activating peptide 78; GROα, growth-related peptide alpha; GCP-2, granulocyte chemotactic protein-2; SDF-1, stromal cell-derived factor 1; PF4, platelet factor 4; TCA3, T cell activation protein 3; RANTES, regulated on activation, normal T cell expressed and secreted; TARC, thymus and activation-regulated chemokine; HCC-1, hemofiltrate-derived CC chemokine 1; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PCR, polymerase chain reaction; PBL, peripheral blood lymphocyte(s); AM, acetoxyethyl-ester; PHA, phytohemagglutinin.
the receptor for the CC chemokine I-309. In stably transfected mouse pre-B cells this receptor mediated intracellular Ca$^{2+}$ mobilization and in vitro migration in response to I-309, whereas no other of 20 human chemokines tested was active. In accordance with the new nomenclature rules for chemokine receptors, we have named the I-309 receptor CCR8.

**EXPERIMENTAL PROCEDURES**

**Peptide Agonists—**The CXC chemokines Mig, IP10, IL-8, GROα, NAP-2, GCP-2, ENA78, SDF-1, PF4, the CC chemokines MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, I-309, HCC-1, TARC, and the neuropeptide NPY were chemically synthesized according to established protocols (15). Somatostatin, substance P, and calcitonin were purchased from Sigma. C5α and C3α were a gift of Dr. C. A. Dahinden (Clinical Immunology, University Hospital, Bern, Switzerland).

CCR8 Transfected 300-19 Cell Clones—CCR8 DNA was generated by PCR with primers TER1SE and TER1AS corresponding to the nucleotide sequence of TER1 (11) and cDNA from IL-2-stimulated PBL as amplification template (16). The primers TER1SE, 5′-TTATGTGTCTCTGTGACAGC, and TER1AS, 5′-TAGTCTCTTGATGCTAC, correspond to positions 327–346 and 1425–1444 in the published sequence (11) as hybridization probe. As internal control, a 2-32P nucleotide was iodinated with Bolton and Hunter reagent (Amersham Corp.) as described (15).

**CCR8 Expression**—10–μg samples of total RNA were examined from freshly isolated human blood monocytes, neutrophils, lymphocytes, cultured macrophages, and activated T cells by Northern blot analysis as described (16, 18) using a 32P-labeled CCR8 DNA insert (1000 cpm/μg DNA) corresponding to the 620-base pair fragment defined by positions 327 and 946 in the published sequence (111) as hybridization probe. As internal control, a 2-μg sample of total RNA from CCR8-transfected 300-19 cells was included.

**Receptor Sequence**—The orphan receptor TER1 shares 39–45% identical amino acids with CC chemokine receptors and only 32–34% with CXC chemokine receptors. The highest similarity of 45% is found with CCR4 (1, 4, 5). Many structural features that distinguish chemokine receptors from other G-protein-coupled serpentine receptors are found in TER1, notably the extended DRY motif in the second intracellular loop feature that distinguishes chemokine receptors from other G-protein-coupled serpentine receptors are found in TER1, notably the extended DRY motif in the second intracellular loop features that distinguish chemokine receptors from other G-protein-coupled serpentine receptors are found in TER1, notably the extended DRY motif in the second intracellular loop.
nomenclature (Fig. 1a). The interaction of CCR8 with I-309 is of high affinity, as judged by the rapid increases in the [Ca\(^{2+}\)]\(_i\) changes and responses to subnanomolar concentrations of agonist. The CXC chemokine SDF-1 is selective for the chemokine receptor CXCR4 and known to induce chemokine responses in cells expressing the murine homologue of CXCR4 (20, 21). Thus, [Ca\(^{2+}\)]\(_i\), responses to SDF-1 in parental (not shown) and CCR8-transfected 300-19 cells that express murine CXCR4 are considered as positive control for functional integrity of these cells to chemokine stimulation. Selectivity of I-309 for CCR8 is further documented in Ca\(^{2+}\) cross-desensitization experiments (Fig. 1b). Stimulation of leukocytes with chemokines generally results in transient unresponsiveness to those chemokines, which bind to the same receptor (1). Stimulation of CCR8-expressing 300-19 cells with 100 nM I-309 completely abolished a second response to I-309. By contrast, stimulation with I-309 did not prevent a second response to 100 nM of SDF-1 and vice versa responsiveness to I-309 was not affected by initial stimulation with SDF-1, indicating that SDF-1 was not interacting with CCR8.

**Chemotaxis**—The prototypical response in vitro of chemokines in leukocytes is chemotactic migration, which is routinely measured in chemotaxis microchambers (1, 2). Results in Fig. 2 demonstrate that CCR8 was very efficient in mediating transfected 300-19 cell migration in response to I-309, whereas, as expected, no effects were seen in parental cells. Responses to increasing concentrations of I-309 were bimodal, which is characteristic for chemokines as opposed to agonists with chemokinetic activity, and maximal migration was observed at 10 nM.

**I-309 Binding to CCR8**—300-19 cells expressing CCR8 bound radiolabeled I-309 with high affinity (Fig. 3). Scatchard analysis of the specifically bound \(^{125}\text{I})\text{I-309} revealed 1.5 \times 10^4 binding sites per cell and a binding constant (K\(_d\)) of 1.2 nM (inset in Fig. 3). Unspecifically bound material was in the range of 30–60% of total cell-associated radioactivity, which could not be reduced by alternative radiiodination protocols or changes in the binding conditions. No specific \(^{125}\text{I})\text{I-309} binding was observed with parental (untransfected) 300-19 cells.

The cDNAs for I-309 and its murine homologue TCA3 were cloned as T cell activation-dependent gene products by subtractive hybridization (22, 23). I-309 and TCA3 were shown to be readily expressed in activated (concanavalin A, PHA, and phorbol myristate acetate) T cell and natural killer cell lines but not in resting T cell lines (22–24). Transcripts for I-309/TCA3 were not detected in B lymphoid and myeloid cell lines and in many organs, suggesting that activated T cells are the primary sites of I-309/TCA3 production.

Recombinant proteins were available for almost 10 years; however, reports on I-309 and TCA3 function are sparse and in part controversial. Chemotactic activity was described with monocytes and the monocytic cell line THP-1 and tissue cells including mesangial and smooth muscle cells (25–28). As opposed to many CC chemokines, I-309 and TCA3 were reported to be inactive on cultured T cell and natural killer cell lines (29, 30). CCR8 expression was examined by Northern blot analysis in total RNA from human blood cells. As shown in Fig. 4, none of the cells was positive for CCR8 transcripts, including freshly isolated blood neutrophils, mono-
cytes, macrophages, and PBL. As positive control, 2 μg of total RNA from a CCR8-expressing 300-19 cell clone was included. The experimental conditions were such that transcripts for other chemokine receptors were readily detected, as previously reported for the IL-8 receptors (CXCR1 and CXCR2) and the RANTES/MIP-1α receptors (CCR1) in neutrophils (16, 17), CCR1 and the MCP-1 receptor (CCR2) in monocytes (16), and CXCR4 in all types of leukocytes (34). Lack of detectable CCR8 transcript expression in neutrophils and lymphocytes is in full agreement with the reported inactivity of I-309 in these cells (25). However, lack of CCR8 transcripts in monocytes is in striking contrast to the reported I-309-induced monocyte responses. This discrepancy was not due to differences in the I-309 preparations used because our synthetic I-309 was identical in sequence to the reported recombinant protein (25), and synthetic and recombinant I-309 were equally potent as stimuli of CCR8-expressing 300-19 cells. The reported chemotactic activity on monocytes (25) is much weaker than the activity observed in the present study on CCR8-expressing 300-19 cells, suggesting that monocytes may express a receptor with lower affinity for I-309.

Except for CXCR4, which is ubiquitously expressed in circulating blood cells and blood cell precursors, freshly isolated PBL have low to undetectable levels of mRNA for any chemokine receptor and do not respond to the corresponding chemokines (16–18). However, culturing of PBL for several days in the presence of IL-2 results in expansion of CD45RO+ T cells with strong expression of receptors, as recently demonstrated for CCR1, CCR2, and CXCR3 (16, 18). By contrast, treatment of PBL with IL-2 did not affect CCR8 expression, and in addition, PHA treatment for 3 days failed to induce receptor expression (Fig. 4). Only faint hybridization signals corresponding to mRNA species of approximately 4 kilobases were occasionally observed in IL-2 cultured PBL, indicating donor to donor variation in CCR8 expression.

Here, we have shown that CCR8-transfected 300-19 cells responded to I-309 with high potency and efficacy, suggesting that natural target cells for I-309 might respond equally well. The primary target cells for chemokines are leukocytes and lymphocytes that express the corresponding receptors in quantities large enough for ready detection in total RNA by Northern blot analysis (1, 4, 5). Similar to CXCR3, the receptor for the CXC chemokines IP10 and Mig (18), we did not find CCR8 transcripts in freshly isolated blood neutrophils, monocytes, or lymphocytes, which therefore may not represent true targets for I-309 action. By contrast to CXCR3 and several other CC chemokine receptors (16, 18), culturing of PBL in the presence of IL-2 did not result in expression of similar levels of CCR8 transcripts. The faint CCR8 mRNA hybridization signals, as observed in the RNA from some IL-2-treated T cell cultures, suggests that I-309 may act on a subpopulation of activated T cells. Molecular probes for CCR8 will now facilitate the identification of the natural target cells and thus help to advance our understanding of I-309 function in inflammation and disease.

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REFERENCES
