Identification of a Promiscuous Inflammatory Peptide Receptor on the Surface of Red Blood Cells*

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Erythrocytes have long been appreciated as transporters and exchangers of O2 and CO2 between the lungs and the tissues. Here we examine the role of erythrocytes as potential mediators of inflammatory processes by assessing their ability to bind to a number of inflammatory peptides of the chemokine (for chemoattractant cytokine) superfamily. Radiolabeled chemokines of either the C-X-C (IL-8, MGSA/gro, NAP-2) or C-C (RANTES, MCP-1) class bind reversibly to red cell surface receptors numbering 1000-9000 sites/cell with a Kd of approximately 5 nm. In contrast to what is seen for chemokine binding to target inflammatory cells, chemokines of either class displace heterologous chemokines, indicating that the proteins are competing for a promiscuous receptor. Chemical cross-linking with radiolabeled chemokines reveals a 30-38-kilodalton protein on the red cell surface, and cross-linking is inhibited in the presence of heterologous unlabeled chemokines. These data show that red blood cells possess a multispecific receptor for the newly identified chemokine superfamily of inflammatory cytokines, and thus the red cell may play a novel role as a regulator of inflammatory processes.

The chemokines are a bipartite superfamily of soluble proteins with chemotactic and proinflammatory properties (1, 2). The superfamily's two branches "C-X-C" and "C-C" are so designated based on whether the first two cysteines in a conserved motif are adjacent or separated by an intervening residue (1, 2). Generally C-X-C chemokines have been reported to be potent chemoattractants and activators of neutrophils but not monocytes (2-6), while the C-C chemokines exhibit chemoattractant potential for monocytes, but not neutrophils (7–10). In addition, various chemokines exhibit specific chemoattractant and activating properties for basophils (11, 12), eosinophils (13, 14), and subpopulations of T cells (7, 15, 16). They may therefore represent a link between distinct populations of immune cells during inflammatory responses.

The specificity of chemokine action between the two branches of the superfamily (such as that seen on monocytes and neutrophils) is reflected by a regulation of receptor/ligand interactions on target inflammatory cells. For example, no cross-competition for binding sites has been observed on either monocytes or neutrophils between members of the C-X-C or C-C branches (6, 17–19). The observation that IL-8, a C-X-C chemokine, could bind erythrocytes in a saturable manner to red blood cells could act directly as a sink for that proinflammatory cytokine in the blood (20). In that study, Darbonne et al. also found evidence that erythrocytes could bind MCP-1. We report here that in fact erythrocytes possess a multispecific chemoattractant peptide on their surface and that this receptor binds various chemokines of both C-X-C and C-C classes. The presence of such a promiscuous chemokine receptor suggests a mechanism by which circulating levels of the chemokines are regulated and may indicate a role for erythrocytes as regulators of inflammatory processes.

EXPERIMENTAL PROCEDURES

Binding Analyses—Recombinant IL-8 ([Ala8]IL-8) was purified and labeled with Na[125]I as described previously (20). Recombinant human RANTES, MCP-1, and MCP-18 were produced in E. coli (21). MIP-la and MIP-lP were produced in Escherichia coli by linking a cDNA encoding the mature, secreted form of the molecules to a bacterial ST II promoter in an expression plasmid as described (16). MCP-1 was obtained commercially (Peprotech, Rocky Hill, NJ). Preparations of the purified chemokines contained less than 1 endotoxin unit/mg of protein as determined by limulus amoebocyte lysate assay. RANTES and MCP-1 were labeled with Na[125]I using the lactoperoxidase method as described (22). [125]I-MIP-la was labeled using the Enzymobead iodination reagent kit (Bio-Rad). Recombinant MGSA/gro was expressed and purified as described elsewhere (23) and labeled by the Bolton-Hunter method (24). For binding studies, purified erythrocytes, prepared as described (25), were incubated at 37 °C for 30 min with 0.5 nm [125]I-labeled cytokine in the presence of increasing concentrations of unlabeled cytokine for the displacement binding experiments or with increasing concentrations of radiolabeled ligand in the presence and absence of excess unlabeled cytokines for saturation experiments. All reactions were performed with 10⁶ cells in a 150-µl total volume. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (24). Nonspecific binding was determined in the presence of 1 µg unlabeled ligand. Equilibrium binding data were analyzed by the LIGAND program (25) as modified for the IBM PC by McPherson (26). Scatchard analysis was performed on the binding data to determine Kd, and rmax values.

Cross-linking Experiments—One-hundred fifty µg of erythrocyte ghosts, prepared as previously described (27), were incubated in the presence of 1 nm [125]I-IL-8 for 1 h at 37 °C in the presence or absence of unlabeled chemokines. At the end of the incubation, the ghosts were pelleted by centrifugation (100,000 × g for 15 min) and chemically cross-linked with EDC at a final concentration of 1 mM for 1 h at 4 °C. The ghosts were then pelleted as described above and solubilized in SDS sample buffer in the presence of dithiothreitol and analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Binding of Chemokines to Red Blood Cells and Red Cell Ghosts—The ability of increasing concentrations of various unlabeled chemokines to displace their radiolabeled counterparts from cell surface receptors was first assessed. Purified radiola beled human recombinant chemokines RANTES, macrophage inflammatory protein-1α (HuMIP-1α), and MCP-1, represent—

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ing the C-C branch, and MGSNgro and IL-8, representing the C-X-C chemokines, were incubated with red blood cells and red cell "ghosts," and binding was assessed by the methods described (20). In each case 0.5 nM 125I-labeled chemokine was incubated with the purified red blood cells, and increasing concentration of the non-labeled identical ligand was added to 1 μM. All binding points were tested in triplicate, and the results from representative experiments are shown in Fig. 1. All of the chemokines tested, with the exception of HuMIP-1α (not shown), bound to the surface of red blood cells, and each of the labeled chemokines could be removed from its binding site by an excess of the same unlabeled molecule. In all cases, the binding was saturable (data not shown), and >75% of the total binding of the 125I-chemokine was eliminated by the presence of 1 μM unlabeled chemokine. The competition binding data were transformed to generate Scatchard plots (insets to the dissociation curves in Fig. 1). The dissociation constant of binding (Kₐ) for each ligand, shown in the corner of each graph, is strikingly similar, about 5 nM in every case. The range of the rₘₚ values was between 0.7 nM (for MCP-1) and 8.1 nM (for RANTES), representing ~800-9000 binding sites/cell for each of the chemokines.

Cross-competition among Chemokines for Erythrocyte Binding—Because the kinetics and dose-response profiles of displacement for all the chemokines were very similar and because previous evidence showed IL-8 would inhibit MCP-1 binding to red blood cells (20), we sought to determine whether all the chemokines were binding to the same or to distinct sites on the surface of the RBC. 125I-RANTES, 125I-MCP-1, 125I-IL-8, or 125I-MGSNgro were added individually to cells, and the ability of the different unlabeled chemokines to displace the labeled proteins was assessed. Data from a representative series of experiments showing the displacement of 125I-RANTES by increasing concentrations of unlabeled RANTES, IL-8, MCP-1, MGSNgro, and a single concentration of NAP-2 are shown in Fig. 2. The data were similar irrespective of what combination of labeled chemokine and cold competitor was used. With the exception of HuMIP-1α (Fig. 2) and HuMIP-1β (not shown), all of the labeled chemokines tested could be displaced by any of the unlabeled molecules regardless of whether the chemokine was of the C-C or C-X-C class. Scatchard analysis was performed for the heterologous ligand displacement data and a Kₐ for each was calculated and is summarized in Table I. The Kₐ for any combination of labeled chemokine displaced by any combination of the same or heterologous chemokines was strikingly similar.

Cross-linking Analysis of the RBC Chemokine Receptor—We next attempted to chemically cross-link the RBC chemokine receptor to radiolabeled chemokines. Membrane preparations of the red blood cells were made, and the C-terminal specific chemical cross-linker EDC was employed in the analysis. A representative experiment using 125I-IL-8 is shown in Fig. 3A, with a separate experiment employing a shorter exposure time of the autoradiogram shown in Fig. 3B. Similar results were obtained using 125I-RANTES as a representative C-C chemokine (not shown). In all cases the labeled chemokines cross-link to a protein which resolves as a diffuse band of Mᵣ ~ 46,000 (Fig. 3, A and B, 125I-IL-8 lane). Assuming the chemokine binds as a monomer (8 kDa) or dimer (16 kDa), the Mᵣ of the chemokine receptor should be ~30,000 or 38,000. The appearance of this cross-linked protein is completely eliminated by the inclusion of an excess of the same unlabeled chemokine in the cross-linking reaction (Fig. 3, A and B, +IL-8 lane). Similarly, when an excess of heterologous unlabeled chemokine such as unlabeled RANTES or unlabeled MGSNgro is present in the reaction employing 125I-IL-8 (Fig. 3, +MGS Ngro and +RANTES lane), the same band is eliminated. The pattern is virtually identical when 125I-RANTES is used as the labeled chemokine (not shown). Thus the chemokines appear to be cross-linking to the same 36-38-kDa protein, which we have designated the red cell chemokine (CK) receptor. 125I-IL-8 cross-linking to the CK receptor was not eliminated in the presence of an excess of unlabeled HuMIP-1α or HuMIP-1β (not shown). The resolution of a cross-linked protein in these analyses supports the idea that the red cell CK receptor is a protein or has an essential

![Fig. 1. Binding characteristics for chemokines on red blood cells.](attachment:image1.png)

![Fig. 2. Cross-competition of multiple chemokines for the same binding site.](attachment:image2.png)

**Table I**

| Cold competitor ligand | RANTES | MCP-1 | IL-8 | MGSNgro |
|------------------------|--------|-------|------|---------|
| 125I-Labeled ligand    |        |       |      |         |
| RANTES                 | 4.76   | 5.50  | 6.40 | 2.38    |
| MCP-1                  | 9.49   | 5.80  | 6.55 | 6.16    |
| IL-8                   | 6.09   | 3.19  | 5.48 | 4.70    |
| MGSNgro                | 5.10   | 4.03  | 5.18 | 5.36    |

**FIG. 2.** Cross-competition of multiple chemokines for the same binding site. Competition displacement curve showing a representative displacement of 125I-RANTES by the heterologous unlabeled chemokines IL-8, MCP-1, MGSNgro, and NAP-2. HuMIP-1α and HuMIP-1β did not displace. Competition curves are similar for 125I-MCP-1, 125I-IL-8, or 125I-MGSNgro displaced by any of the other unlabeled chemokines.
protein component. These observations are also consistent with the finding that the red cell binding site for IL-8 is protease-sensitive (20).

These studies show that a cell surface receptor on erythrocytes has the capacity to bind to a wide variety of inflammatory peptides of both the C-X-C and C-C groups within the chemokine superfamily. The RBC CK receptor is unlike the cell surface receptors for chemokines on neutrophils, which bind only C-X-C molecules, and monocytes, which recognize only C-C molecules, in that it will accommodate chemokines of both classes. Thus, the binding and cross-linking studies presented here suggest that the red blood cell binding site originally postulated to be a "sink" for IL-8 is likely to be a promiscuous receptor for the newly identified chemokine inflammatory peptide superfamily. The only chemokines tested that failed to bind were human MIP-1α and MIP-1β. The reasons for this are not clear, but the accessibility of the MCP-1 proteins may be essential for their roles in the regulation of hematopoietic precursor cells (28-30).

The broad specificity for chemokine binding suggests that the RBC chemokine receptor is likely to be distinct from the receptors reported for IL-8 on neutrophils (17) or those for MCP-1 on monocytes (18). Indeed, studies in our laboratory indicate that neither of the two IL-8 receptors encoded by the cloned cDNAs (31, 32) will recognize purified recombinant C-C chemokines. Moreover, the C-C chemokine receptor identified in our laboratory (19) binds C-C chemokines, including HuMIP-1α, to the exclusion of C-X-C chemokines. Thus, based on both binding and molecular cloning studies, the red cell CK receptor appears to be distinct from the chemokine receptors on inflammatory cells. Further experiments are under way to more fully characterize the biochemical properties of this new multispecific chemokine receptor. Its presence on RBCs suggests a newly appreciated role for these cells in the regulation of inflammatory processes, and its molecular elucidation could provide clues to the development of anti-inflammatory therapeutics.

In summary we have demonstrated that red blood cells carry multispecific receptors that can bind a wide array of chemokines. Other experiments show that the RBC-bound IL-8 (and likely other chemokines) does not induce signaling (Ca2+ ion flux) in target cells and that chemokines bound to the red cell surface are inaccessible to their normal target inflammatory cells (20). Thus, the major role of the red cell chemokine receptor may be one of a clearance receptor for chemotactic and inflammatory peptides in the blood. This role might be complemented by the circulating specific antibodies to IL-8 that have been recently reported (33).

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