Chemokine Class Differences in Binding to the Duffy Antigen-Erythrocyte Chemokine Receptor*

(Received for publication, July 24, 1995, and in revised form, August 24, 1995)

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The Duffy blood group antigen-erythrocyte chemokine receptor has been shown to bind to chemokines of both the C-X-C and C-C classes and to the malarial parasite Plasmodium vivax and Plasmodium knowlesi. We performed experiments to evaluate the binding properties of this receptor for the newly appreciated "C" and "non-ELR C-X-C" classes of chemokines. Binding to mouse erythrocytes was also evaluated for the first time. Whereas ELR C-X-C and C-C chemokines bound to human erythrocytes with high affinity, differences in the ability of non-ELR chemokines to act as competitive inhibitors were noted. While non-ELR chemokines were unable to displace C-X-C chemokines on human cells, they exhibited a low affinity interaction with the C-C chemokine binding site. The newly discovered C-chemokine, lymphotactin, was unable to displace either C-X-C or C-C chemokines. On mouse erythrocytes, non-ELR chemokines exhibited a low affinity for both the C-X-C and C-C chemokines binding sites; again lymphotactin failed to bind. Binding competition studies using an anti-Duffy monoclonal antibody and chemokines suggested a common binding domain. These data show that the chemokine superfamily has at least four functional subdivisions, each interacting differently with the Duffy antigen-erythrocyte chemokine receptor. In addition, the chemokine binding function is conserved between mouse and man. Unlike other proteins in the superfamily, C and non-ELR C-X-C chemokines do not efficiently bind red blood cells, thus their role may not require clearance from circulation.

The chemokine superfamily of pro-inflammatory cytokines has traditionally been divided into the two structural branches: C-C and C-X-C (1–3). This classification has recently been expanded to include the C branch represented by the T cell co-stimulatory lymphotactin (Ltn) (4, 5). Structurally, these three classes are distinguished based on whether the first two cysteines in the motif are adjacent (C-C), separated by an intervening residue (C-X-C), or whether the first of the two cysteines is missing (C). The C-X-C branch may be further subdivided into those that contain the amino acid motif "ELR" preceding the initial cysteine residue and those that do not ("non-ELR" C-X-C). Biologically, the C-C and C-X-C chemokines tend to act primarily, but not exclusively, on monocytes and neutrophils, respectively (2), whereas the C chemokine Ltn currently appears to be a lymphocyte-specific chemotactant (4, 5).

It has been reported previously that erythrocytes possess a "promiscuous" receptor, which, unlike other known chemokine receptors, binds chemokines of both the C-C and C-X-C classes (6, 7). C-C and C-X-C chemokines bind this receptor, present at about 5000–15,000 sites per cell, with an affinity of $K_d \sim 5$ M (6, 7). While the function of this erythrocyte receptor has not been directly demonstrated, chemokine clearance from the circulation has been postulated. It was later shown that this erythrocyte chemokine receptor is serologically indistinct from the Duffy antigen which is used by Plasmodium vivax and knowlesi to bind to and invade erythrocytes (8–10). The recent finding that the Duffy antigen-erythrocyte chemokine receptor (DFA-ECKR) is also expressed by endothelial cells lining post-capillary venules and splenic sinuositites (11) suggests additional unelucidated roles for this protein.

Here we dissect the binding functions of the human DFA-ECKR by testing its ability to bind to the newly discovered C chemokine, lymphotactin; by assessing the ability of non-ELR C-X-C chemokines to act as competitive inhibitors of binding, and by examining receptor-ligand interactions using an anti-Duffy mAb. In addition we ascertain whether the binding functionality is conserved between mouse and men. We provide evidence for a functional division of the chemokine superfamily into at least four classes based on the differential binding interactions of the C-C, ELR C-X-C, non-ELR C-X-C, and C chemokines tested.

EXPERIMENTAL PROCEDURES

Materials—125I-RANTES (specific activity, 2200 Ci/mmmol) and 125I-MGSA/gro (melanoma growth stimulatory activity; specific activity, 2200 Ci/mmmol) were obtained from DuPont NEN. Recombinant human RANTES, MGSA/gro, monocyte chemotactic protein (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) were purchased from R&D (Minneapolis, MN). Recombinant human I-309 and interferon-γ-inducible protein-10 (IP-10) were kindly provided by Monica Tsang (R&D) and platelet factor 4 (PF-4) was from Sigma. Recombinant human and mouse Ltn were produced as described previously (4, 5). The mAb anti-Fy3 (CRC-S12) is specific for the Duffy antigen (12) and was the kind gift of Makato Uchikawa (Japanese Red Cross, Tokyo, Japan).

Binding Analyses—Whole blood was obtained from normal donors by venipuncture or from genetically homogeneous BALB/c mice by cardiac puncture. The blood of each donor was kept separate and multiple donors were used for each assay. Human and mouse erythrocytes were isolated by a standard protocol (6). Flow cytometric analysis of the purified erythrocytes indicated a population that was >96% CD58- and

* DNAK is supported by Schering Plough Corp. The costs of publica-

† tion of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Immunology Fellowship AI-07290.

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** The abbreviations used are: Ltn, lymphotactin; DFA-ECKR, Duffy antigen-erythrocyte chemokine receptor; mAb, monoclonal antibody; MIP-1α, macrophage inflammatory protein-1α; IP-10, interferon-γ-in-

†† dulible protein-10; FITC, fluorescein isothiocyanate; MGSA, melanocyte growth stimulatory activity.
Cross-competition of multiple chemokines for the same binding site on human red blood cells. Competition binding curve showing a representative displacement of $^{125}\text{I}-\text{RANTES}$ (A) or $^{125}\text{I}-\text{MGSA/gro}$ (B) by the unlabeled chemokines RANTES, MGSAGro, MCP-1, IP-10, PF-4, I-309, human Ltn (HuLtn), and mouse Ltn (MoLtn). (The experiment is representative of $n=5$).

Cross-competition of multiple chemokines for binding to mouse red blood cells. Competition binding curve showing a representative displacement of $^{125}\text{I}-\text{RANTES}$ (A) or $^{125}\text{I}-\text{MGSA/GRO}$ (B) by the unlabeled chemokines RANTES, MGSA/Gro, MCP-1, IP-10, PF-4, I-309, human Ltn (HuLtn), and mouse Ltn (MoLtn). (The experiment is representative of $n=3$).

CD44$, <3% CD2+ and CD19+, and <1% CD64+ (data not shown). For the displacement binding studies, purified human or mouse red blood cells were incubated at 37°C for 30 min with 0.5 nm $^{125}\text{I}$-labeled chemokine in the presence of increasing concentrations of unlabeled chemokine. For inhibition of binding, purified human erythrocytes were incubated with or without various concentrations of the anti-Fy3 or a control anti-CD44 mAb at 4°C for 2.5 h prior to the addition of 0.5 nm $^{125}\text{I}$-labeled chemokine (a 1-h incubation period yielded identical results; data not shown). All reactions were performed in duplicate and repeated two to five times. Each reaction contained $10^6$ red blood cells in a total volume of 150 μl of binding buffer (1 × phosphate-buffered saline containing 1% bovine serum albumin). The assay was terminated by layering the cell suspension over a 150 μl of 20% sucrose/binding buffer solution, and the cells were separated from buffer by centrifugation as described previously (6). The bottom of the tube containing the cell pellet was cut into a fresh vial and the radioactivity quantified in a Packard Cobra 5010 γ counter (Packard Instrument Co., Downers Grove, IL). Nonspecific binding was determined in the presence of 250 nM unlabeled ligand. The binding data were analyzed by the LIGAND computer program (13) to determine displacement binding, $K_d$, and the number of sites.

Flow Cytometry—Purified red blood cells in binding buffer were incubated with or without various concentrations of unlabeled chemokines at 4°C for 30 min. Aliquots containing $10^6$ cells were removed and incubated with control antibodies or the anti-Fy3 mAb at 4°C for 30 min in 100 μl of binding buffer. The cells were centrifuged, washed twice in 100 μl of binding buffer, and incubated for an additional 30 min at 4°C with a secondary antibody (FITC-conjugated goat-anti-mouse IgG F(ab)2 fragment; Sigma). A nonspecific anti-human IgG2a mAb was used as the isotype-matched control. Additional controls included unstained cells and secondary antibody alone (data not shown). The erythrocytes were centrifuged, washed three times in 100 μl of binding buffer, and analyzed by flow cytometry on a Becton Dickinson FACScan (San Jose, CA).

RESULTS AND DISCUSSION

Cross-competition Binding of Chemokines to Human and Mouse Red Blood Cells—The ability of increasing concentrations of different unlabeled chemokines of the four subclasses to displace radiolabeled chemokines from the cell surface was explored. In this study, RANTES, MCP-1, and I-309 represented the C-C class, MGSA/Gro represented the ELR C-X-C branch, IP-10 and PF4 the non-ELR C-X-C branch, and human and mouse Ltn comprised the C chemokines. For each reaction, 0.5 nm $^{125}\text{I}$-RANTES or $^{125}\text{I}$-MGSA/Gro was incubated with purified erythrocytes and increasing concentrations of various unlabeled chemokines up to 250 nM.

The results from representative experiments using human erythrocytes showed the displacement of $^{125}\text{I}$-RANTES (Fig. 1A) or $^{125}\text{I}$-MGSA/Gro (Fig. 1B) by an excess of unlabeled chemokines. The displacement curves for RANTES, MGSA/Gro, and MCP-1 on human erythrocytes were very similar to those reported previously (7), with RANTES showing a slightly weaker binding affinity on the human red blood cells than MGSA/Gro or MCP-1. Labeled RANTES could be removed from its human erythrocyte binding sites by unlabeled I-309 and the non-ELR C-X-C chemokines PF4 and IP-10, which showed inefficient but consistent displacement of RANTES binding at high concentrations (Fig. 1A). By contrast, unlabeled PF4, IP-10, or I-309 did not compete for binding with $^{125}\text{I}$-MGSA/Gro on human erythrocytes, even though efficient displacement was observed with MCP-1 and RANTES (Fig. 1B). The C chemokine lymphotactin was unable to displace either radiolabeled RANTES or MGSA/Gro from these cells (Fig. 1, A and B). Similar results were obtained in multiple experiments using the red blood cells of different normal donors. Thus, the binding displacement data do not seem to be a function of DFA-ECKR heterogeneity.

Direct assessment of the in vivo function of DFA-ECKR awaits the characterization of this marker in non-human species. We therefore tested directly whether mouse red blood cells could bind chemokines and how this binding compared with the human system. Data from a series of homologous and heterologous binding displacement on mouse erythrocytes are presented in Fig. 2. As with human erythrocytes (Fig. 1), both C-C and C-X-C chemokines seemed to compete for binding to a single site on the surface of mouse red blood cells (Fig. 2). However, the principle difference from the human system is that the non-ELR C-X-C chemokines, as well as I-309, displace both radiolabeled RANTES (Fig. 2A) and MGSA (Fig. 2B) from
Chemokine Class Differences in Erythrocyte Receptor Binding

**TABLE I**

| 125I-Labeled ligand | Cold competitor Kd |
|---------------------|--------------------|
|                     | RANTES | MGSA/gro | MCP-1 | IP10 | PF4 | 1309 | HuLtn | MoLtn |
| Human               |        |          |       |      |     |      |       |       |
| RANTES              | 5.69   | 1.81     | 5.98  | –a   | 200 | 104  | –a    | –a    |
| MGSA/gro            | 9.72   | 2.37     | 6.79  | –a   | –a  | –a   | –a    | –a    |
| Mouse               |        |          |       |      |     |      |       |       |
| RANTES              | 8.58   | 6.14     | 4.43  | –a   | 83.4| 90.8 | –a    | –a    |
| MGSA/gro            | 5.10   | 4.62     | 4.23  | 49.7 | –a  | 21.8 | –a    | –a    |

*a Dashes indicate no displacement of radiolabeled chemokines occurred, therefore Kd values could not be generated.

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**FIG. 3.** Flow cytometric analysis of human erythrocytes for labels of DFA-ECKR expression. Purified human erythrocytes were preincubated with or without various concentrations of MCP-1 and MIP-1α prior to staining with control antibodies or the anti-Fy3 mAb followed by FITC-conjugated goat-anti-mouse IgG F(ab)2 fragment. (n = 2).

**FIG. 4.** Percent binding of radiolabeled chemokines to human erythrocytes after incubation with the anti-Fy3 mAb. Purified human erythrocytes were pre-incubated with various concentrations of the anti-Fy3 or an anti-CD44 mAb prior to the addition of 0.5 nM 125I-RANTES or 125I-MGSA/gro. The percent inhibition of radiolabeled chemokine binding versus mAb concentration is shown. (n = 2).

Scatchard analysis was performed for the ligand displacement data using the LIGAND program (13), and where possible the Kd for each of the represented curves in Figs. 1 and 2 were generated and summarized in Table I. The table reveals several displacement patterns. First, the C chemokine was unable to displace 125I-RANTES or 125I-MGSA/gro on both human and mouse. Second, the Kd and number of binding sites per cell (data not shown) derived from any combination of labeled RANTES and MGSA/gro displaced by unlabeled RANTES, MGSA/gro or MCP-1 were similar to each other (2–10 nM) (7). Third, 125I-RANTES displaced by unlabeled I-309 (Kd = 104 nM) and PF4 (Kd = 200 nM) revealed low binding affinity with those two chemokines for the C-C binding site on human erythrocytes, but strikingly no displacement of the C-X-C chemokine occurred. Finally, in the mouse system, the binding affinities of I-309 and non-ELR C-X-C chemokines were slightly higher for 125I-RANTES as represented by their Kd values, and in contrast to human, mouse 125I-MGSA/gro was displaced by I-309 (Kd = 22 nM) and IP-10 (Kd = 50 nM). Thus, the heterologous displacement studies indicated that non-ELR C-X-C chemokines did not bind the red blood cell as efficiently as the C-C and ELR C-X-C chemokines, and the C chemokine did not bind at all.

Chemokines and Anti-DFA mAb Compete for Binding—The ability of the anti-DFA mAb anti-Fy3 (12) to bind human erythrocytes was assessed. Purified erythrocytes were incubated with various concentrations of the C-C chemokine MCP-1, which binds DFA-ECKR, or MIP-1α, which has been shown not to bind DFA-ECKR (7). The cells were then incubated with the anti-Fy3 mAb followed by a FITC-conjugated second stage antibody and analyzed by flow cytometry. In Fig. 3 (left panel) human erythrocytes were clearly positive for DFA-ECKR expression and negative for the control isotype-matched antibody. The middle panel of Fig. 3 shows that when increasing concentrations of MCP-1 were added (10−8 to 10−6 M), a reduction in the fluorescence occurred, indicating that the chemokine prevented anti-Fy3 mAb binding to red blood cells in a dose-dependent fashion. Similar results were obtained using RANTES as the competitor (not shown). In contrast, MIP-1α (right panel), which is known not to interact with DFA-ECKR in ligand displacement experiments (7), failed to interfere with the ability of anti-Fy3 mAb to bind.

In a converse experiment, human erythrocytes were incubated with the indicated concentrations of the anti-Fy3 or control mAbs and then 0.5 nM radiolabeled RANTES or MGSA/gro were added to determine their binding potential. Fig. 4 shows that the preincubation of erythrocytes with increasing concentrations of mAb Fy3 dramatically inhibited the binding of 125I-RANTES and 125I-MGSA/gro, whereas a control mAb specific for CD44 did not. These data together indicate that the anti-Fy3 mAb was a competitive antagonist for chemokine binding.

In summary, we have characterized the DFA-ECKR on red blood cells, revealing new distinctions in its binding properties. The data show for the first time that positive C-C and C-X-C proteins do indeed compete for binding to a shared site with equal affinities, the precise nature of the binding interaction is subtly different in that the C-C proteins are displaced by non-ELR C-X-C chemokines, whereas the C-X-C are not. We also show for the first time that the mAb anti-Fy3 is a competitive
inhibitor of chemokine binding and that the newly discovered C chemokine does not bind to the DFA-ECKR. Last, we show that mouse erythrocytes also possess a promiscuous chemokine receptor, allowing for the initiation of further studies of erythrocyte chemokine binding in vivo. The data thus provide the basis for a functional division of the chemokine superfamily into four distinct classes, where binding interactions with the DFA-ECKR are ELR C-X-C > C-C > non-ELR C-X-C; no binding with the C class. This understanding should assist in the refinement of experimental models to more accurately test the physiological roles of both the chemokine ligands and the promiscuous receptor.

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