Chemokine Receptor CCR3 Function Is Highly Dependent on Local pH and Ionic Strength*

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The CC chemokine receptor 3 (CCR3) plays an important role in the regulation of the migration of eosinophils, a leukocyte population involved in many inflammatory pathologies including asthma. CCR3 binds to the CC chemokine eotaxin, a promigratory cytokine originally isolated as the key component in a model of eosinophil-induced airway inflammation. We show here that eotaxin/CCR3 binding interactions exhibit a marked sensitivity to relatively small changes in the extracellular environment. In particular, modest variations in the pH and the level of sodium chloride over a range of physiologic and near physiologic conditions had dramatic effects on eotaxin binding and CCR3-mediated cytoplasmic Ca\textsuperscript{2+} mobilization. These biochemical indicators were reflected at the functional level as well; small changes in pH and salt also resulted in striking changes in the migration of primary human eosinophils in vitro. These results reveal that relatively small perturbations in extracellular buffer conditions can yield widely disparate interpretations of CCR3 ligand binding and affinities and suggest that modulation of the tissue microenvironment might be utilized to control the affinity and efficacy of chemokine-mediated cell migration.

Eosinophils are involved in many inflammatory pathologies including asthma, urticaria, and hypereosinophilic syndrome (1–3). The CC chemokine receptor 3 (CCR3)\textsuperscript{1} and its ligands, most notably eotaxin, have been shown to play a central role in controlling eosinophil migration (4–12). Eotaxin was originally isolated as a protein responsible for eosinophil chemotraction in the bronchoalveolar fluid of allergen-sensitized guinea pigs (10). Subsequently, the human and mouse homologs were identified and also shown to be chemotaxant for eosinophils (10, 12). Eotaxin has been shown to be a primary ligand for the seven-transmembrane G protein-coupled receptor CCR3. The genes for CCR3 and the related receptors CCR1–CCR5 are encoded within a 130-kilobase region of human chromosome 19 indicating recent gene duplication and divergence and perhaps explaining the partial overlap of chemokine ligand repertoires. CCR3 is highly expressed on primary human eosinophils (50,000–400,000 sites/cell) (5–7), consistent with the potent ability of eotaxin to selectively induce the migration of these cells.

The mechanisms by which specific cells are attracted by specific chemokines have been a topic of intense interest and clinical relevance. The question is complicated by the fact that a number of closely related chemokine receptors with overlapping specificities are expressed on many different classes of leukocytes. In addition, the affinity of a specific receptor/ligand interaction can vary depending upon the specific cell type expressing the chemokine receptor, perhaps due to posttranslational modification events or G protein coupling. Thus it appears that cells can modulate their responses to chemokines, but how this is done is not yet clear. While most investigations have focused on factors intrinsic to the cell, G proteins, receptor modifications, etc., we wished to investigate whether extracellular factors could act to modify chemokine receptor action. We report here that relatively modest changes in pH and salt over a physiologic range can dramatically alter both the binding affinity and signaling efficacy of eotaxin/CCR3 interactions. Moreover, these biochemical changes are mirrored by functional changes induced by eotaxin on primary human eosinophils.

MATERIALS AND METHODS

CCR3 Transfectant—A cell line stably expressing the human CCR3 chemokine receptor was generated using standard protocols. Briefly, the CCR3 open reading frame was cloned into the pME eukaryotic expression vector and transfected into the rat Y3 cell line using electroporation. Stable transfecteds in Y3 cells were generated by selection in medium containing 1 mg/ml G418 (Life Technologies, Inc.). CCR3 expression was confirmed using calcium signaling with the fluorescent calcium-sensitive indicator fluo-3.

Binding Analysis—A ligand binding protocol using wheat germ agglutinin-coated scintillation proximity assay (SPA) beads (Amersham Corp.) (14) was employed. Membranes were prepared from the CCR3 transfectant by using hypotonic lysis of the cells with multiple centrifugation steps. Added to 200 μg of wheat germ agglutinin-coated SPA beads in a 200-μl reaction volume were 14 μg (protein units) of the transfectant membrane preparation, ~0.05 nm \textsuperscript{125}I-eotaxin (specific activity, 2200 cpn/Ci), and the stated concentration of unlabeled eotaxin. The binding reaction buffers contained systematically varied concentrations of NaCl (120, 140, or 160 mM) and final pH levels (7.0, 7.2, 7.4, or 7.6) for a total of 12 individual buffers. However, all reaction buffers additionally contained 25 mM Hepes, 1 mM CaCl\textsubscript{2}, 5 mM MgCl\textsubscript{2}, and 0.5% bovine serum albumin. The reactions were incubated from 2 to 5 h at 22 °C with gentle agitation. The reaction binding data were assessed using a Topcount scintillation counter (Packard). The ligand binding data were then analyzed using IgorPro software (WaveMetrics).

Signaling Analysis—The Ca\textsuperscript{2+} response to eotaxin in the presence of different reaction buffers was measured using standard protocols. Briefly, the CCR3 transfectant (and the wild type Y3 cells) was loaded with 1 μM INDO-1 AM (Molecular Probes, Eugene, OR). Calcium mobilization was measured using a Photon Technology International dual wavelength spectrophotometer with excitation at 350 nm and dual simultaneous recording of fluorescence emission at 400 and 490 nm. Relative intracellular calcium levels are expressed as the 400 nm/490 nm emission ratio. Experiments were performed at 37 °C with constant mixing in cuvettes each containing 10\textsuperscript{6} cells in 2 ml of the indicated
buffer. The reaction buffers were the same as those used for the binding analyses with the one exception that no bovine serum albumin was added. For the EC$_{50}$ determinations, the maximal amplitude of the signaling response (400/490 ratio) was quantified, and the data were analyzed using IgorPro software (Wavemetrics).

**Eosinophil Preparation**—Eosinophils were isolated from the buffy coats of normal volunteers. Briefly, erythrocytes were removed by hypotonic lysis from the dextran sedimentation-isolated granulocyte fraction. The granulocytes were then incubated with anti-CD16 microbeads (Mitenyi Biotec Inc., Sunnyvale, CA) for 30 min (15) and were then passed through a MACS column (Mitenyi Biotec Inc.). The eosinophils (Mitenyi Biotec Inc.) for 30 min (15) and were then passed through a MACS column (Mitenyi Biotec Inc.). The eosinophils collected in the flow-through were shown immunohistochemically to be greater than 99% pure.

**Chemotaxis Analysis**—The migration of eosinophils was measured using a modified Boyden chamber with 5-μm pore size polyvinylpyrrolidone-free filters (16).

### RESULTS

**Binding Affinity as a Function of pH and Salt**—We examined the effects of relatively small changes in the extracellular buffer on the binding affinity of eotaxin for CCR3. Membrane preparations of a human CCR3 transfectant were used in a SPA bead binding format (14) to allow the direct measurement of eotaxin binding and the assessment of dissociation constants ($K_d$) of binding. A high throughput format was employed to ensure sufficient data points to accurately reflect changes in binding affinity.

Initial trials indicated that two critical reaction parameters were the level of sodium chloride and the final pH of the reaction buffers. We therefore varied these two conditions over a fairly narrow physiologic range while other aspects of the ligand binding reaction were kept constant. Displacement profiles observed with the different buffer conditions varied markedly (Fig. 1A). Specifically, total binding as well as the “steepness” of the competitor curves increased as a function of higher pH (7.0–7.6) but lower salt (160–120 mM), as summarized in Fig. 1B. The comparison includes reactions performed in a “standard” condition with 140 mM NaCl and a final pH of 7.4 (7.4/140), a “high affinity” condition with 120 mM NaCl and pH 7.6 (7.6/120), and a “low affinity” condition with 160 mM NaCl and pH 7.0 (7.0/160). A nearly 5-fold difference of total CCR3 bound $^{125}$I-eotaxin is seen between the 7.0/160 and the 7.6/120 conditions, suggesting a profound influence on binding by salt and pH. Scatchard analysis indicated that the differences in the eotaxin/CCR3 interaction observed under different buffer conditions was due to an alteration in the binding affinity ($K_d$), with the number of binding sites per reaction remaining relatively constant (x axis intercept on Scatchard plots, Fig. 1, A and B). The 7.6/120 conditions resulted in a binding constant ($K_d$) of 0.03 nM, whereas the 7.0/160 conditions resulted in a 20-fold lower affinity interaction ($K_d$ = 0.60 nM).

**Ca$^{2+}$ Signaling Is Sensitive to Salt and pH**—To examine whether the differences in binding affinities were also reflected at the level of eotaxin-induced signal transduction, we examined CCR3-mediated Ca$^{2+}$ mobilization under these different buffer conditions. Several aspects of the Ca$^{2+}$ response profiles are noteworthy (Fig. 2). While the magnitude of maximum Ca$^{2+}$ mobilization was equivalent, there were striking differences in the efficacy of signaling under the three different conditions. As shown with representative traces in Fig. 2A, stimulation of the CCR3 transfectant with 1.6 nM eotaxin evoked essentially no Ca$^{2+}$ flux response with the 7.0/160 conditions, a minimal response with the 7.4/140 conditions, and nearly a half-maximal response with 7.6/120 conditions. Likewise, 16 nM eotaxin stimulation evoked essentially a maximal response with the 7.4/140 and 7.6/120 conditions whereas it only evoked a half-maximal response in the 7.0/160 conditions. These results were further borne out in the full dose-response profiles (Fig. 2B) from which were calculated the concentration of eotaxin giving rise to a half-maximal Ca$^{2+}$ response (EC$_{50}$) values. While the standard conditions resulted in an EC$_{50}$

![Fig. 1](image-url)

**FIG. 1.** Affinity of eotaxin binding to CCR3 varies as a function of salt and pH. Shown are $^{125}$I-eotaxin equilibrium binding experiments using CCR3-transfectant membranes in the SPA format. A, displacement and Scatchard diagrams of results obtained from a matrix of pH and NaCl conditions (120 (●), 140 (□), and 160 (△) mM NaCl at stated pH of 7.0, 7.2, 7.4, and 7.6). A total of 50,000 cpm of $^{125}$I-eotaxin were added to each reaction. B, representative diagrams highlighting the differences in the eotaxin/CCR3 interactions due to the conditions of 7.6/120 (●), $K_d$ = 0.03 nM; 7.4/140 (□), $K_d$ = 0.14 nM; and 7.2/160 (△), $K_d$ = 0.60 nM.
The determination of 5 nM, the other two conditions gave rise to values of 2 and 26 nM. These two values, like the respective binding constants, are over an order of magnitude apart. Thus signaling efficacy is also a function of pH and salt and directly correlates with binding affinity.

**Effects on Native Human Eosinophils**—We next wished to assess if the marked changes on binding and signaling seen with the CCR3 transfectant were relevant to eotaxin-induced responses in native human eosinophils. Using freshly isolated human eosinophils from a normal donor, we examined the Ca\(^{2+}\) response to eotaxin stimulation at the three different conditions previously used. As shown in Fig. 3, eosinophils responded to eotaxin stimulation with the same sensitivities as seen with the CCR3 transfectant. When tested at the 7.0/160, 7.4/140, or 7.6/120 conditions, 1 nM eotaxin stimulation of the eosinophils resulted in no response, minimal response, or half-maximal responses, respectively. Likewise, 10 nM eotaxin stimulation resulted in half-maximal, maximal, and maximal responses at the same conditions. Therefore, eotaxin/CCR3-mediated Ca\(^{2+}\) signaling appears to be a function of microenvironmental conditions for both transfected cells and primary native human eosinophils.

**Eosinophil Migration Is Sensitive to Environmental Conditions**—Eotaxin binding to CCR3 and subsequent signal transduction result ultimately in the migration of eosinophils. We therefore wished to assess whether this effector function was also dependent on relatively subtle microenvironmental conditions. Freshly isolated human eosinophils were tested in a chemotaxis assay for their ability to respond to eotaxin stimulation under the three different conditions used with the CCR3 transfectant. As shown in Fig. 4, two different observations can be made. First, within the experimental range tested, significantly more eosinophils responded at the 7.6/120 conditions than at the 7.0/160 or even 7.4/140 conditions to each level of eotaxin stimulation, in particular 3, 10, and 30 nM. Second, by comparing submaximal response levels (i.e., comparing results from 7.6/120 and 7.0/120 conditions at 3 and 30 nM, or 10 and 100 nM) an order of magnitude difference in response was again observed. Therefore, at least in vitro, the 7.6/120 conditions served to allow greater eosinophil migration mediated through the eotaxin/CCR3 interaction than did the 7.0/160 conditions.

**DISCUSSION**

The direct measure of the interaction between eotaxin and CCR3 has been marked with difficulties, including low expression of CCR3 in transfected cells and peculiarities in the biophysical characteristics of chemokines in ligand binding studies. In this report, we investigate other factors that may also add to the complexity of interactions between chemokines and their receptors by assessing how subtle changes in the extracellular environment may alter profoundly the affinity of ligand/receptor binding. For eotaxin/CCR3 interactions, we show that ligand binding, Ca\(^{2+}\) signaling, and primary eosinophil
migration varied over an order of magnitude as a function of net changes of 40 mM (160–120 mM) sodium chloride and 0.6 pH unit (pH 7.0–7.6). Optimum CCR3/eotaxin interactions occurred at pH 7.6 and 120 mM NaCl, and the biochemical effects were directly mirrored at the level of function, with far more primary human eosinophils migrating to eotaxin under these conditions than under any other.

While small variations in the physiologic buffer alone can profoundly influence subsequent eosinophil responses mediated via the eotaxin/CCR3 interaction, the role this possible control mechanism might play in the trafficking of eosinophils in vivo remains to be firmly established. Recently, Helminger et al. (17) measured mean interstitial pH values between adjacent blood vessels using fluorescent ratio imaging. These high resolution measurements identified pronounced pH gradients, with values dropping to 7.0 just 50 μm (~5 cell lengths) from the 7.4 values at the vessel walls. This observation establishes the paradigm of significant pH variations occurring within the microenvironment and frames the possibility of altered trafficking patterns of cells under the influence of chemokines. It also is possible that some more widespread local modifications (lower pH and/or higher salt) might help dampen eosinophil recruitment to certain organs (such as the thymus) that constitutively express eotaxin and yet remain eosinophil-free (7, 10, 11). It is interesting to note that the majority of eosinophil granule proteins, such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase, are extremely basic in nature, all having isoelectric points around 10.8 (18). Degranulation of these primarily tissue-localized leukocytes, whether as part of an inflammatory response or even as part of eosinophil turnover, might sufficiently increase the local pH levels at those sites to enhance additional eosinophil recruitment mediated through a higher affinity eotaxin/CCR3 interaction, possibly leading to a positive feedback cycle.

The effects of salt and pH on receptor/ligand interactions in general can often be correlated with important aspects of biology. In many instances, however, the responses to these variables are the inverse of those seen with the CCR3/eotaxin interactions. For example, neonatal Fc receptors bind maternal IgG from ingested milk in the gut (pH 6.0–6.5) with a high affinity and deliver them to the bloodstream (pH 7.0–7.5) where the binding affinity drops 2 orders of magnitude (19). There are several examples also where the difference in binding affinity at acidic pH, such as would be present in subcellular compartments, might have a biological significance. The affinity of ligand binding to the epidermal growth factor receptor at acidic pH correlates with the final outcome of endosomal sorting (20). A similar link to biology is seen with major histocompatibility complex interactions with specific peptide ligands (21). While the effects of salt variations tend to be qualitatively consistent, there are even examples of increases of sodium chloride concentrations increasing ligand function, specific agonists of the somatostatin receptor being one example (22). While it is likely that pH and salt-dependent variations occur with other chemokine/receptor pairs the magnitude of these has not yet been fully characterized. However, our experience to date indicates these variations are not as marked as those observed with the CCR3/eotaxin interactions.

In summary, while the precise physiologic implications of alterations in extracellular environment remain to be worked out, we have shown that small compositional changes in the extracellular environment can have profound effects upon chemokine action. Potentially, the modulation of extracellular fluids represents an additional physiologic control mechanism for regulating chemokine/receptor interactions and hence specific aspects of cellular migration and trafficking. With the particular case of eotaxin/CCR3 interactions, the possibility is strong that the extracellular environment has a significant role in governing eosinophil trafficking.

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