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Differential Regulation of Eosinophil Chemokine Signaling Via CCR3 and Non-CCR3 Pathways

Ian Sabroe, Adele Hartnell, Louise A. Joppel, Sandra Bel, Paul D. Ponath, James E. Pease, Paul D. Collins, and Timothy J. Williams

To investigate eosinophil stimulation by chemokines we developed a sensitive assay of leukocyte shape change, the gated autofluorescence/forward scatter assay. Leukocyte shape change responses are mediated through rearrangements of the cellular cytoskeleton in a dynamic process typically resulting in a polarized cell and are essential to the processes of leukocyte migration from the microcirculation into sites of inflammation. We examined the actions of the chemokines eotaxin, eotaxin-2, monocyte chemotactic protein-1 (MCP-1), MCP-3, MCP-4, RANTES, macrophage inflammatory protein-1α (MIP-1α), and IL-8 on leukocytes in mixed cell suspensions and focused on the responses of eosinophils to C-C chemokines. Those chemokines acting on CCR3 induced a rapid shape change in eosinophils from all donors; of these, eotaxin and eotaxin-2 were the most potent. Responses to MCP-4 were qualitatively different, showing marked reversal of shape change responses with agonist concentration and duration of treatment. In contrast, MIP-1α induced a potent response in eosinophils from a small and previously undescribed subgroup of donors via a non-CCR3 pathway likely to be CCR1 mediated. Incubation of leukocytes at 37°C for 90 min in the absence of extracellular calcium up-regulated responses to MCP-4 and MIP-1α in the majority of donors, and there was a small increase in responses to eotaxin. MIP-1α responsiveness in vivo may therefore be a function of both CCR1 expression levels and the regulated efficiency of coupling to intracellular signaling pathways. The observed up-regulation of MIP-1α signaling via non-CCR3 pathways may play a role in eosinophil recruitment in inflammatory states such as occurs in the asthmatic lung. The Journal of Immunology, 1999, 162: 2946–2955.

The inflammatory response associated with asthma is characterized by the recruitment of eosinophils from the bronchial microcirculation in response to the regulated local production of chemoattractant molecules (1, 2). Although several chemical mediators are active upon eosinophils in vitro, a central role in eosinophil accumulation in vivo is emerging for the C-C chemokine eotaxin, which was first identified in a guinea pig model of allergic airway inflammation using protein purification and microsequencing (3). Eotaxin is the dominant eosinophil-selective chemoattractant in this model, acting to cause both local eosinophil recruitment to the lung (4) and the release of a rapidly mobilizable pool of bone marrow eosinophils in cooperation with IL-5 (5, 6).

Using primers based on the guinea pig sequence, human eotaxin has been cloned and its synthesis (mRNA and protein) demonstrated in allergic diseases including asthma (7–10). Recently, another human eosinophil-selective C-C chemokine has been described and named eotaxin-2 on the basis of its functional homology with eotaxin (11, 12). Eotaxin and eotaxin-2 are potent stimulators of eosinophils, signaling exclusively via high affinity binding to the receptor CCR3 (13). C-C chemokines, including MCP-3, MCP-4, and RANTES, also stimulate eosinophils via CCR3, although they show less leukocyte selectivity as they additionally signal via other chemokine receptors expressed on a range of leukocytes, including lymphocytes and monocytes (14–17). CCR3 is expressed in high numbers on eosinophils (13, 18) and is thought to be the major eosinophil chemokine receptor. Blockade of CCR3 in vivo inhibits eosinophil recruitment in response to eotaxin in both the guinea pig (19) and mouse (20). More recently, CCR3 expression has been demonstrated on basophils (16) and Th2-type T cells (21–24), suggesting possible roles for CCR3 in the genesis and maintenance of allergic inflammation. CCR3 is therefore a major target for anti-inflammatory drug development (25).

There is evidence that eosinophils express low levels of CCR1 (13, 18), a receptor for MIP-1α, RANTES, and MCP-3. MIP-1α was first isolated from LPS-stimulated macrophages (26) and is thought to be a preferential monocyte chemoattractant (27), acting via CCR1 (28) or CCR5 (29). Although MIP-1α is among the chemokines produced in the human asthmatic lung in response to allergen (30, 31), there is controversy over its ability to induce functional responses in eosinophils (32–34).

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4 Abbreviations used in this paper: CCR, C-C chemokine receptor; MCP, monocyte chemotactic protein; MIP-1α, macrophage inflammatory protein-1α; GAFS, gated autofluorescence/forward scatter; FSC, forward scatter; VLA-4, very late Ag-4; PMNL, polymorphonuclear leukocytes (comprising eosinophils and neutrophils); SSC, side scatter; MHR, MIP-1α-highly responsive; MPR, MIP-1α-poorly responsive; PTX, pertussis toxin.
Leukocyte chemoattractants such as eotaxin initiate a coordinated sequence of adhesive interactions between cells in the circulation and the microvascular endothelium. The phases of leukocyte migration comprising leukocyte adhesion, spreading, penetration of the vessel wall, and tissue infiltration all depend on a continual reorganization of cytoskeletal elements associated with specific changes in cell morphology (35–37). The formation of microvilli, uropod, and lammelipodia is important in regulating the distribution and accessibility of cell surface adhesion molecules that are involved in cell migration (37, 38).

We postulated that analysis of the leukocyte shape change response to chemoattractant agonists would be a powerful tool for analyzing receptor usage by chemokines in human leukocytes. We have developed a sensitive flow cytometric system to quantitate shape changes in eosinophils and neutrophils simultaneously in mixed cell suspensions, the gated autofluorescence/forward scatter (GAFS) assay. This system uses differential autofluorescence to identify leukocyte type and changes in forward scatter (FSC) to measure shape change in response to agonists. We have characterized the shape change responses of eosinophils, neutrophils, and monocytes in response to a range of chemokines. Using the GAFS assay we have discovered considerable variations in the responses of eosinophils from individual donors to MIP-1α and demonstrated that these responses may be up-regulated by a simple preincubation of the leukocytes in the absence of calcium. A group of donors was also identified whose eosinophils consistently responded to MIP-1α with a potency comparable to that of eotaxin.

The existence of these signaling pathways is of significance in the development of small molecule chemokine receptor antagonists.

Materials and Methods

Reagents

BSA, EDTA, and glucose were purchased from Sigma (Poole, U.K.). RPMI 1640, FCS, PBS, and HEPES were purchased from Life Technologies (Paisley, U.K.). Dextran T-500 and Percoll were purchased from Pharmacia (St. Albans, U.K.). FITC-labeled goat anti-mouse monoclonal F(ab′)2 were obtained from Dako (High Wycombe, U.K.). IgG1 (clone MOPC 21) and IgG2a (clone UPI 10) negative control Abs were obtained from Sigma. Human recombinant RANTES, MIP-1α, MCP-1, MCP-3, and MCP-4 were purchased from PeproTech (London, U.K.). Anti-CD14 monoclonals were purchased from Milltenyi-Biotec (Bergisch Gladbach, Germany). Cellix was obtained from Becton Dickinson (Mountain View, CA). Anti-human CCR3 mAb 2B11 (13, 34), anti-human CCR1 mAb 2D4, and recombinant human eotaxin were produced by LeukoSite (Boston, MA).

Cellix containing human IL-8 and eotaxin-2 were from Dr. J. White, SmithKline Beecham (King of Prussia, PA); recombinant human C5a was from Dr. J. van Oostrum (Ciba-Geigy, Summit, NJ); anti-human IL-8RA (CXCRI) and IL-8RB (CXCRII) mAbs were from Dr. C. A. Hébert (Genentech, South San Francisco, CA); and anti-VLA-4 mAb was from Dr. R. R. Lobb (Biogen, Cambridge, MA).

Cell preparation

Volunteer blood donors were healthy normal subjects or atopics, as defined by a history of asthma, eczema, or hayfever, and symptoms on exposure to common aero-allergens, including pollens and house dust mites. The donors were taking no systemic medication. Blood was sampled according to a Royal Brompton Hospital ethical committee-approved protocol and was prepared as previously described (39). Briefly, platelet-rich plasma was removed by centrifugation of citrated whole blood, after which the erythrocytes were removed by dextran sedimentation. Polymorphonuclear leukocytes (PMNL; containing neutrophils and eosinophils) were then separated from PMNL by a discontinuous gradient and purified by negative magnetic selection, where the leukocytes were incubated with anti-CD16-coated microbeads in RPMI 0.5% BSA, 20 mM HEPES, and 5 mM EDTA, which selectively bound neutrophils in the PMNL suspension. The eosinophils were separated from the neutrophils by passage of the cell suspension through a magnetic field (41), resulting in eosinophil populations of >97.5% purity; the contaminating cells were PBMCs.

Measurement of changes in leukocyte shape using flow cytometry

For the standard GAFS assay, purified PMNL were washed in buffer (10 mM PBS containing Ca2+ and Mg2+), 10 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.2–7.4) and preincubated for 30 min at 37°C. In additional experiments as indicated in Results, the cells were preincubated for 30 or 90 min in the presence or the absence of Ca2+ and Mg2+.

In all experiments the cells were washed again in buffer containing Ca2+ and Mg2+, resuspended in buffer containing Ca2+ and Mg2+, and held for 5 min at room temperature to allow equilibration of intracellular and extracellular calcium. Aliquots of cells (5 × 106 PMNL) were mixed with agonists or buffer in 1.2-ml polypropylene cluster tubes (Costar, Cambridge, MA) in a final volume of 10 μL. The tubes were placed in a 37°C shaking water bath for 6 min (or as indicated), after which they were transferred to an ice-water bath, and 250 μL of ice-cold optimized fixative (a 1/4 dilution of 1× Cellix-buffered formaldehyde solution in PBS) was added to terminate the reaction and maintain the cell shape change until analysis. Initial experiments demonstrated that basal cell FSC and agonist-induced changes in cell FSC were not affected by fixation using this regimen, but were maintained without alteration for up to 2 h poststimulation on ice. In the absence of fixation, changes in cell FSC were identical with those seen in the fixed cell populations, but persisted for only 40 min when the cells were held on ice. In some experiments the cell aliquots or agonists (5 × 105 PMNL) were incubated for 10 min with Abs or buffer in 50 μL before addition of the other components of the assay (also in 50 μL to a final volume of 100 μL as above). The samples were then analyzed immediately on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA) whose FSC detector had been optimized using control 2-μm beads and restimulated leukocytes. Data was acquired using the FL-2 fluorescence channel on a sensitive setting, allowing eosinophils to be distinguished from neutrophils by their higher autofluorescence. FSC, side scatter (SSC), and FL-2 data were acquired, and acquisition was terminated after 500 high fluorescence (eosinophil) events.

In experiments investigating the effects of cation chelation during the preincubation, PMNL were preincubated with buffer (containing Ca2+ and Mg2+) and either 5 mM EDTA or 2 mM EGTA for 90 min in parallel with PMNL preincubated in buffer both with and without Ca2+ and Mg2+. After the preincubation, all cells were washed twice in buffer to remove the chelating agents. All cells were finally washes once in buffer containing Ca2+ and Mg2+ before performance of the GAFS assay as described above. Buffer pH was checked before and after the preincubation and did not vary over the course of the experiment (data not shown).

All data presented here show the responses of PMNL and PBMC in the presence of Ca2+ and Mg2+: manipulation of responses by removal of cations occurred during the preincubation phases only.

To measure monocyte shape change in response to chemokines, a similar protocol was used. However, since monocytes could not be identified separately from other cells by autofluorescence, an additional labeling step was incorporated. PBMCs were incubated for 10 min at room temperature with FITC-labeled anti-CD14 mAb (PharMingen, San Diego, CA) at 1 × 107/μL and washed once. After labeling, determination of monocyte shape change proceeded as described for PMNL above, except that agonist stimulation was for 10 min at 37°C, and data were acquired using the FITC FL-1 channel to identify monocytes. One thousand monocyte events were counted for each sample.

Immunofluorescence flow cytometry

To confirm eosinophil and neutrophil identity as predicted by autofluorescence, mixed PMNL were suspended in buffer at 5 × 105 cells/ml. Samples were incubated on ice for 60 min with saturating concentrations of anti-CXCRI mAb, anti-CXCR2 mAb, and anti-VLA-4 mAb; washed; and then incubated with FITC-conjugated polyclonal goat-anti-mouse F(ab′)2 Abs on ice for 30 min. Nonbinding control mAbs of IgG1 and IgG2a subclasses were used to determine nonspecific binding. Samples were held on ice until FACScan analysis. For staining of eosinophils in whole blood, aliquots of EDTA-anticoagulated blood (100 μL) were incubated for 1 h on ice with 10 μg/ml anti-CR1 mAb 2D4, 3 μg/ml anti-CR3 mAb 7B11, or relevant isotype-matched control Abs. The blood was then washed with buffer (PBS and 0.25% BSA) and resuspended in 100 μL of FITC-conjugated polyclonal goat-anti-mouse F(ab′)2 Abs on ice for 30 min. After one additional
FIGURE 1. Eosinophil and neutrophil separation by autofluorescence and specific mAb labeling, and the principles of the GAFS assay. A shows a representative dot plot of FSC vs SSC of 10,000 unstimulated mixed PMNL. Autofluorescence was measured using a sensitive FL-2 (585 nm) setting, and regions of high (R1) and low (R2) autofluorescence are defined. The resulting two cell populations are displayed on separate dot plots, where the high autofluorescence cells are labeled R1 (eosinophils) and the low autofluorescence cells are labeled R2 (neutrophils). Mean FSC values were derived for each population. B shows the FL-1 (530 nm) fluorescence histogram of cells labeled with an anti-VLA4 mAb, anti-IL-8RA mAb, or anti-IL-8RB mAb in mixed PMNL populations. The dotted line in each histogram shows the fluorescence of PMNL labeled with a negative control Ab, and on each histogram a region gate has been applied to a discrete cell population. The adjacent dot plots show the scatter characteristics of these defined regions. C shows the quantitation of agonist-induced changes in the FSC of eosinophils and neutrophils. FSC vs SSC dot plots of mixed PMNL preparations treated with buffer (i), 0.3 nM eotaxin (ii), 0.3 nM IL-8 (iii), and 1 nM C5a (iv) for 4 min at 37°C are shown. In each panel the separated dot plots for the eosinophil and neutrophil populations (determined by autofluorescence; A) are shown, and the mean FSC values are inset in the panels.

Measurement of cytosolic calcium
Eosinophils purified by immunomagnetic selection (1 x 10^7/ml) were loaded with 1 μM fura-2 in PBS without Ca^2+ for 30 min at 37°C, washed in PBS and 0.1% BSA, and resuspended at a final concentration of 1 x 10^6 cells/ml in PBS, 0.1% BSA, 10 mM HEPES, and 10 mM glucose. Aliquots of cells (2 ml) were dispensed into quartz cuvettes and equilibrated with 1 mM calcium at 37°C for 12 min before use. Changes in fluorescence were measured in a Perkin-Elmer LS-50 fluorescence spectrophotometer (Norwalk, CT) as previously described (40). Calculation of intracellular free calcium was derived from the fluorescence spectra (excitation wavelengths, 340 and 380 nm; emission wavelength, 510 nm) in accordance with established methods (40).

Statistical analysis
Data were analyzed using analysis of variance and Bonferroni’s posttest.

Results
Discrimination of eosinophils and neutrophils by autofluorescence and measurement of shape change using flow cytometry in mixed, unlabeled PMNL populations (the GAFS assay)
Fig. 1A shows a dot plot of SSC vs FSC and the FL-2 fluorescence histogram of unstained, unstimulated PMNL (eosinophils and neutrophils). The cells were divided into two distinct populations of high and low autofluorescence (R1 and R2, respectively) on the fluorescence histogram, correlating with the proportion of eosinophils and neutrophils, respectively, as determined in Kimura-stained preparations (data not shown). The light scatter characteristics of the eosinophils and neutrophils within the population (as identified by autofluorescence) are shown separately, and from these plots the mean FSC of each population is determined. We confirmed the identity of these leukocyte populations using Abs to specific cell surface molecules. Fig. 1B shows the fluorescence histograms of PMNL populations stained with anti-VLA-4, anti-CXCR1, or anti-CXCR2 mAbs, adjacent to which the light scatter characteristics of the stained cell populations are shown. The predominant VLA-4-positive cell population (R3) showed light scatter characteristics indistinguishable from those of the high autofluorescence cells (R1; Fig. 1A), confirming that these were eosinophils. Similarly, a single population of cells (R4) was stained with anti-CXCR1 Ab, which showed the same light scatter characteristics as the low autofluorescence cells, confirming their identity as neutrophils (R2; Fig. 1A). Anti-CXCR2 labeled two populations of cells. The most intense fluorescence was associated with neutrophils (data not shown), while eosinophils also stained weakly with this Ab (R5).

The eosinophil and neutrophil populations in the mixed PMNL preparation (identified according to their autofluorescence) responded independently to agonists with marked changes in their mean FSC. Fig. 1Ci, shows the control unstimulated population for comparison (eosinophil mean FSC = 208; neutrophil mean FSC = 214; as in Fig. 1A). Fig. 1Cii, shows that after stimulation with 0.3 nM eotaxin there was a selective increase in eosinophil mean FSC (348), but not neutrophil mean FSC (190) compared with those in the unstimulated cells. Conversely, there was an increase in neutrophil mean FSC (331) but not in eosinophil mean FSC (186) following addition of 0.3 nM IL-8 (Fig. 1Ciii), while Fig. 1Civ, shows that C5a (1 nM) increased both eosinophil and neutrophil mean FSC (252 and 373, respectively). There were no significant...
changes in the mean SSC following any treatment, and leukocyte autofluorescence was unaffected by chemokine stimulation. Eosinophil shape change was not inhibited when the agonist stimulation was performed in the absence of Ca$^{2+}$ and Mg$^{2+}$ (data not shown).

In other experiments performed simultaneously with the investigation of PMNL responses to chemokines, PBMCs from the same donors were labeled with an FITC-conjugated anti-CD14 mAb to distinguish monocytes from lymphocytes before measurement of their FSC as described for PMNL above.

![FIGURE 2. Eosinophil, monocyte, and neutrophil shape changes induced by chemoattractants. Mixed PMNL or PBMC were treated for 4 or 10 min, respectively, at 37°C with varying concentrations of eotaxin (open squares), eotaxin-2 (open diamonds), MCP-1 (triangles), MCP-3 (half-shaded squares), MCP-4 (half-shaded diamonds), or IL-8 (stars). The resulting shape change of the leukocyte populations was measured simultaneously as described. The results are expressed as the percent increase in FSC induced by each agonist compared with that of buffer-stimulated cells. The data represent the mean ± SEM of a minimum of four experiments, each using cells from a separate donor. Dose-response curves to eotaxin, eotaxin-2, MCP-4, and MCP-3 were significant on eosinophils. MCP-1, -3, and -4 induced significant changes on monocytes; neutrophils only showed a significant shape change in response to IL-8.](http://www.jimmunol.org/)

![FIGURE 3. Time courses of eosinophil shape changes induced by eotaxin, eotaxin-2, MCP-1, and MCP-4. Mixed PMNL preparations (A) or PBMC populations (B) were treated at 37°C for varying times with eotaxin (open squares; 2.5 nM), eotaxin-2 (open diamonds; 2.5 nM), MCP-1 (triangles; 10 nM), MCP-4 (half-shaded diamonds; 2.5 nM on PMNL, 10 nM on PBMC), or buffer (solid squares), and the resulting shape changes were measured. Results are expressed as the percent increase in FSC induced by the agonist compared with the FSC of cells 0 min after the addition of that agonist. The data represent the mean ± SEM of four experiments, each using cells from a separate donor. Significant shape changes were induced on eosinophils by eotaxin, eotaxin-2, and MCP-4 and on monocytes by MCP-1 and MCP-4. Significant differences between eotaxin-2- and MCP-4-induced shape change responses on eosinophils are shown (*, p < 0.05; ***, p < 0.01.](http://www.jimmunol.org/)

Eosinophil, neutrophil, and monocyte shape change induced by chemokines

In parallel experiments, the responses of eosinophils, neutrophils, and monocytes to chemokines were determined using the standard GAFS assay described above. Fig. 2A shows the percent change in mean FSC of eosinophils from a mixed PMNL preparation in response to the C-C chemokines eotaxin, eotaxin-2, MCP-1, MCP-3, and MCP-4 compared with that in response to the C-X-C chemokine IL-8. Eotaxin, eotaxin-2, and MCP-4 were the most potent inducers of eosinophil shape change, followed by MCP-3, while MCP-1 and IL-8 were inactive. MCP-4 alone showed a bell-shaped eosinophil dose-response curve over the dose range tested. Eotaxin and eotaxin-2 were inactive on monocytes (Fig. 2B), whereas MCP-1, -3, and -4 all induced monocyte shape change, with MCP-3 exhibiting the highest potency. Of the chemokines tested, only IL-8 induced a change in neutrophil FSC (Fig. 2C). Eosinophil shape change in response to eotaxin was abolished by pretreatment of the cells for 10 min at room temperature with 10 μg/ml of anti-CCR3 mAb, 7B11 (see Fig. 6). Neutrophil and eosinophil shape change responses to all agonists were abolished by...
Intracellular calcium flux in response to MIP-1α was assessed in purified eosinophils prepared by negative magnetic selection from one MPR and one MHR individual. Shape change responses were measured as described. The data represent the mean ± SEM of three experiments (MHR individuals) or four to nine experiments (MPR individuals), each using cells from a separate donor. In MPR individuals, significant differences between eotaxin and both RANTES and MIP-1α (A) are shown (***, p < 0.001). In MHR individuals significant differences were only seen between eotaxin and RANTES (B; †, p < 0.05; ††, p < 0.01).

**Time course of eosinophil and monocyte shape change**

To investigate the time course of agonist-induced changes in cell shape, PMNL and anti-CD14-labeled PBMC were incubated at 37°C with eotaxin (2.5 nM), eotaxin-2 (2.5 nM), MCP-1 (10 nM), or MCP-4 (2.5 nM for PMNL, 10 nM for PBMC) for varying time periods from 0–20 min. Fig. 3 shows that changes in eosinophil and monocyte FSC occurred rapidly and could be detected by flow cytometry within 30 s. For eosinophils stimulated by eotaxin or eotaxin-2, maximal shape change was obtained after 4 min of chemokine treatment and remained constant thereafter. The increase in eosinophil FSC induced by MCP-4 gradually reversed on prolonged stimulation. This reversal of shape change with time was observed with MCP-4 only. Both MCP-1 and MCP-4 induced a rapid-onset, persistent, monocyte shape change, with no reversal of stimulated cells to baseline shape up to the 20 min point.

**Identification of donors with eosinophils highly responsive to MIP-1α**

Investigation of atopic individuals using the GAFS assay (incorporating the standard 30-min preincubation step in the presence of Ca²⁺ and Mg²⁺) identified a group whose eosinophils showed strong MIP-1α responses (designated MIP-1α-highly responsive (MHR) individuals). These responses were in marked contrast to the majority of individuals who showed absent or poor MIP-1α responses under standard conditions (designated MIP-1α-poorly responsive (MPR) individuals). Eosinophils from the MHR individuals showed a shape change in response to MIP-1α of similar potency to that of eotaxin (Fig. 4). There was no difference in responses to eotaxin or MCP-4 between MPR and MHR individuals (Figs. 4, A and B, and 5). Sixteen individuals were investigated for the ability of their eosinophils to respond to MIP-1α, of whom 13 (81%) were MPR and 3 (19%) were MHR individuals. In contrast, there was no significant difference in MIP-1α responsiveness in the monocyte populations from either group screened on the same day as the PMNL populations (Fig. 4, C and D).

To identify the receptor usage by MIP-1α in the MHR group of individuals, PMNL populations from each group were pretreated with the blocking anti-CCR3 mAb 7B11 or a control mAb (MOPC 21) following the 30 min preincubation step. 7B11 treatment completely blocked the eosinophil responses to eotaxin (see Fig. 6) and MCP-4 (data not shown) from both the MPR and MHR individuals. In contrast, MHR eosinophils showed a potent response to MIP-1α that was not blocked by anti-CCR3 mAb. The shape change response to RANTES was completely inhibited by anti-CCR3 mAb pretreatment in the MPR individuals; however, in the MHR group there was only partial inhibition of the response to RANTES by CCR3 blockade (Fig. 6, A and B). Flow cytometric analysis of eosinophil CCR1 and CCR3 expression was performed in whole blood from four MPR donors and one MHR donor. These data showed low levels of CCR1 expression on eosinophils from all donors, in contrast to the expression of CCR3, which was at high levels that were remarkably consistent between donors. Levels of eosinophil CCR1 expression appeared higher in the MHR donor than in the MPR donors (Fig. 7).

**Correlation of shape change response with intracellular calcium mobilization**

Intracellular calcium flux in response to MIP-1α and eotaxin was assessed in purified eosinophils prepared by negative magnetic selection from one MPR and one MHR individual. Shape change
was simultaneously measured in the purified eosinophils to confirm the MIP-1α responsiveness of the donors (data not shown). Fig. 8 shows that eosinophils from both MPR and MHR individuals responded with similar calcium flux to 3 nM eotaxin (Fig. 8, A and B). Eosinophils from the MPR individual showed only a minimal calcium response to 10 nM MIP-1α (Fig. 8C), whereas in contrast, eosinophils from the MHR individual showed a pronounced calcium flux in response to 10 nM MIP-1α (Fig. 8D). Pretreatment with the anti-CCR3 mAb 7B11 (10 μg/ml) blocked eotaxin signaling in both individuals, but did not block MIP-1α signaling in the MHR eosinophils (Fig. 8, E and F). MOPC-21 pretreatment did not affect intracellular calcium signaling in either group in response to these ligands (Fig. 8, C and D). Eosinophils from both the MPR and MHR individuals showed similar intracellular calcium signaling responses to C5a, and two additional MPR individuals showed similar overall patterns of C-C chemokine responsiveness in assays of intracellular calcium flux (data not shown).

**Up-regulation of eosinophil responses to MIP-1α**

Pilot data suggested that eosinophil responses to chemokines might be affected by variations in the preincubation conditions. Fig. 9 shows the eosinophil responses to eotaxin, MCP-4, and MIP-1α after variations in these conditions. Mixed PMNL were preincubated according to the standard GAFS assay protocol (30 min in the presence of Ca2+ and Mg2+). In parallel, PMNL from the same preparations were also preincubated for 30 min in the absence of cations and for 90 min in both the presence and the absence of cations. Subsequently, all GAFS assays were performed as described previously, the only variations between cell treatments being in the preincubation steps, and data were acquired immediately after stimulation of the cells with agonists. Fig. 9 shows that responses to eotaxin, MCP-4, and MIP-1α were affected by the preincubation conditions. Fig. 9B shows that responses to 1.25 nM MCP-4 were greatest in the cells preincubated for 90 min without Ca2+ and Mg2+ (p < 0.001). The order of responsiveness of the eosinophils to MCP-4 for the different preincubation conditions was 90 min without cations > 30 min without cations > 30 min with cations > 90 min with cations. Fig. 9C shows that eosinophil responses to MIP-1α were absent or poor in this donor group after the 30-min incubation with Ca2+ and Mg2+. These responses to MIP-1α were significantly up-regulated after preincubation for 90 min without cations (p < 0.01 at the 0.6- and 1.25-nM concentrations), although with greater variability between donors than was seen with MCP-4 or eotaxin. An enhancement of eosinophil responses to MIP-1α of varying magnitude occurred in seven of eight donors tested, with one donor only showing no increase in MIP-1α responsiveness after the extended preincubation step. Eosinophil responses to MIP-1α were similarly enhanced when either EDTA (5 mM) or EGTA (2 mM) were added to the cells during the 90-min preincubation with Ca2+ and Mg2+ (n = 4). Shape change responses to both eotaxin and MIP-1α were abolished by pretreatment of the PMNL with pertussis toxin (PTX; 0.1 μg/ml) during the 90-min preincubation step. Staining of leukocytes with an anti-CCR1 mAb (2D4) and the anti-CCR3 mAb (7B11) in two donors showed no changes in eosinophil CCR1 and CCR3 expression after any preincubation condition compared with that in cells stained immediately after purification.
Discussion

Using the GAFS assay, we have demonstrated that C-C chemokines known to bind to CCR3 induce an eosinophil shape change response. Of these, eotaxin, eotaxin-2, and MCP-4 were the most potent, in keeping with their high affinities for CCR3 (12, 13, 16). Eosinophil and neutrophil shape change measured in the GAFS assay occurred rapidly (within 30 s of agonist stimulation), consistent with a relationship between these chemokine-induced responses and the earliest phases of leukocyte recruitment from the microcirculation. IL-8 has been shown to be chemotactic for eosinophils from atopic donors in vitro (42). Although we demonstrated low levels of CXCR2 expression on eosinophils, we were unable to demonstrate IL-8-induced eosinophil shape change at concentrations of \(100 \text{ nM}\) (data not shown). This may reflect a lower level of in vivo priming in our mildly atopic donors than those used by other studies. Monocytes, in contrast, showed chemokine-induced shape change responses to MIP-1\(\alpha\), MCP-1, MCP-3, and MCP-4 only.

The association between FSC in flow cytometry and cell size is well recognized, but it has also been shown that changes in this parameter can provide a measure of neutrophil polarization and agonist-induced shape change (43–45). Neutrophil shape change in response to FMLP and PMA has been measured using flow cytometry (44, 45), and using laser turbimetry, eosinophils have been shown to undergo a brief shape change response to platelet-activating factor (PAF) and C5a (46). This shape change requires cytoskeletal rearrangement through the polymerization of cytoplasmic monomeric actin (G-actin) to microfilamentous actin (F-actin) (47, 48), and eotaxin has been shown to induce eosinophil actin polymerization (49). Eosinophil shape change in response to eotaxin was completely abolished by PTX, consistent with studies showing that eotaxin-induced actin polymerization is inhibited by pretreatment of cells with PTX (49). In the studies by Kernen et al. the eosinophil responses to PAF and C5a were significantly, but not completely, inhibited by PTX (46), suggesting that for some chemoattractants eosinophil shape change may be partially mediated by PTX-resistant G proteins. The response measured by the GAFS assay was abolished by pretreatment of PMNL with cychotralasin B, supporting the dependence of the response on actin polymerization (50). The GAFS response is therefore most likely to represent a direct measure of leukocyte shape change, but may also involve contributions from changes in cell size (volume). Further studies using electron microscopy are underway to investigate the nature of leukocyte shape responses measured in the GAFS assay.
Assays investigating eosinophil responses to chemoattractants are hampered by the low numbers of these cells in the circulation and the relative complexity of their purification. The GAFS assay is sensitive, requires small numbers of cells, and exploits the characteristic autofluorescence of human eosinophils (51), avoiding cell purification procedures that may themselves modulate leukocyte responses. The assay proved to be reliable and robust over time, but required periodic optimization of the FSC detector to maximize consistency of the results.

In contrast to eotaxin and eotaxin-2, the eosinophil response to MCP-4 showed time-dependent reversal to baseline values and a markedly bell-shaped dose-response curve. The reversal of the MCP-4-induced shape change only occurred in eosinophils; monocytes from the same donors assayed simultaneously showed shape change responses to MCP-4 that reached stable plateau levels with time and increasing agonist concentration. Eosinophil responses to both MCP-4 and eotaxin were up-regulated by preincubation of the PMNL without Ca\(^{2+}\) and Mg\(^{2+}\), although the up-regulation of responses was more marked for MCP-4 than for eotaxin. MCP-4 is thought to act on human eosinophils solely through CCR3 (15, 16). This suggests that either MCP-4 causes different eosinophil responses compared with eotaxin despite signaling through the same receptor, or that MCP-4 may modulate its own signaling through CCR3 by selective actions on an additional and as yet undescribed receptor signaling pathway. The significance of the reversal of the MCP-4-induced shape change is not clear, but may represent a potential mechanism to enhance the specificity of this chemokine in vivo, favoring eosinophil accumulation at low concentrations and monocyte accumulation at high concentrations.

Using the GAFS assay we identified three individuals whose eosinophils showed shape change responses to MIP-1\(\alpha\) that were equipotent to eotaxin under the standard preincubation conditions (30 min in the presence of Ca\(^{2+}\) and Mg\(^{2+}\)). These were designated MHR individuals. Eosinophil shape change responses to eotaxin, RANTES, and MCP-4 were not different between MHR and other donors. The responses to MIP-1\(\alpha\) observed in the GAFS assay correlated with the ability of MIP-1\(\alpha\) to induce an intracellular calcium flux in purified eosinophils from an MHR individual. In contrast, eosinophils from the majority of donors showed only weak MIP-1\(\alpha\)-induced eosinophil shape change responses and poor intracellular calcium flux in response to this chemokine (designated MPR).

Our data showed that pretreatment of cells from MHR individuals with 10 \(\mu\)g/ml of a blocking anti-human CCR3 mAb (34) completely inhibited the eosinophil responses to eotaxin, but did not inhibit eosinophil responses to MIP-1\(\alpha\) and only partially neutralized eosinophil shape change responses to RANTES. Thus, these data provide evidence for the existence of a functional shared MIP-1\(\alpha\)/RANTES receptor distinct from CCR3 in the MHR donors that is most likely to be CCR1. When used at a concentration of 50 \(\mu\)g/ml, the anti-CCR1 mAb, 2D4, blocks the CCR1-mediated intracellular calcium flux of monocytes and the chemotactic response of activated T cells to MIP-1\(\alpha\) in vitro (S. Qin, unpublished observation). In the GAFS assay, pretreatment of cells from MHR individuals with 10 \(\mu\)g/ml 2D4 had no effect on their eosinophil shape change responses to MIP-1\(\alpha\) or eotaxin. Pretreatment of PMNL with 50 \(\mu\)g/ml 2D4 caused variable suppression of eosinophil shape change responses to both MIP-1\(\alpha\) and, to a lesser extent, eotaxin. A degree of nonspecific inhibition of eosinophil chemokine responses was also seen when PMNL were pretreated with 50 \(\mu\)g/ml of an isotype-matched control Ab (data not shown). The failure of the anti-CCR1 mAb to reliably block responses in the GAFS assay is likely to be assay and Ab concentration dependent and suggests that the affinity of 2D4 for CCR1 may be less than the affinity of the 7B11 Ab for CCR3.

FACS analysis of CCR1 and CCR3 expression on eosinophils in whole blood showed high levels of CCR3 expression that were consistent between donors and much lower levels of CCR1 expression. This is consistent with previous data showing low levels of \(^{125}\)I-labeled MIP-1\(\alpha\) binding to human eosinophils (13, 18). There was a higher level of CCR1 expression on eosinophils from an MHR donor than on those from donors whose eosinophils were poorly responsive to MIP-1\(\alpha\). However, even in the MHR donor the levels of eosinophil CCR1 expression were considerably less than those of CCR3. These data additionally show that an absence of MIP-1\(\alpha\) responsiveness is not due to a complete absence of CCR1 expression.

Monocyte responses to MIP-1\(\alpha\) were similar in the MHR and MPR groups (EC\(_{50}\), 0.3 vs 0.5 nM). However, it is possible that the enhanced MIP-1\(\alpha\) responsiveness in the eosinophils of MHR individuals may be due to a mutation in CCR1 enhancing MIP-1\(\alpha\) binding or signaling, and this is the subject of further studies.

There were no clear differences in the clinical presentations of the MHR and MPR groups. The MHR individuals were atopic nonasthmatics, and only one of the three experienced regular symptoms to allergens (rhinitis on exposure to house dust mites). Therefore, it is unlikely that the MHR population represents a more severe group of atotics showing in vivo priming to MIP-1\(\alpha\) as has been described for IL-8 (42).

Similar to the modulation of eotaxin and MCP-4 responses, preincubation of PMNL for 90 min without Ca\(^{2+}\) and Mg\(^{2+}\) up-regulated eosinophil MIP-1\(\alpha\) responses in the MPR group of donors. This up-regulation of MIP-1\(\alpha\) responsiveness was not associated with changes in CCR1 or CCR3 expression levels. These data together with the immunofluorescence data reported above suggest that the MIP-1\(\alpha\) receptor is present on eosinophils at low levels in a nonfunctional state in the majority of individuals. Regulation of receptor responsiveness might be mediated through receptor phosphorylation, although receptor phosphorylation is generally thought to occur in response to direct activation of receptors by agonist (52). However, it has been shown that receptors are not required to be occupied by agonist to be phosphorylated, as phosphorylation of unstimulated chemoattractant receptors occurs during the process of heterologous receptor desensitization (40, 53).

Alternatively, the eosinophil MIP-1\(\alpha\) receptor in most individuals may be held in an inactivated complex together with molecules such as arrestins (54). In IL-2-activated NK cells MIP-1\(\alpha\) signaling is mediated via different G proteins than RANTES, as demonstrated by a selective resistance of MIP-1\(\alpha\) signaling to PTX (55); however, in our study eosinophil responses to MIP-1\(\alpha\) were fully blocked by PTX.

Controversy has existed over the ability of MIP-1\(\alpha\) to cause a chemotactic response in human eosinophils; some groups have shown MIP-1\(\alpha\)-induced eosinophil chemotaxis (33), while others have not (32). Our data suggest that MIP-1\(\alpha\) responsiveness is likely to be a function of both CCR1 expression levels and the regulated efficiency of CCR1 coupling to intracellular signaling pathways. Although in our experiments classification of donors as MHR or MPR has been consistent over a 2-yr period, we cannot exclude the possibility that both expression levels and coupling efficiency may vary independently within individuals over time and with disease activity (e.g., in asthma). Further studies of much larger populations of individuals over a long period of time are required to investigate these possibilities fully. Although our experiments have not investigated eosinophil chemotaxis directly, they provide an explanation for the variations seen in eosinophil responses by different research groups.
MIP-1α is one of several chemokines whose synthesis is up-regulated in the human asthmatic lung, together with eotaxin and RANTES (9, 10, 30, 31). CCR3 antagonists may prove to be valuable drugs targeting eosinophilic allergic inflammation. However, such drugs may be of reduced effectiveness in individuals whose eosinophils are highly responsive to MIP-1α. Furthermore, we have shown that eosinophil responses to MIP-1α can be up-regulated in MPR individuals in vitro. Pathways are therefore likely to exist allowing the up-regulation of eosinophil MIP-1α responsiveness to occur in vivo; the endogenous stimuli may be present at sites of acute inflammation associated with asthma. Screening of individuals using functional techniques such as the GAFS assay will be vital during recruitment of individuals for clinical trials of novel anti-chemokine receptor therapies. Ongoing studies should elucidate whether MHR status correlates with a clinical phenotype in the setting of asthma and allergic disease.

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References


