Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3– and CC chemokine receptor 4–independent manner

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Background: Chemokines are believed to contribute to selective cell recruitment. Macrophage-derived chemokine (MDC) is a CC chemokine that causes chemotaxis of dendritic cells, monocytes, and activated natural killer cells. MDC binds to CC chemokine receptor 4 (CCR4) but not to CCR1, CCR2, CCR3, CCR5, CCR6, or CCR7.
Objective: Our aim was to determine the in vitro activity of MDC on human eosinophils by using chemotaxis and calcium flux assays.
Methods: Eosinophils were purified from peripheral blood of allergic donors, and chemotactic activity of MDC and other CC chemokines was compared in microchemotaxis chamber assays. The role of CCR3 in these assays was determined by using a CCR3-blocking antibody. Measurements of cytosolic Ca^{++} mobilization were performed by using fura-2AM labeling, with eosinophils and cell lines transfected with CCR3 or CCR4. Eosinophil expression of CCR3 and CCR4 mRNA was determined by using RT-PCR.
Results: MDC (0.1 to 100 nmol/L) caused dose-dependent chemotaxis of purified human eosinophils (maximum ~3-fold control). Compared with other CC chemokines, the potency and efficacy for eosinophil chemotaxis were similar for MDC and eotaxin but were less than that observed for RANTES, monocyte chemoattractant protein (MCP)-4, and eotaxin-2. Although MDC can act by means of CCR4, RT-PCR analysis failed to reveal CCR4 mRNA in eosinophils. Effects of MDC on eosinophils was also independent of CCR3, as a blocking mAb to CCR3 failed to inhibit MDC-induced chemotaxis. Furthermore, CCR3-transfected human embryonic kidney cells labeled with Fura-2AM exhibited a rapid rise in intracellular free calcium after stimulation with eotaxin, eotaxin-2, or MCP-4, but not with MDC. Eosinophils cultured for 72 hours in 10 ng/mL IL-5 also demonstrated increased intracellular free calcium after stimulation with eotaxin-2 or MCP-4, but not with up to 100 nmol/L MDC.
Conclusion: MDC is a CCR3- and CCR4-independent activator of eosinophil chemotaxis, but it does not appear to elicit measurable cytosolic calcium elevations during these responses. MDC appears to act by means of another receptor in addition to CCR4 and may therefore contribute to eosinophil accumulation without working through CCR1 to CCR7. (J Allergy Clin Immunol 1999;103:527-32.)

Key words: *Eosinophils, chemokines, chemotaxis, macrophage-derived chemokine*

Eosinophils accumulate in increased numbers at sites of inflammation in chronic allergic diseases, such as bronchial asthma, and after the instillation of allergen into the skin and airways.[1,2] Because these cells are preferentially attracted, mechanisms must exist that favor their preferential recruitment into the affected tissue site where they subsequently contribute to allergic disease pathogenesis. Leukocyte recruitment into the airway is known to result from a process that involves the interaction of cell-surface adhesion molecules (eg, integrins, selectins, and immunoglobulin-like molecules) on the circulating leukocyte with receptors on vascular endothelial cells, epithelial cells, extracellular matrix proteins, and other tissue structures.[1,3] In addition, within the last few years it has become clear that chemokines also play an important role in inflammatory cell recruitment. With respect to allergic diseases, interest has focused on chemokines belonging to the CC family, such as RANTES, eotaxin, eotaxin-2, monocyte chemoattractant protein (MCP)-3, and MCP-4, because of their ability to cause migration of human eosinophils in vitro and in vivo.[4-11] Their ability to selectively activate human eosinophil migration appears to be primarily a result of their activation of CCR3, a 7 transmembrane–spanning chemokine receptor found on these cells, as well as on basophils and T_{H2} lymphocytes.[11-15]

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Abbreviations used
CCR: CC chemokine receptor
HEK: Human embryonic kidney
MCP: Monocyte chemoattractant protein
MDC: Macrophage-derived chemokine
NK: Natural killer
PAF: Platelet-activating factor

Macrophage-derived chemokine (MDC) is a CC chemokine that was originally identified by mass sequencing of a cDNA library from 6-day cultured monocytes and was shown to cause chemotaxis of dendritic cells, monocytes, and IL-2–activated natural killer (NK) cells.[16] Subsequently, MDC was also identified as a CD8+ T cell–derived factor capable of interfering with HIV-1 infection.[17] MDC has closest homology to the CC chemokines RANTES, macrophage inflammatory protein-1α, and TARC,[7,16] and like TARC it has been shown to bind to CC receptor 4 (CCR4), but not to CCR1, CCR2, CCR3, CCR5, CCR6, or CCR7.[18,19] As part of ongoing efforts to characterize CC chemokines and their effects on human eosinophils, the ability of MDC to cause migration and activation of human eosinophils was examined.

METHODS

Chemokines

RANTES and eotaxin were purchased from R&D Systems (Minneapolis, Minn), and MCP-4 and eotaxin-2 were generously provided by Dr John White (SmithKline Beecham, King of Prussia, Pa). MDC, TARC, and a mutant form of MDC lacking the first 8 N-terminal amino acids were synthesized by Gryphon Sciences (San Francisco, Calif) by using an automated peptide synthesizer (Applied Biosystem, San Francisco, Calif).

Eosinophil purification and chemotaxis

Human eosinophils were purified from mildly allergic donors by using a combination of Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation and immunomagnetic removal of CD16-positive cells (neutrophils) with Dynabeads (Dynal Inc, Lake Success, NY), as previously described.[20] Eosinophil purity and viability always exceeded 95%.

Leukocyte chemotaxis was measured with a Neuroprobe 48-well microchemotactic chamber (Neuroprobe) as described.[10] In some experiments chemotaxis was performed with cells preincubated in saturating concentrations of the CCR3-blocking murine mAb 7B11[13] (generously provided by Dr Charles Mackay, LeukoSite, Inc, Cambridge, Mass).

Measurements of cytosolic Ca^{++} mobilization

Measurements of intracellular calcium in CCR4 transfectants was performed by using fluorometry, as described elsewhere.[19] Measurements of intracellular calcium in CCR3 transfectants or eosinophils[13,21,22] were performed by using fura-2AM labeling. Briefly, cells were incubated in 1 mmol/L fura-2AM for 25 minutes at 37°C in RPMI-1640 containing 2% FBS and 0.3 mmol/L EDTA, washed once with Ca^{++}-free buffer, and kept on ice before loading into a Dvorak-Stotler chamber for observation under the microscope. Fifteen microliters of cell suspension was loaded into the microscope chamber, allowed to settle, and overlayed with 0.7 mL PAGCM buffer (composed of 25 mmol/L piperazine-N,N´-bis-2-ethanesulfonic acid [PIPES; Sigma Chemical Co, St Louis, Mo], 110 mmol/L NaCl, 5 mmol/L KCl, 40 mmol/L NaOH, 0.003% human serum albumin, 0.1% glucose, 1 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$ adjusted to pH 7.3). Stimulus in 0.7 mL PAGCM was then added. A field of 50 to 100 cells was monitored by sequential dual excitation at 352 and 380 nm, and ratios of the images were converted to calcium concentrations, according to methods and parameters described elsewhere.[22] Ratio images were acquired every 3 seconds early in the reaction and every 10 seconds later in the reaction. Platelet-activating factor (PAF; Sigma) was used as a positive control.

Eosinophil studies were performed on freshly isolated cells or after culture for 72 hours in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (High Clone Laboratories, Inc, Logan, Utah), 100 U/mL penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Gibco BRL), and 10 ng/mL IL-5 (R&D Systems). Cultured eosinophils were used because freshly isolated eosinophils frequently failed to demonstrate calcium fluxes, even with known CCR3-active chemokines[13] (data not shown). Viability after culture, as determined by erythrosin B dye exclusion, was greater than 90%.[23] Cells were cultured at a density of 5×10^5/mL at 37°C in a humidified atmosphere containing 5% CO_2 in 24-well, flat-bottom plates (Costar Corp, Cambridge, Mass) previously coated with 1% BSA.

PCR analysis of CCR3 and CCR4 from eosinophil RNA

First-strand cDNA was generated from human eosinophil RNA with both oligo-dT and random primers by using the First Strand cDNA Synthesis Kit (Boehringer-Mannheim, Indianapolis, Ind). Full-length chemokine receptor sequences were amplified by PCR with primers specific for CCR3 and CCR4. The same receptor sequences were also amplified from genomic DNA purified from human blood by using the Puregene kit (Gentra Systems, Inc, Research Triangle Park, NC). The primers used were as follows: CCR3 sense: 5´-ATGACAACCTCACTAGATACAG; CCR3 antisense: 5´-CTGACCTAAAACACAATAG; CCR4 sense: 5´-ATGAACCCCACGGATATAGCAG; and CCR4 antisense: 5´-TTTTCTACAGAGCATCATGG. The PCR conditions included a 4-minute denaturation at 94°C followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute. PCR products were then separated on a 1% agarose gel.

Statistical analysis

Data are presented as means ± SEM. Statistical significance of MDC effects was determined by paired *t* test. Two-way ANOVA with repeated measures was used for comparisons between chemokine dose-response curves. Both tests were considered significant at P values less than .05.

RESULTS

Initial experiments were performed to determine whether MDC was chemotactic for human eosinophils. As shown in Fig 1, MDC induced statistically significant concentration-dependent eosinophil chemotaxis, reaching values of migration about 3-fold above buffer control at 10 nmol/L MDC. No chemokinetic activity of MDC was observed (Fig 1). In contrast, a truncated form of MDC lacking the first 8 N-terminal amino acids lacked chemotactic activity (n = 2, data not shown).

To examine relative potencies, MDC and other

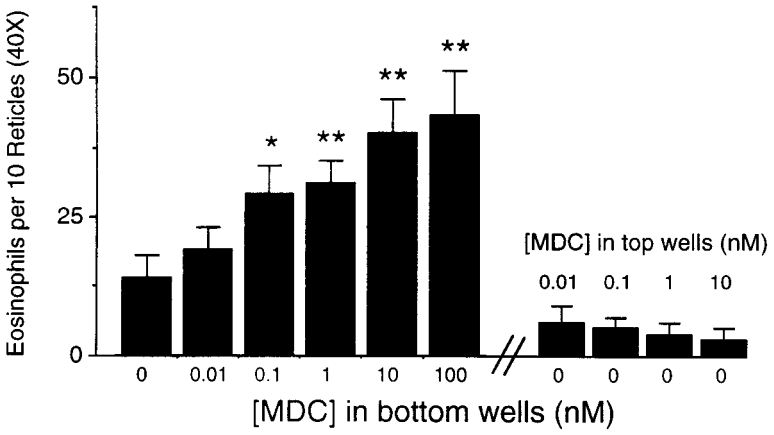

FIG 1. MDC induces chemotaxis, but not chemokinesis, of human eosinophils. Values are means ± SEM from 5 or more experiments for chemotaxis. Data displayed for the chemokinesis experiment (right portion of the figure) are from a single experiment representative of 2 separate experiments with similar results. **P* < .001; ***P* < .0001 (as determined by paired *t* test).

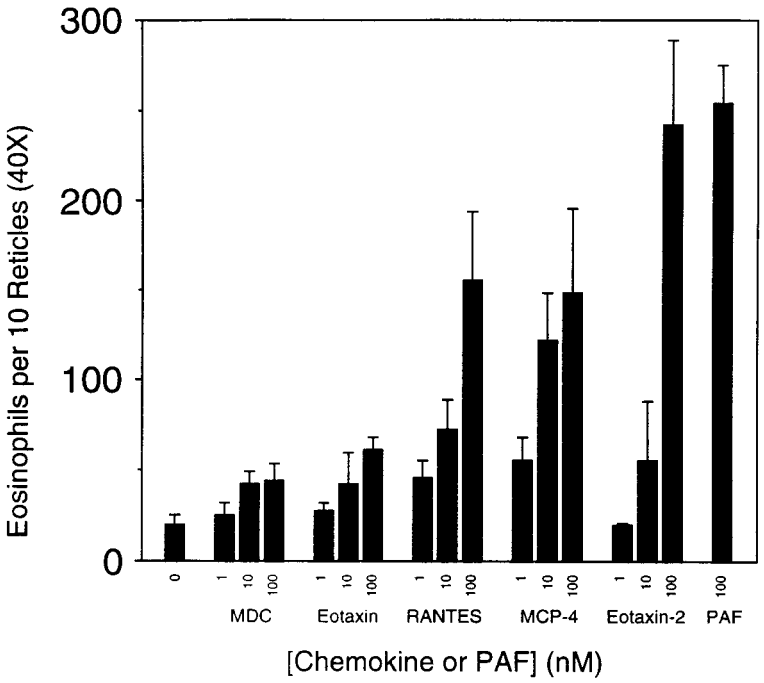

FIG 2. Simultaneous comparison of chemotactic effects of MDC, eotaxin, RANTES, MCP-4, eotaxin-2, MCP-4, and PAF on human eosinophils. Values are means ± SEM (n ≥ 3). As determined by 2-way ANOVA with repeated measures, the MDC and eotaxin dose-response curves were not significantly different from each other but were different from those obtained with the other chemokines (*P* < .05).

eosinophil-active CC chemokines[10,11] were simultaneously compared for their ability to cause migration of purified human eosinophils. All were active in inducing eosinophil migration (Fig 2). By means of 2-way ANOVA with repeated measures, the effects of MDC were not significantly different from those seen with eotaxin. However, RANTES, MCP-4, and eotaxin-2 were more potent than MDC or eotaxin; these differences in dose-response curves reached statistical significance ($P < .05$).

Subsequent studies were performed to characterize the receptor use of MDC in eosinophils. Previous studies with CCR transfectants suggest that CCR4 functions as a receptor for MDC, whereas CCR1, CCR2b, CCR3, CCR5, CCR6, and CCR7 do not.[19] In other studies with human eosinophils, CCR3 was identified as a critical receptor for a variety of CC chemokines, including eotaxin, RANTES, MCP-4, and eotaxin-2.[6,9-11] We therefore determined whether CCR3 or CCR4 might be involved in eosinophil migration induced by MDC.

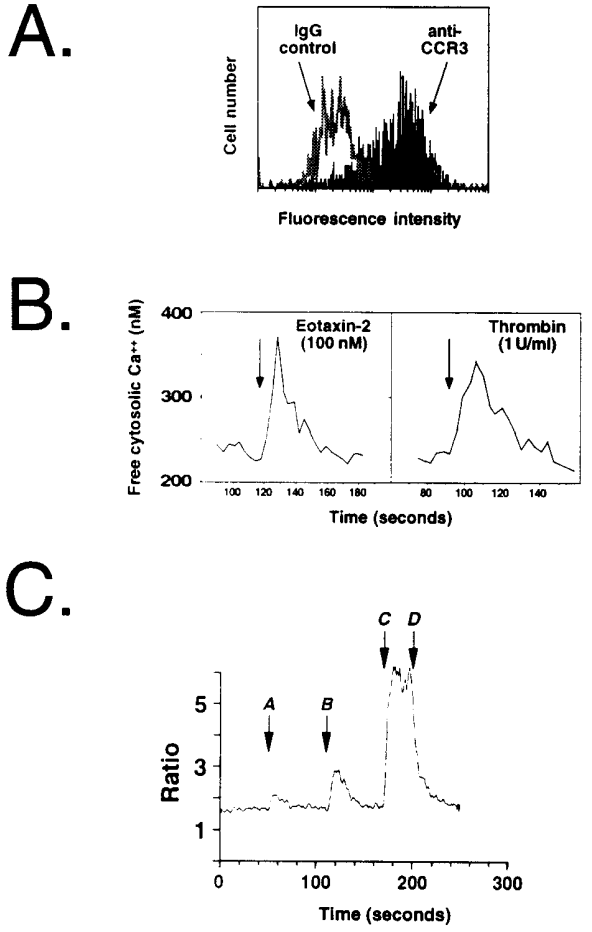

FIG 3. A, Surface expression of CCR3 on transfected HEK cells detected by indirect immunofluorescence and flow cytometry. Data displayed are from a single experiment representative of 2 separate experiments with similar results. **B,** Eotaxin-2 induces calcium flux by means of CCR3. Shown are averaged single cell Ca^{++} tracings of fura-2AM–labeled CCR3-transfected HEK cells. *Arrows* indicate when the stimulus was added. Thrombin was used as a positive control because HEK cells constitutively express 7 transmembrane thrombin receptors. Data displayed are from a single experiment representative of 3 separate experiments with similar results. **C,** MDC induces a calcium flux by means of CCR4. Shown are suspension cell Ca^{++} tracings of Fura-2AM–labeled CCR4-transfected HEK cells as measured by fluorometry. *Arrows* indicate when stimuli were added. Stimuli were as follows: *A,* 70 nmol/L MDC; *B,* 1 U/mL thrombin; *C,* 1 μg/mL ionomycin; and *D,* 5 mmol/L ethylene-bis (oxyethylenenitrilo) tetraacetic acid. Data displayed are from a single experiment representative of 2 separate experiments with similar results.

HEK293 cells stably transfected with CCR3 (Fig 3, *A*) displayed a rise in intracellular calcium on stimulation with eotaxin-2 (Fig 3, *B*) or eotaxin (data not shown), but not on exposure to MDC (up to 100 nmol/L concentrations, data not shown). In contrast, both MDC (Fig 3, *C*) and TARC (data not shown) induced a rise in intracellular calcium in HEK293 cells stably transfected with CCR4. In addition, a CCR3-blocking mAb inhibited eotaxin- and eotaxin-2–induced eosinophil chemotaxis, but not chemotaxis induced by MDC or PAF (Fig 4). As expected, RT-PCR analysis of mRNA from human eosinophils readily detected mRNA for CCR3. In contrast, mRNA for CCR4 could not be detected, even though these methods readily detected CCR4 mRNA from dendritic cells (Fig 5 and data not shown).

To obtain additional information about the characteristics of the MDC receptor on human eosinophils, changes in cytosolic free Ca^{++} were examined. Eosinophils demonstrated no detectable rise in intracellular free calcium after stimulation with up to 100 nmol/L MDC or TARC (Fig 6 and data not shown). In contrast, eotaxin, eotaxin-2, or MCP-4 induced a rapid increase (Fig 6 and data not shown), as previously reported.[9-11,24] Finally, pretreatment of eosinophils with up to 100 nmol/L concentrations of MDC failed to prevent eotaxin-2–induced increases in intracellular free calcium (data not shown).

DISCUSSION

In this study MDC was shown to induce chemotaxis of human eosinophils, expanding the list of cells (monocytes, monocyte-derived dendritic cells, and activated NK cells and T cells) known to respond to this chemokine.[16,25] MDC was as potent and efficacious as eotaxin in this respect, but much less so than RANTES, MCP-4, or eotaxin-2. Although the relative potencies observed with MCP-4, RANTES, and eotaxin-2 for induction of eosinophil chemotaxis were similar to previous reports, the lower potency seen with eotaxin differs from that reported previously.[5,10,11] The reason for these differences is unknown. MDC induced eosinophil migration even though it did not elicit measurable cytosolic calcium elevations during these responses. Previous studies with human eosinophils have suggested that they express CCR1 and CCR3,[12,26-28] and competition studies with chemokines and CCR3-blocking mAb suggest that CCR3 is especially important for selective eosinophil recruitment.[7,12,13,29] However, a CCR3-blocking mAb did not inhibit MDC-induced eosinophil chemotaxis, even though it blocked chemotaxis induced by other CC chemokines. This information is consistent with data showing the lack of effect of MDC on CCR3-transfected cells described elsewhere.[19] MDC reportedly binds to CCR4, a receptor found on basophils, lymphocytes, and monocytes, but it does not bind to CCR1, CCR2, CCR3, CCR5, CCR6, or CCR7.[19] We were unable to detect CCR4 mRNA in eosinophils by RT-PCR nor were we able to detect chemotactic or calcium responses to TARC, another known CCR4 ligand.[30] Lack of CCR4 expression is also characteristic of dendritic cells and NK cells, 2 other cell types responsive to MDC.[16,19,31] Furthermore, MDC failed to desensitize the eosinophil calcium response to eotaxin-2. Taken together, these data suggest that MDC acts on eosinophils by means of an as yet unidentified receptor that does not include any of the first 7 known CCRs (ie, CCR1 to CCR7).

Dissociation of calcium flux responses from cell migration, as was observed with MDC and eosinophils, was an unexpected observation. Because chemokines

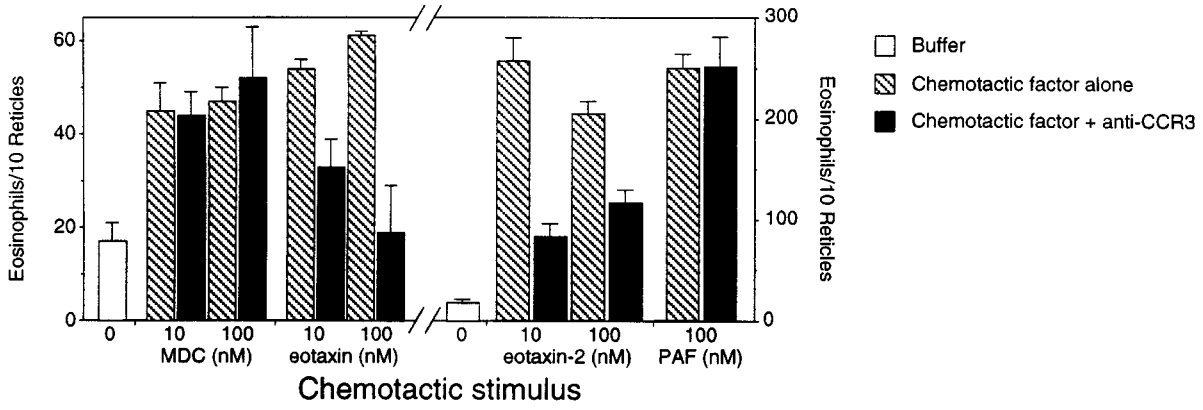

FIG 4. Effect of a blocking mAb to CCR3 on eosinophil chemotaxis in response to various CC chemokines or PAF. Values represent means ± SEM of triplicate determinations from a single experiment representative of 2 separate experiments with similar results. Note differences in the magnitude of the chemotaxis responses listed on the 2 vertical axes.

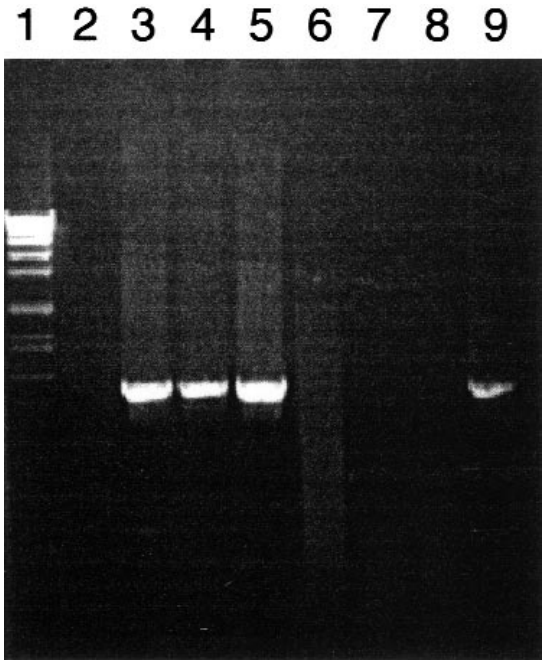

FIG 5. PCR analysis of eosinophil mRNA for CCR3 and CCR4. CCR3 was readily amplified from eosinophil cDNA generated with oligo-dT or random primers. CCR4 could not be detected in either the oligo-dT or randomly primed eosinophil cDNA, although the same primers amplified CCR4 from genomic DNA. *Lane 1,* Molecular weight markers; *lanes 2* to *5,* CCR3 analysis; *lanes 6* to *9,* CCR4 analysis; *lanes 2* and *6,* negative controls; *lanes 3* and *7,* oligo-dT cDNA; *lanes 4* and *8,* random cDNA; *lanes 5* and *9,* genomic DNA.

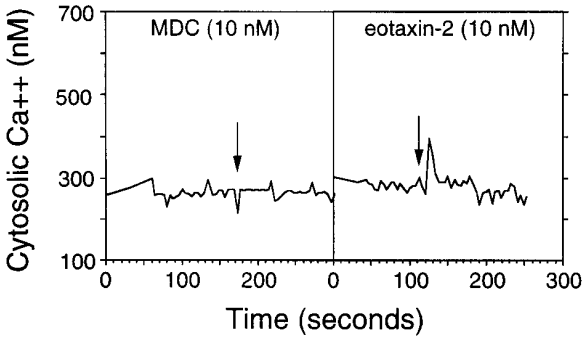

FIG 6. Purified eosinophils were cultured for 72 hours in 10 ng/mL IL-5 and labeled with fura-2AM. Cells were stimulated with MDC or eotaxin-2 *(arrows).* Eosinophils demonstrated no detectable rise in intracellular free calcium after stimulation with MDC, whereas eotaxin-2 induced a rapid, transient increase. Values represent averaged signals for greater than 50 cells per field, and results are representative of at least 3 experiments with each stimulus.

typically act by means of G protein-coupled 7-transmembrane receptors, it is common to use changes in intracellular calcium to screen for cellular effects, as well as to monitor receptor specificity and desensitization.[28,32] Indeed, MDC has been shown to induce calcium mobilization in CCR4-transfected cells, as well as activated T cells.[17,19] However, the lack of intracellular calcium alterations seen with MDC and eosinophils suggests that this approach may miss some chemokine-mediated responses. Precedence for this exists in T cells, in which chemotactic responses can be observed without detectable calcium mobilization.[28] The reverse has also been reported, namely that some chemokines can elicit calcium mobilization in neutrophils but fail to induce their chemotaxis.[26,33] These data are also consistent with the observed lack of MDC effect on eosinophils by any of the 7 CCRs (CCR1 to CCR7) because, in other cells, effects by means of these receptors are associated with transient elevations of intracellular calcium. Although many other "orphan" chemokine receptors have been discovered as the result of mass cDNA screening and other

approaches,[7,28] the identity of the additional MDC receptor or receptors remains to be determined.

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