

Chemokine-induced Eosinophil Recruitment

Evidence of a Role for Endogenous Eotaxin In An In Vivo Allergy Model in Mouse Skin

Mauro M. Teixeira,* Timothy N.C. Wells,† Nicholas W. Lukacs,§ Amanda E.I. Proudfoot,‡ Steven L. Kunkel,§ Timothy J. Williams,* and Paul G. Hellewell*

*Applied Pharmacology, Imperial College School of Medicine at the National Heart and Lung Institute, London, SW3 6LY, United Kingdom; †Geneva Biomedical Research Institute, Geneva, Switzerland; and ‡Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Abstract

Selective eosinophil recruitment into tissues is a characteristic feature of allergic diseases. Chemokines are effective leukocyte chemoattractants and may play an important role in mediating eosinophil recruitment in various allergic conditions in man. Here, we describe a novel mouse model of eosinophil recruitment in which we have compared the in vivo chemoattractant activity of different C-C chemokines. Furthermore, we describe the use of antibodies to chemokines and receptor blockade to address the endogenous mechanisms involved in eosinophil recruitment in a late-phase allergic reaction in mouse skin. Intradermal injection of mEotaxin and mMIP-1 α , but not mMCP-1, mRANTES, mMCP-5, or mMIP-1 β , induced significant ¹¹¹In-eosinophil recruitment in mouse skin. Significant ¹¹¹In-eosinophil recruitment was also observed in an active cutaneous anaphylactic reaction. Pretreatment of skin sites with antieotaxin antiserum, but not an antiMIP-1 α antibody, suppressed ¹¹¹In-eosinophil recruitment in this delayed-onset allergic reaction. Similarly, desensitization of the eosinophil eotaxin receptor CCR3 with mEotaxin, or blockade of the receptor with metRANTES, significantly inhibited ¹¹¹In-eosinophil recruitment in the allergic reaction. These results demonstrate an important role for endogenous eotaxin in mediating the ¹¹¹In-eosinophil recruitment in allergic inflammation, and suggest that blockade of the CCR3 receptor is a valid strategy to inhibit eosinophil migration in vivo. (*J. Clin. Invest.* 1997. 100:1657–1666.) Key words: chemokines • chemokine receptors • eosinophils • allergy • late-phase response

Introduction

Tissue eosinophilia in the absence of a concomitant increase in the number of neutrophils is a characteristic feature of allergic and parasitic diseases (1, 2). This preferential accumulation of

eosinophils in tissue suggests that there are specific pathways used by eosinophils for their accumulation in vivo. Understanding these mechanisms would aid in developing pharmacological therapies that would block eosinophil recruitment, but not that of other leukocytes (3). Such therapies may be of benefit in allergic diseases where eosinophil recruitment inhibition is desirable, and may have considerable advantage over existing treatments (e.g., steroids) that inhibit leukocyte recruitment indiscriminately (3) and have other deleterious actions.

Recently, it has become clear that a family of chemoattractants, the chemokines, may play an important role in activation and subsequent recruitment of leukocytes in vivo (4–6). Chemokines are proteins usually ranging from 8 to 10 kD, having amino acid sequence identity of between 20 and 90% (7). These proteins generally have four conserved cysteine residues, and, depending on the presence of one amino acid between the first two cysteines, are classified as C-C (no amino acid) or C-X-C (one intervening amino acid) chemokines (8). A member (lymphotactin) of a third subfamily possessing just two cysteine residues (8), C chemokines, has also been identified, and more recently, a novel chemokine family C-X₃-C has been described (9). The main function of chemokines appears to be activation and recruitment of particular leukocyte subsets, although a number of different roles have been ascribed to these proteins (8, 10).

Chemokine action on leukocytes is mediated by a family of G protein-coupled, seven-transmembrane receptors. There are five known receptors that mediate the actions of C-C chemokines, and these receptors are differentially expressed on different leukocyte subsets (7, 10, 11). Human eosinophils have been shown to express high levels of the CCR3 receptor (40,000–400,000 receptors per cell) and this receptor appears to mediate most of the actions of C-C chemokines on eosinophils (12, 13). Eosinophils also express the CCR1 receptor, but only at 1–5% of the levels of CCR3 (13). In agreement with their ability to bind and activate these two receptors, regulated upon activation in normal T cells expressed and secreted (RANTES),¹ macrophage inflammatory protein (MIP)-1 α , monocyte chemoattractant protein (MCP)-3, MCP-4, and eotaxin have been shown to activate eosinophils in vitro (14–17). In contrast to the wealth of data demonstrating the effects of chemokines on eosinophil function in vitro, however, there has been relatively little attention paid to testing comparatively the efficacy and potency of chemokines as eosinophil

Address correspondence to Mauro M. Teixeira, Ph.D., Applied Pharmacology, Imperial College School of Medicine at the National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, United Kingdom. Phone: 44-171-352-8121 ext. 3108; FAX: 44-171-351-8270; E-mail: mauro.teixeira@ic.ac.uk

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1. *Abbreviations used in this paper:* ACA, active cutaneous anaphylactic reaction; LTB₄, leukotriene B₄; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; OVA, ovalbumin; PAF, platelet activating factor; RANTES, regulated upon activation in normal T cells expressed and secreted.

chemoattractants in vivo. In addition, only a few studies have demonstrated a role for endogenous chemokines in mediating eosinophil recruitment in response to antigen challenge in vivo (18–21). This fact is particularly important since there is clinical evidence to suggest an important role for chemokines in mediating eosinophil recruitment in various allergic conditions in man (for review see references 4 and 6). In this study, we describe a novel mouse model of eosinophil recruitment in which we have compared the in vivo chemoattractant activity of different C-C chemokines. Furthermore, we describe the use of antibodies to chemokines and receptor blockade in addressing the endogenous mechanisms involved in eosinophil recruitment in a late-phase allergic reaction in mouse skin.

Methods

Animals. Female CBA/Ca mice (18–20 g) were purchased from Harlan (Bicester, United Kingdom). CBA/Ca mice overexpressing the murine IL-5 gene (Tg1 mice [22]) were obtained from GlaxoWellcome (Stevenage, United Kingdom) and were bred in-house.

Reagents. The following compounds were purchased from Sigma Chemical Company (Poole, United Kingdom): ovalbumin (OVA), BSA, and 2-mercaptopyridine-*N*-oxine. Dulbecco's PBS (calcium- and magnesium-free, pH 7.4), and HBSS were from Life Technologies Ltd. (Paisley, United Kingdom). Percoll and dextran (T500) were from Pharmacia (Milton Keynes, United Kingdom). C16 platelet-activating factor (PAF) was from Bachem U.K. (Saffron Walden, United Kingdom), and leukotriene B₄ (LTB₄) was from Cascade (Reading, United Kingdom). Human recombinant C5a (C5a) was a gift from Dr. J. van Oostrum, Ciba Geigy (Summit, NJ). ¹²⁵I-HSA and ¹¹¹InCl₃ were obtained from Amersham International (Little Chalfont, United Kingdom). mEotaxin, hEotaxin, hMIP-1α, hRANTES, hMCP-3, hMCP-4, mMCP-5, and affinity-purified rabbit IgG were purchased from PeproTech Inc. (London, United Kingdom). mMIP-1α, mMCP-1, mKC, mMIP-2, mMIP-1β, anti-CD2, anti-B220, and affinity-purified anti-MIP-1α polyclonal antibody were purchased from R & D Systems (Abingdon, United Kingdom). mRANTES was a kind gift of Dr. I. Clark-Lewis, University of British Columbia, Vancouver, Canada. metRANTES was synthesized by GlaxoWellcome.

Anti-eotaxin antiserum. Rabbit anti-mEotaxin antibodies were prepared by multiple-site immunization of New Zealand White rabbits with recombinant mEotaxin in complete Freund's adjuvant. Rabbits were boosted with mEotaxin in incomplete Freund's adjuvant at 2-wk intervals for 1 mo after the original immunization, and were boosted when titers began to fall. Polyclonal antibodies were titered by direct ELISA, and were specifically verified by their failure to cross-react with mIL-3, mIL-1α/β, mTNF-α, mMIP-1α, IL-6, mJE, mMIP-1β, mC10, hMCP-1, hIL-8, hRANTES, hMIP-1α, hTNF-α, hEotaxin, and hMIP-1β. The ability of these antibodies to neutralize eosinophil chemotaxis was verified using in vitro chemotactic assays at a dilution of 1:1,000. This dilution was able to inhibit the eosinophil chemotactic response to 30 ng/ml of eotaxin by ~80% (data not shown).

Purification and radiolabeling of mouse eosinophils. Eosinophils were purified from the blood of CBA/Ca mice overexpressing the IL-5 gene. In our transgenic mouse colony, eosinophils accounted for ~60% of circulating blood leukocytes (data not shown). Blood was obtained by cardiac puncture (three to four donor mice per experiment), and red blood cells were sedimented using Dextran (T500, one part blood to four parts Dextran 1.25%). The leukocyte-rich supernatant was removed, centrifuged (300 g, 7 min), and layered onto a discontinuous four-layer Percoll gradient (densities: 1.070, 1.075, 1.080, and 1.085 g/ml). The gradients were centrifuged at 1,500 g for 25 min at 20°C, and eosinophils and lymphocytes were collected from the 1.080/1.085 interface. Lymphocytes were removed by using negative immunoselection with rat anti-mouse CD2 and B220 mAbs on a MACS BS col-

umn according to guidelines set by the manufacturers (Miltenyi Biotec Inc., Camberley, United Kingdom). In brief, the eosinophil and lymphocyte pellet was resuspended in PBS/BSA (10⁷ cells in 500 μl), and was incubated with 10 μg/ml of anti-CD2 and 7.5 μg/ml of anti-B220 for 20 min on ice. The cells were washed and resuspended in PBS/BSA (80 μl of PBS/BSA per 10⁷ cells). 20 μl of goat anti-rat IgG microbeads (Miltenyi Biotec Inc.) per 10⁷ cells were added, and the cells were incubated for a further 20 min at 6–8°C. The cell suspension was run through an immunomagnetic selection column, and the eosinophils were collected with the column effluent. The eosinophils purified this way were >95% pure and >98% viable. Flow cytometric analysis of purified eosinophils showed these cells to express similar amounts of CD11/CD18, very late activation antigen 4 (VLA-4), and L-selectin as granulocytes (~97% eosinophils) in whole blood of IL-5 transgenic mice (data not shown).

For the in vivo experiments, eosinophils were radiolabeled as previously described for guinea pig cells (23, 24). In brief, purified mouse eosinophils were incubated with ¹¹¹In (~100 μCi in 10 μl) chelated to 2-mercaptopyridine-*N*-oxine (40 μg in 0.1 ml of 50 mM PBS, pH 7.4) for 15 min at room temperature. Cells were then washed twice in PBS/BSA, and were finally resuspended at a final concentration of 10⁷ ¹¹¹In-eosinophils/ml. Eosinophils taken through the labeling procedure without addition of ¹¹¹InCl₃ exhibited no detectable changes in cell adhesion molecule expression (data not shown).

For the in vitro experiments measuring intracellular calcium, eosinophils were loaded with Fura-2 as previously described for guinea pig cells (25). Purified eosinophils (5 × 10⁶ cells/ml in PBS with 0.25% BSA) were loaded with fura-2-acetoxymethyl ester (1.0 μM, 30 min at 37°C). After two washes, eosinophils were resuspended at 10⁶ cells/ml in PBS buffer containing 10 mM Hepes, 0.25% BSA, and 1 mM calcium, and were stored on ice.

Immunization procedure. Animals were immunized with ovalbumin (OVA) adsorbed to aluminium hydroxide gel as previously described (26). In brief, mice were injected subcutaneously on days 1 and 8 with 0.2 ml of a solution containing 100 μg of OVA and 70 μg of aluminium hydroxide (Reheiss, Dublin, Ireland). 7–8 d after the last immunization, the animals were anaesthetized and shaved, and antigen (OVA 0.1 and 1.0 μg per site) was injected intradermally. The allergic reaction in mouse skin will be referred to as an active cutaneous anaphylactic (ACA) reaction.

Evaluation of eosinophil recruitment in mouse skin. 10 min after intravenous injection of ¹¹¹In-eosinophils (10⁶ ¹¹¹In-eosinophils/mouse), each animal received up to six intradermal injections (50 μl vol) of recombinant chemokines (1–30 pmol/site), PAF (1.5–500 pmol/site), LTB₄ (1.5–500 pmol/site), or hC5a (1.5–50 pmol/site). Recruitment of ¹¹¹In-eosinophils was allowed to occur over a period of 4 h, after which the animals were killed, and the number of ¹¹¹In-eosinophils per skin site was quantified after counting on a gamma counter (Canberra Packard, Berks, United Kingdom). In some experiments, ¹²⁵I-human serum albumin (¹²⁵I-HSA, ~5 μCi) was added to the ¹¹¹In-eosinophils before the cell suspension was injected intravenously. In these experiments, skin sites were counted in the gamma counter, and the counts for each isotope were cross-channel corrected for spillover. Extravasation of ¹²⁵I-HSA was expressed as μl of plasma, and was calculated by dividing the number of counts in each skin site by the number of counts in 1 μl of plasma.

For the experiments assessing ¹¹¹In-eosinophil recruitment in ACA reactions, animals were injected intradermally with antigen 4 h before the intravenous injection of radiolabeled cells, and ¹¹¹In-eosinophil recruitment was measured over a period of 4 h. Thus, ¹¹¹In-eosinophil recruitment in the ACA reaction was measured from 4 to 8 h after intradermal injection of antigen. At the end of the 4-h measurement period, blood was obtained by cardiac puncture, and the number of circulating ¹¹¹In-eosinophils was calculated.

Time course experiments were carried out to evaluate the optimal measurement periods for ¹¹¹In-eosinophil recruitment induced by eotaxin and LTB₄. Animals were given an intradermal injection of the chemoattractant 4 h, 3 h, 2 h, 1 h, and just before intravenous injection.

tion of radiolabeled cells. ^{111}In -eosinophil recruitment was assessed over a period of 1 h. Thus, the following measurement periods were considered in the time course: 0–1 h, 1–2 h, 2–3 h, 3–4 h, and 4–5 h.

Treatment with anti-MIP-1 α polyclonal antibody, antieotaxin antiserum, and metRANTES. To test the efficacy of anti-MIP-1 α antibody and antieotaxin antiserum, these agents were mixed with chemokines before intradermal injection of the mixture in mouse skin. To block the activity of endogenously generated chemokines, both the anti-MIP-1 α polyclonal antibody and antieotaxin antiserum were given intradermally into sites of 4-h-old ACA reactions just before intravenous injection of radiolabeled eosinophils. Anti-MIP-1 α was used at a dose of 50 μg per site when used with MIP-1 α , and at 100 μg per site when used in sites of ACA reactions. Affinity-purified rabbit IgG was used as control. Antieotaxin antiserum was used as a 5 and 20% dilution in PBS when used with eotaxin, and as a 20% dilution when used in sites of ACA reactions. Nonimmune rabbit serum was used as control. MetRANTES (5 $\mu\text{g}/\text{mouse}$) or saline (100 μl) were given subcutaneously at a remote site 30 min before injection of ^{111}In -eosinophils. metRANTES had no significant effect on the levels of circulating ^{111}In -eosinophils measured at 2 or 4 h after their intravenous injection (data not shown). This dose of metRANTES was chosen based on its ability to induce maximal inhibition of the recruitment of eosinophils in the lungs of allergen challenged mice (T.N.C. Wells, unpublished observations). In some experiments, ^{111}In -eosinophils were pretreated with 10^{-8} M mEotaxin at 37°C before their intravenous administration.

Measurement of changes in intracellular calcium. 10 min before their use, Fura-2-loaded eosinophils were warmed to 37°C, and 300- μl aliquots were dispensed into quartz cuvettes. Changes in fluorescence after activation with mEotaxin (10^{-10} – 10^{-8} M), mMIP-1 α (10^{-10} – 10^{-8} M), or LTB $_4$ (10^{-7} M) were monitored at 37°C using a fluorimeter (LS50; Perkin-Elmer Corp., Beaconsfield, Bucks, United Kingdom) at excitation wavelengths 340 and 380 nm, and emission wavelength 510 nm. MetRANTES was used at a concentration of 10^{-6} M. For cross-desensitization experiments, the trace was allowed to return to baseline levels before addition of a further stimulus. $[\text{Ca}^{2+}]_i$ levels were calculated using the ratio of the two fluorescence readings and a K_d for Ca^{2+} binding at 37°C of 224 nM (27).

Statistical analysis. All results are presented as the mean \pm SEM. Normalized data were analyzed by one-way ANOVA, and differences between groups was assessed using the Student-Newman-Keuls post-test. A P value < 0.05 was considered significant. Percent inhibition of ^{111}In -eosinophil recruitment in skin was calculated by subtracting background values obtained in response to intradermal PBS injection.

Results

Effects of lipid mediators and hC5a on ^{111}In -eosinophil recruitment in mouse skin. Initial experiments were designed to assess the effects of chemoattractant agents previously shown to induce the direct recruitment of eosinophils in vivo (24, 28). The intradermal injection of PAF, LTB $_4$, and hC5a induced a dose-dependent ^{111}In -eosinophil recruitment when measured over a 4-h period (Fig. 1). PAF-induced ^{111}In -eosinophil recruitment was maximal at 50 pmol/site, and there was significant cell recruitment at 5 pmol/site. LTB $_4$ was more effective than PAF, but significant ^{111}In -eosinophil recruitment was only observed at doses ≥ 15 pmol/site (Fig. 1). C5a induced significant ^{111}In -eosinophil recruitment at 50 pmol/site, but it was less effective than were the other two mediators tested at similar doses (Fig. 1). PAF, LTB $_4$, and hC5a induced significant oedema formation in mouse skin (for example: PBS, 2.4 ± 0.5 μl of plasma; PAF, 50 pmol/site, 7.4 ± 1.2 μl ; LTB $_4$, 150 pmol/site, 4.5 ± 0.2 μl ; C5a, 50 pmol/site, 4.9 ± 1.5 μl ; $n = 5$).

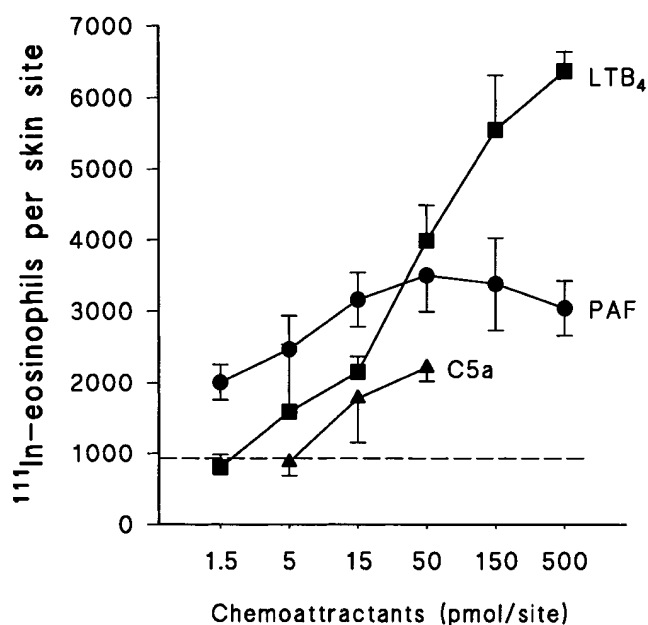


Figure 1. Recruitment of ^{111}In -eosinophils to the skin of mice injected with LTB $_4$, PAF, and hC5a. Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ^{111}In and ^{106}In -eosinophils injected intravenously into nontransgenic CBA/Ca mice. 10 min later, the animals received intradermal injections of LTB $_4$ (1.5–500 pmol/site), PAF (1.5–500 pmol/site), or hC5a (5–50 pmol/site). After 4 h, the animals were killed, and ^{111}In -eosinophils accumulating at skin sites were quantified in a gamma counter. The dashed line represents background recruitment of ^{111}In -eosinophils in sites injected with PBS. Results are expressed as the mean \pm SEM for 5–6 animals.

Time-course experiments showed that maximal recruitment of ^{111}In -eosinophils in response to LTB $_4$ occurred over the first 2 h with little ^{111}In -eosinophil over the next h (Fig. 2). PAF- and hC5a-induced ^{111}In -eosinophil was also maximal over the first 2 h (data not shown).

Comparative effects of C-C chemokines on ^{111}In -eosinophil recruitment in mouse skin. The following recombinant murine C-C chemokines were tested for their ability to induce ^{111}In -eosinophil recruitment in mouse skin: eotaxin, MIP-1 α , MIP-1 β , RANTES, MCP-5, and MCP-1/JE. Intradermal injection of mEotaxin and mMIP-1 α , but not mMIP-1 β , mRANTES, mMCP-1, or mMCP-5, resulted in significant ^{111}In -eosinophil recruitment over the 4-h measurement period (Fig. 3). mEotaxin-induced ^{111}In -eosinophil recruitment was significant at 1.0 pmol/site, and was not maximal at the highest dose tested (Fig. 3). mMIP-1 α -induced ^{111}In -eosinophil recruitment was significant at 3.0 pmol/site, and peaked around 30 pmol/site (Fig. 3). Comparable doses of mEotaxin were significantly more effective than was mMIP-1 α when compared in the same animal (Fig. 4a). None of the chemokines at the doses tested above induced any significant oedema formation as assessed by extravasation of ^{125}I -HSA (for example: PBS, 2.4 ± 0.3 μl of plasma; mEotaxin, 30 pmol/site, 2.5 ± 0.7 μl ; mMIP-1 α , 30 pmol/site, 2.8 ± 0.4 μl , $n = 4$ –5). In contrast to LTB $_4$, mEotaxin-induced ^{111}In -eosinophil recruitment was more protracted, and significant cell recruitment was observed even when measured from 4–5 h after intradermal injection (Fig. 2).

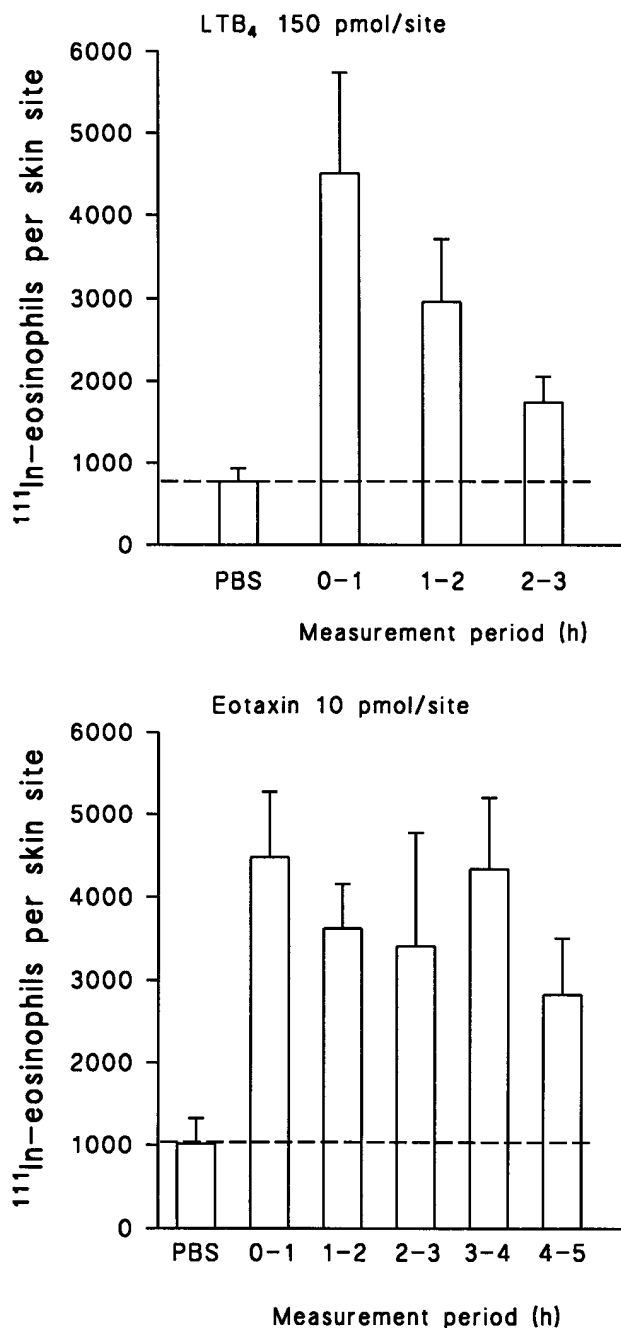


Figure 2. Time course of ¹¹¹In-eosinophil recruitment after intradermal administration of LTB₄ and mEotaxin in mouse skin. Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ¹¹¹In and ¹⁰⁶In-eosinophils injected intravenously into nontransgenic CBA/Ca mice. Animals were given an intradermal injection of LTB₄ (150 pmol/site) or mEotaxin (10 pmol/site) 4 h, 3 h, 2 h, 1 h, and just before intravenous injection of radiolabelled cells, and ¹¹¹In-eosinophil recruitment was assessed over a period of 1 h. The animals were then killed, and ¹¹¹In-eosinophils accumulating at skin sites quantified in a gamma counter. The *dashed line* represents background recruitment of ¹¹¹In-eosinophils in sites injected with PBS. Results are expressed as the mean ± SEM for four animals.

The following human recombinant chemokines were tested for their ability to induce ¹¹¹In-eosinophil recruitment in mouse skin: eotaxin, MIP-1α, MCP-3, MCP-4, and RANTES. Similar to the results described above, hEotaxin and hMIP-1α (Fig. 4 b),

but not hMCP-3, hMCP-4, or hRANTES, induced significant ¹¹¹In-eosinophil recruitment (data not shown). Fig. 4 b depicts the effects of similar doses of hEotaxin and hMIP-1α when injected intradermally into mouse skin. Similar to its murine counterparts, hEotaxin was more effective than was hMIP-1α, and significant ¹¹¹In-eosinophil recruitment was observed at doses as low as 1.0 pmol/site of hEotaxin (Fig. 4 b).

The murine recombinant C-X-C chemokines KC and MIP-2 induced no significant recruitment of ¹¹¹In-eosinophils when injected intradermally in doses of up to 30 pmol/site (data not

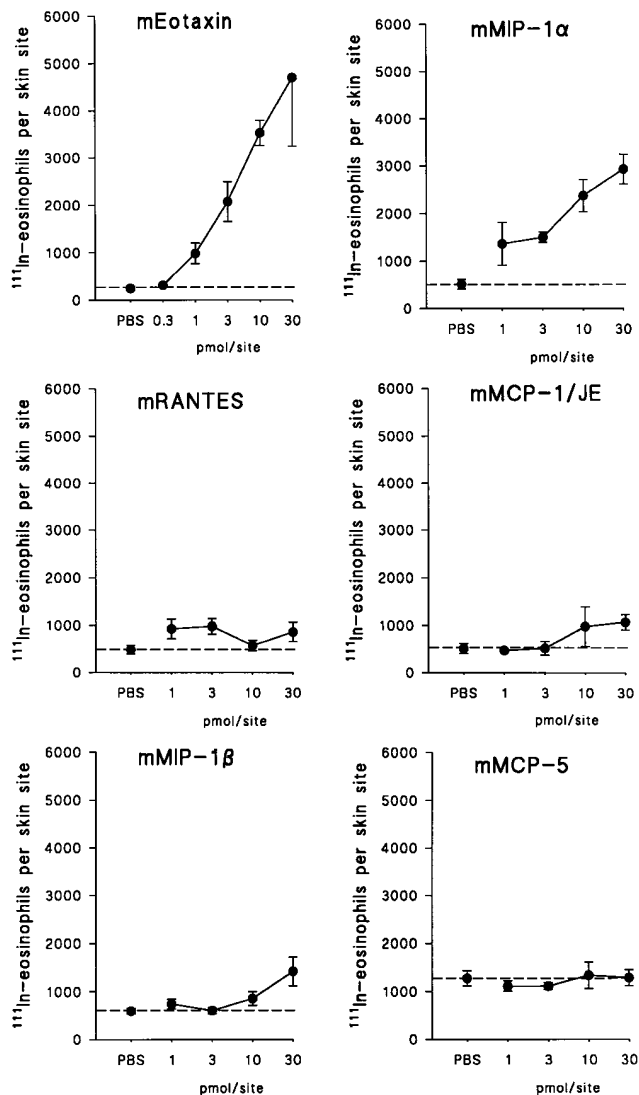


Figure 3. Comparison of eosinophil-recruiting activities of murine C-C chemokines in mouse skin. Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ¹¹¹In and ¹⁰⁶In-eosinophils injected intravenously into nontransgenic CBA/Ca mice. 10 min later, the animals received intradermal injections of mEotaxin (0.3–30 pmol/site), mMIP-1α (1–30 pmol/site), mMIP-1β (1–30 pmol/site), mMCP-1/JE (1–30 pmol/site), mRANTES (1–30 pmol/site), and mMCP-5 (1–30 pmol/site). After 4 h, the animals were killed, and ¹¹¹In-eosinophils accumulating at skin sites were quantified in a gamma counter. The *dashed lines* represent background recruitment of ¹¹¹In-eosinophils in sites injected with PBS. Results are expressed as the mean ± SEM for 4–6 animals.

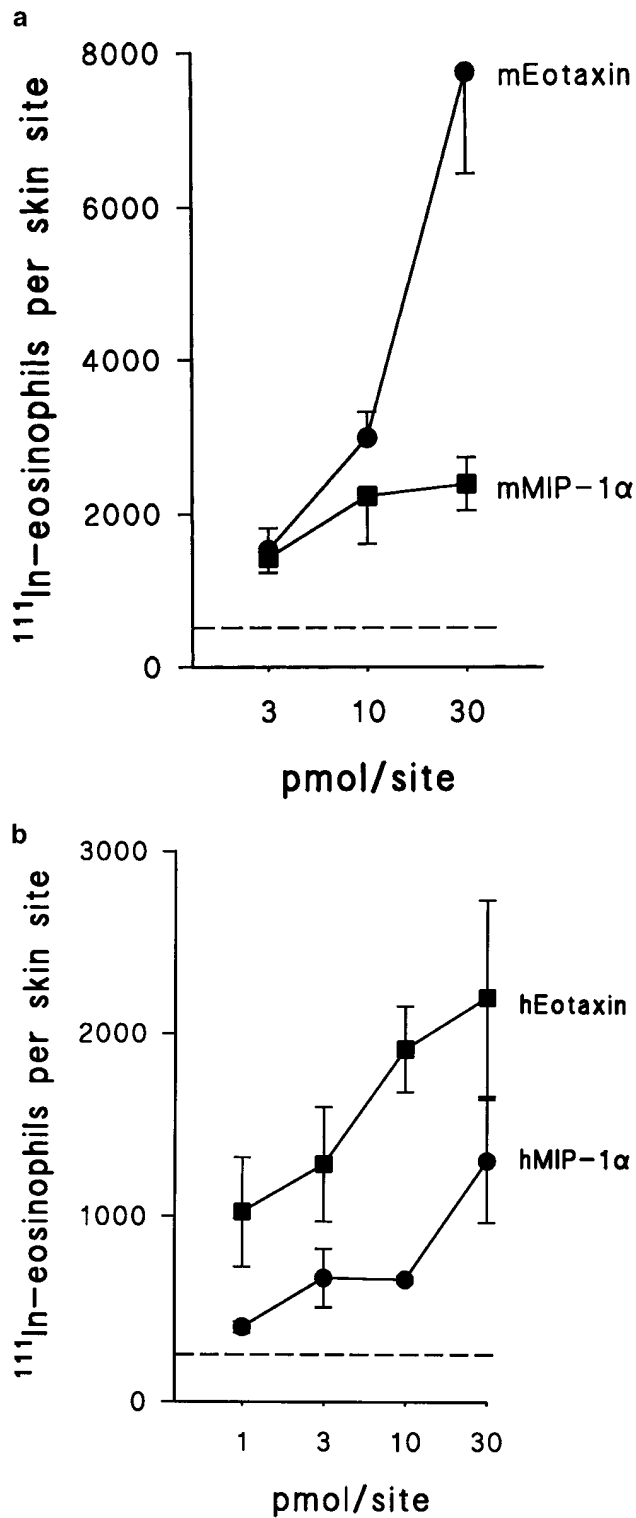


Figure 4. Eosinophil recruitment in mouse skin induced by injection of murine or human eotaxin and MIP-1 α . Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ^{111}In and 10^6 ^{111}In -eosinophils injected intravenously into nontransgenic CBA/Ca mice. 10 min later, (a) mEotaxin (3–30 pmol/site) and mMIP-1 α (3–30 pmol/site) or (b) hEotaxin (1–30 pmol/site) and hMIP-1 α (1–30 pmol/site) were injected intradermally in the same animals. After 4 h, the animals were killed, and ^{111}In -eosinophils accumulating at skin sites were quantified in a gamma counter. The dashed line represents background recruitment of ^{111}In -eosinophils in sites injected with PBS. Results are expressed as the mean \pm SEM for 4–6 animals.

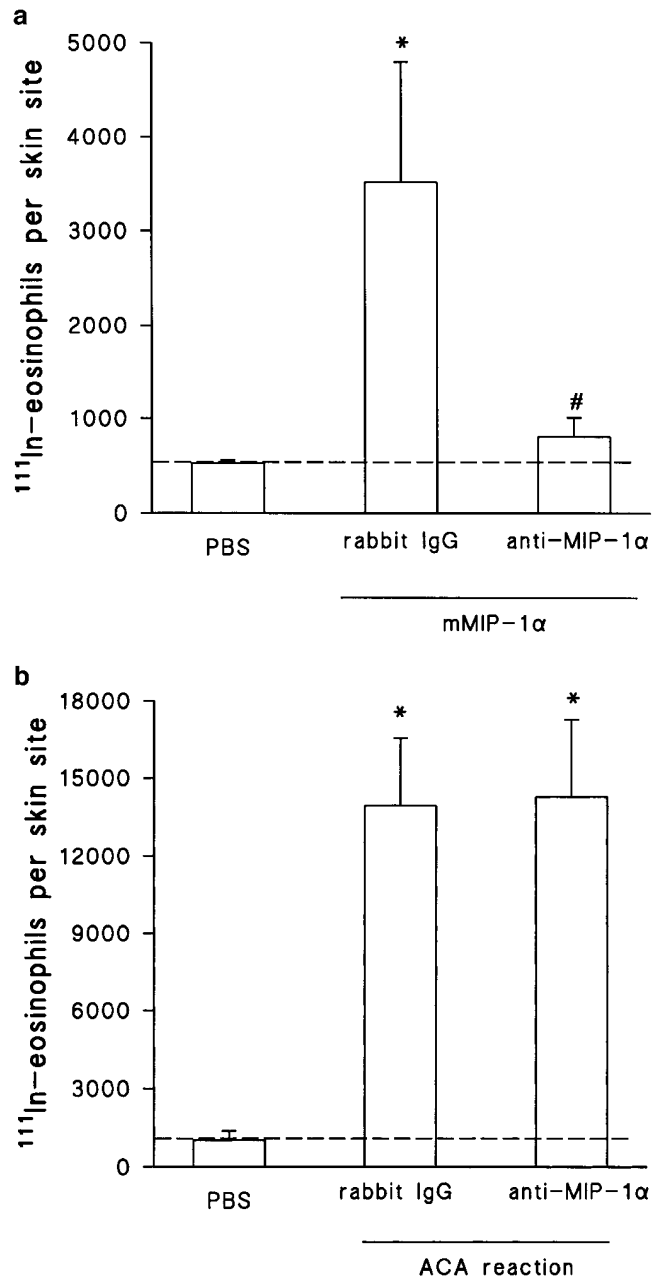


Figure 5. Effects of anti-MIP-1 α polyclonal antibody on ^{111}In -eosinophil recruitment induced by (a) mMIP-1 α and (b) in a delayed-onset allergic reaction in mouse skin. Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ^{111}In and 10^6 ^{111}In -eosinophils injected intravenously into nontransgenic CBA/Ca mice. OVA (1 $\mu\text{g}/\text{site}$) was administered intradermally 4 h before, and mMIP-1 α (10 pmol/site) 10 min after, intravenous injection of ^{111}In -eosinophils. Rabbit anti-mMIP-1 α polyclonal antibody or purified rabbit IgG were coinjected with mMIP-1 α (50 $\mu\text{g}/\text{site}$), or injected intradermally into sites of 4–8 h ACA reactions (100 $\mu\text{g}/\text{site}$) just before the intravenous injection of cells. All experiments were performed in sensitized animals. After 4 h, the animals were killed, and ^{111}In -eosinophils accumulating at skin sites were quantified in a gamma counter. The dashed line represents background recruitment of ^{111}In -eosinophils in sites injected with PBS. Results are expressed as the mean \pm SEM for five animals. * $P < 0.05$ when compared with PBS, and # $P < 0.05$ when compared with sites treated with rabbit IgG.

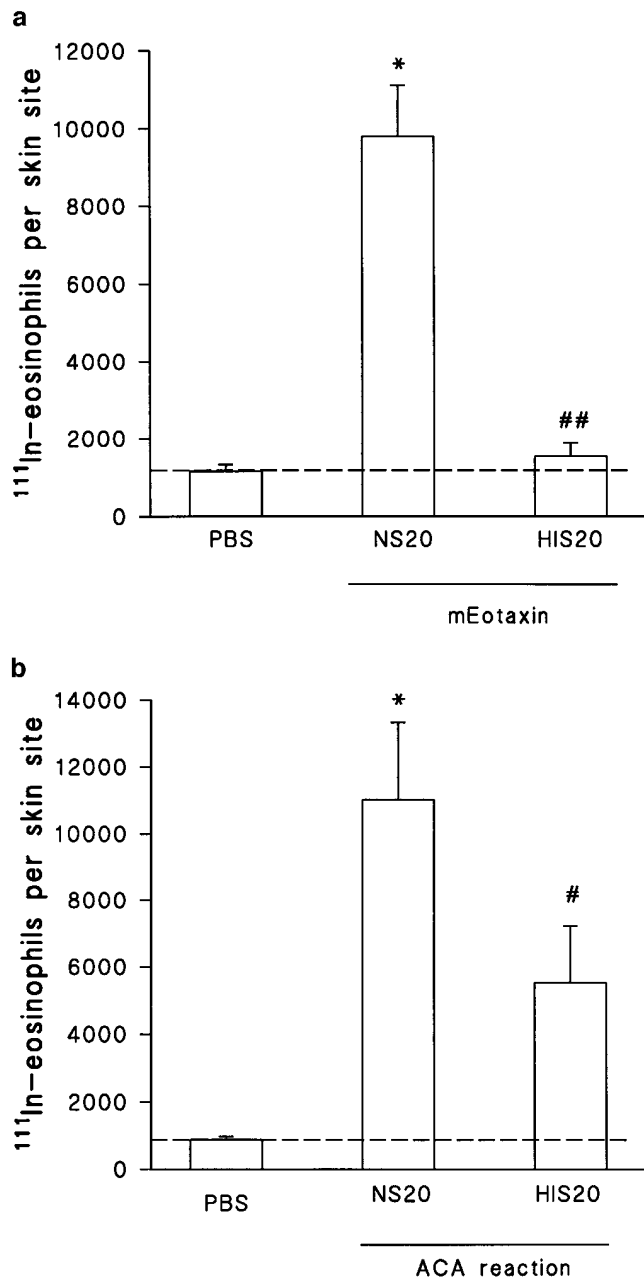


Figure 6. Suppression of ¹¹¹In-eosinophil recruitment induced by (a) mEotaxin and (b) in a delayed-onset allergic reaction in mouse skin by an antieotaxin antiserum. Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ¹¹¹In and 10⁶ ¹¹¹In-eosinophils injected intravenously into nontransgenic CBA/Ca mice. OVA (1 µg/site) was administered intradermally 4 h before, and mEotaxin (10 pmol/site) 10 min after intravenous injection of ¹¹¹In-eosinophils. Rabbit antieotaxin antiserum (HIS, 20% dilution in PBS) or rabbit nonimmune serum (NS, 20% dilution in PBS) was coinjected with mEotaxin, or injected intradermally into sites of 4–8 h ACA reactions just before intravenous injection of cells. After 4 h, the animals were killed, and ¹¹¹In-eosinophils accumulating at skin sites were quantified in a gamma counter. The dashed line represents background recruitment of ¹¹¹In-eosinophils in sites injected with PBS. Results are expressed as the mean ± SEM for five animals. **P* < 0.05 when compared with PBS. # and ## denote *P* < 0.05 and *P* < 0.01, respectively, when compared with sites treated with rabbit serum (NS).

shown). In contrast, histological analysis of skin sites injected with both C-X-C chemokines revealed a marked neutrophil infiltrate without any infiltrating eosinophils (data not shown).

Effects of anti-mMIP-1α polyclonal antibody and antieotaxin antiserum on ¹¹¹In-eosinophil recruitment in a delayed-onset allergic reactions in mouse skin. To examine the role of MIP-1α and eotaxin in allergic inflammation in mouse skin, we evaluated the effects of antibodies that bind and neutralize these chemokines. Ovalbumin-sensitized mice were challenged with OVA and ¹¹¹In-eosinophil recruitment assessed from 4–8 h after antigen challenge, a period at which maximal ¹¹¹In-eosinophil recruitment occurs (M.M. Teixeira and P.G. Hellewell, unpublished observations). At a dose of 50 µg/site, the rabbit anti-mMIP-1α polyclonal antibody reduced ¹¹¹In-eosinophil recruitment induced by mMIP-1α to basal levels (Fig. 5 a). In contrast, the anti-mMIP-1α antibody used at 100 µg/site failed to modify ¹¹¹In-eosinophil recruitment in the 4–8 h ACA reaction (Fig. 5 b).

We then examined the effects of a rabbit anti-mEotaxin antiserum on ¹¹¹In-eosinophil recruitment induced by mEotaxin and in the 4–8 h ACA reaction. Dilutions of 5 and 20% of the antiserum in PBS blocked ¹¹¹In-eosinophil recruitment induced by mEotaxin by 45% and 94%, respectively (Fig. 6 a).

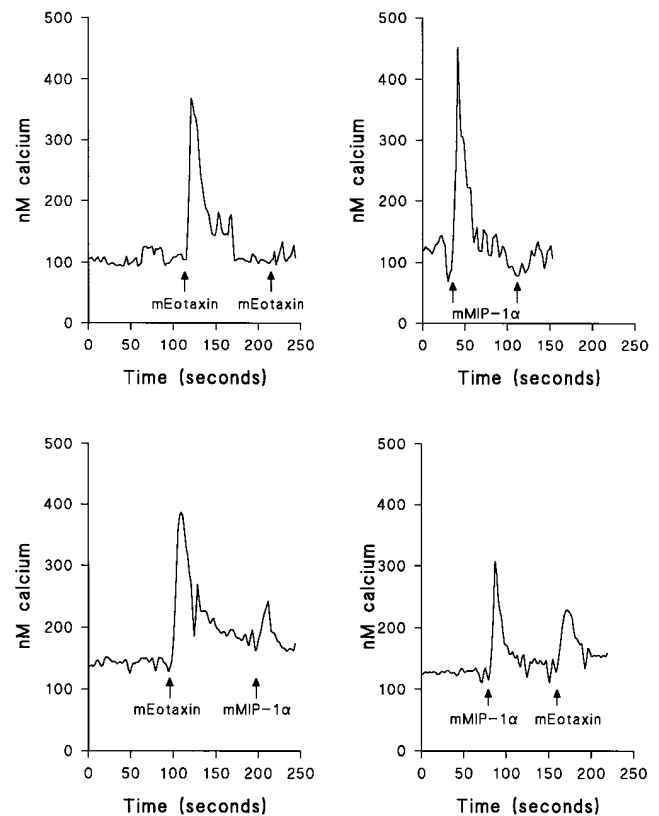


Figure 7. Changes in the intracellular calcium levels in murine eosinophils in response to mEotaxin and mMIP-1α. Eosinophils were purified from the blood of IL-5-transgenic mice and labelled with Fura-2. Changes in fluorescence after activation with eotaxin (10⁻⁸ M) and MIP-1α (10⁻⁸ M) were monitored at 37°C using a fluorimeter. The arrows indicate the time of addition of the stimulus. Results are representative of at least four experiments using cells from different donors.

The 20% antiserum dilution was then chosen to be tested against the ACA reaction. As shown in Fig. 6 *b*, intradermal injection of anti-eotaxin antiserum into sites of ACA reaction just before intravenous injection of ^{111}In -eosinophils, inhibited the recruitment of these cells by 55%. Together, these results suggest an important role for eotaxin, but not for MIP-1 α , in mediating eosinophil migration into sites of delayed-onset allergic inflammation in mouse skin.

Effects of mEotaxin and mMIP-1 α on intracellular calcium levels in eosinophils. Activation of eosinophils with mEotaxin induced a significant elevation in the intracellular calcium levels in eosinophils (Fig. 7). mEotaxin-induced calcium elevation was detected at concentrations $> 10^{-10}$ M eotaxin, and was maximal at 10^{-8} M (data not shown). Similarly, mMIP-1 α -induced intracellular calcium elevation in eosinophils (Fig. 7) was detected at concentrations greater than 2×10^{-9} M, and was maximal at 2×10^{-8} M (data not shown). As shown in Fig. 7, mEotaxin completely desensitized responses to further stimulation with mEotaxin, and significantly inhibited by 73% ($n = 5$) responses to a further stimulation with mMIP-1 α . Similarly, mMIP-1 α desensitized responses to itself, and partially inhibited by 52% ($n = 4$) the intracellular calcium elevation induced by a second stimulation with mEotaxin (Fig. 7). Neither mEotaxin or mMIP-1 α modified responses to a subsequent stimulation with LTB_4 (data not shown; see also Fig. 8 *a*).

Effects of desensitization and blockade of the eotaxin receptor on ^{111}In -eosinophil recruitment in a delayed-onset allergic reaction in mouse skin. As shown in Fig. 7, mEotaxin desensitized eosinophils to a further stimulation by mEotaxin or mMIP-1 α . To establish whether desensitized eosinophils would be impaired in their capacity to recruit *in vivo*, aliquots of the same batch of ^{111}In -eosinophils were pretreated with buffer or 10^{-8} M mEotaxin for 10 min at 37°C , and were injected intravenously into recipient animals. Compared with buffer pretreatment, pretreatment with mEotaxin significantly attenuated ^{111}In -eosinophil recruitment into skin sites induced by mEotaxin, mMIP-1 α , and in the 4–8 h ACA reaction by 51, 60, and 65%, respectively (Table I). In contrast, ^{111}In -eosinophil recruitment induced by LTB_4 was not altered (Table I). Pretreat-

ment of eosinophils with mEotaxin did not reduce significantly the number of ^{111}In -eosinophils circulating at 4 h (control, $7.2 \pm 0.9\%$ of total ^{111}In -eosinophils injected; mEotaxin-treated, $5.4 \pm 1.6\%$, $n = 4$).

Extension of hRANTES by retaining the initiating methionine produces a potent antagonist at the CCR1 receptor (29). To assess whether metRANTES would also inhibit the murine eotaxin receptor, we evaluated the effects of metRANTES on elevation of intracellular calcium induced by mEotaxin. As seen in Fig. 8 *a*, metRANTES significantly reduced mEotaxin- but not LTB_4 - induced intracellular calcium elevation in eosinophils. Thus, in addition to blocking the action of chemokines on the human CCR1 receptor (29), metRANTES also blocks the action of chemokines on the murine CCR3 receptor. Next we examined the effects of systemic treatment with metRANTES on the ^{111}In -eosinophil recruitment induced by mEotaxin and in the 4–8 h ACA reaction; metRANTES (5 $\mu\text{g}/\text{mouse}$) was administered subcutaneously 30 min before the intravenous injection of ^{111}In -eosinophils. As seen in Fig. 8 *b*, metRANTES blocked ^{111}In -eosinophil recruitment induced by eotaxin and in the ACA reaction by 45 and 68%, respectively, but had no effect on the response to LTB_4 .

Discussion

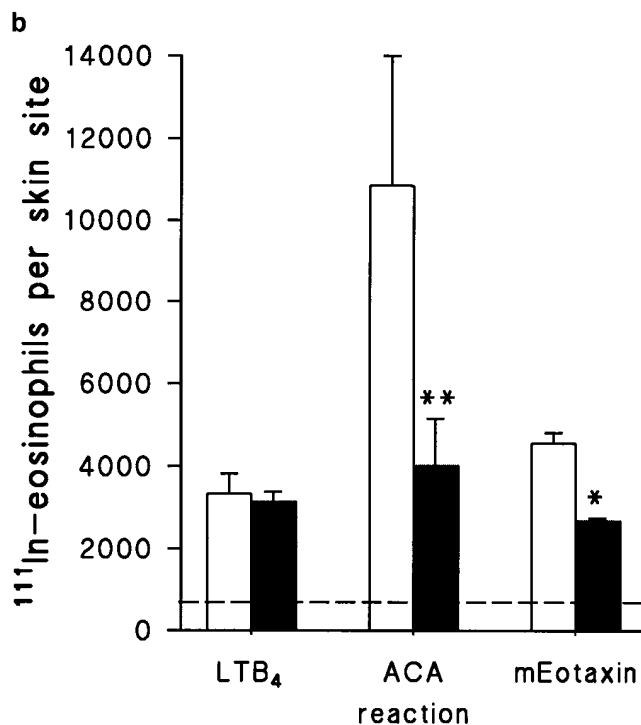
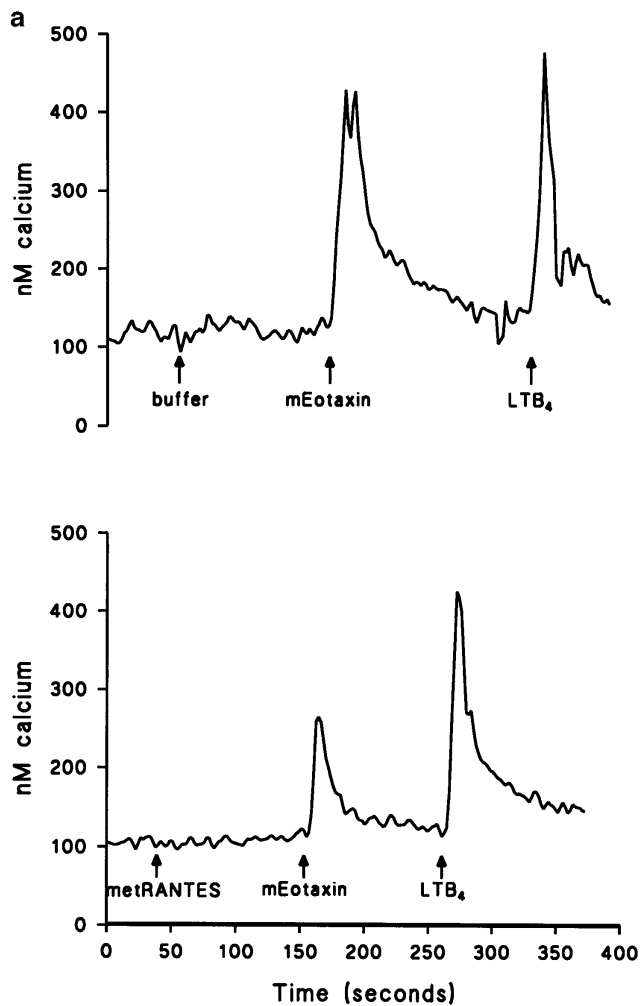
There is considerable evidence in support of an important role for eosinophils in the pathophysiology of allergic diseases such as asthma and atopic dermatitis (2, 30, 31). A detailed understanding of the molecular mechanisms that govern the eosinophil recruitment into tissues during inflammation is essential if eosinophil-specific pharmacological therapies are to be developed for treatment of allergic diseases (3). In this study, using eosinophils purified from the blood of IL-5 transgenic mice, we have evaluated and compared the *in vivo* capacity of C-C chemokines to induce eosinophil recruitment, and have assessed the role of endogenous eotaxin and of the eotaxin receptor in mediating eosinophil migration into sites of delayed-onset allergic inflammation in mouse skin.

Initial studies were carried out to investigate the chemoattractant effects of mediators previously shown to induce eosinophil recruitment when injected intradermally in the skin of guinea pigs, namely PAF, LTB_4 , and C5a (24, 28). These inflammatory mediators induced significant cell recruitment and oedema formation when injected intradermally. Although LTB_4 appeared to be more effective than the other mediators, PAF induced significant migration of cells at doses as low as 5 pmol/site. Next, we examined the effects of a range of chemokines previously shown to stimulate various eosinophils functions *in vitro* (14–17, 32). Of the chemokines tested, only eotaxin and MIP-1 α induced significant recruitment of ^{111}In -eosinophils, but eotaxin was consistently more effective than was MIP-1 α . In agreement with studies evaluating the *in vitro* activation of murine eosinophils (33, 34), both human and murine recombinant proteins induced significant recruitment of ^{111}In -eosinophils. Although RANTES appears to activate human eosinophils via the eotaxin receptor CCR3 (6, 14), neither mRANTES nor hRANTES induced significant recruitment of ^{111}In -eosinophils in mouse skin. This result is consistent with the lack of effect of hRANTES on the levels of intracellular calcium in murine and guinea pig eosinophils (16, 35, 36), but contrasts with the capacity of hRANTES to induce eosinophil recruitment in dog (37) and Rhesus monkey

Table I. Desensitization of the Eotaxin Receptor Suppresses ^{111}In -eosinophil Recruitment in a Delayed-onset Allergic Reaction in Mouse Skin

Stimulus	^{111}In -eosinophils per skin site	
	Control	mEotaxin-pretreated
PBS	772 \pm 56	662 \pm 103
LTB_4 150 pmol	3166 \pm 210	3170 \pm 294
mEotaxin 10 pmol	2881 \pm 434	1705 \pm 617*
mMIP-1 α 10 pmol	2160 \pm 350	1212 \pm 117*
ACA reaction (1 μg of OVA)	15441 \pm 6219	5823 \pm 1010*

^{111}In -eosinophils were pretreated with buffer (control) or 10^{-8} M mEotaxin for 10 min at 37°C and injected intravenously into recipient animals. OVA was administered intradermally into sensitized animals 4 h before, and direct-acting chemoattractants just after, the intravenous injection of ^{111}In -eosinophils. Their recruitment was measured after a further 4 h. Results are mean \pm SEM for four animals in each group. * $P < 0.05$.



(38) skin. Moreover, the chemokines mMIP-1 β , mMCP-1/JE, hMCP-3, and hMCP-4 failed to trigger calcium flux in eosinophils (36), and failed to induce significant ¹¹¹In-eosinophil recruitment in our in vivo model. Finally, mMCP-5 failed to induce significant recruitment of ¹¹¹In-eosinophils when injected intradermally in mouse skin. This result is consistent with the lack of ability of this chemokine to activate murine eosinophils in vitro (39).

The lipid mediators and C5a induced significant oedema formation at the doses tested, but none of the chemokines had this effect. These results are in contrast to the swelling-inducing activity of hMIP-1 α and hMCP-1 when injected into the footpad of mice (40). In the latter study, immediate swelling was accompanied by mast cell degranulation and cell recruitment, although a more direct measure of increased vascular permeability was not assessed (40). Thus, it is unclear whether chemokine-induced swelling was due to oedema formation, or just to local cell infiltration. As we failed to observe any oedema formation in response to the intradermal injection of chemokines and mast cell degranulation, our results suggest that mast cell degranulation is unlikely to explain the observed recruitment of ¹¹¹In-eosinophils. Thus, our results suggest that the chemokines eotaxin and MIP-1 α are potent and effective direct inducers of ¹¹¹In-eosinophil recruitment in mouse skin.

Like human eosinophils (12), eosinophils purified from IL-5 transgenic mice have been shown to possess two receptors that mediate the action of chemokines CCR1 and CCR3 (41). To investigate whether eotaxin and MIP-1 α could activate eosinophils purified from the blood of IL-5 transgenic mice via a distinct or the same receptor, we assessed the ability of these chemokines to induce cross-desensitization of the calcium response. Both eotaxin and MIP-1 α desensitized the intracellular calcium elevation when cells were activated by the same chemokine subsequently, and markedly desensitized responses to each other at the concentrations used. As eotaxin appears not to bind to the CCR1 receptor, it is possible that both eotaxin and MIP-1 α induce intracellular calcium elevation in eosinophils by activating the CCR3 receptor. This possibility is

Figure 8. Modulation by metRANTES of (a) mEotaxin-induced intracellular calcium elevation in eosinophils, and (b) ¹¹¹In-eosinophil recruitment induced by mEotaxin, LTB₄, and in a delayed-onset allergic reaction in mouse skin. (a) Eosinophils were purified from the blood of IL-5-transgenic mice and labeled with Fura-2. Eosinophils were pretreated with buffer or MetRANTES (10⁻⁶ M) for 2 min, and were then activated with eotaxin (10⁻⁸ M). Changes in fluorescence were monitored at 37°C using a fluorimeter. The arrows indicate the time of stimulus addition. Results are representative of two experiments using cells from different donors. (b) Eosinophils were purified from the blood of IL-5-transgenic mice, labelled with ¹¹¹In and 10⁶ ¹¹¹In-eosinophils injected intravenously into nontransgenic CBA/Ca mice. OVA (1 μ g/site) was administered intradermally 4 h before, and mEotaxin (10 pmol/site) and LTB₄ (150 pmol/site) 10 min after intravenous injection of ¹¹¹In-eosinophils. MetRANTES (5 μ g/mouse, closed bars) or saline (open bars) was given subcutaneously 30 min before intravenous injection of ¹¹¹In-eosinophils. After 4 h, the animals were killed, and ¹¹¹In-eosinophils accumulating at skin sites were quantified in a gamma-counter. The dashed lines represent background recruitment of ¹¹¹In-eosinophils in sites injected with PBS. Results are expressed as the mean \pm SEM for 4–8 animals. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, when compared with saline-treated animals.

in agreement with the ability of murine and human MIP-1 α to bind the mCCR3 receptor (41), but not the hCCR3 receptor (12, 13). An alternative explanation for the data, however, is that there is cross-desensitization between the CCR1 and CCR3 receptors. In this respect, it has recently been reported that MIP-1 α and eotaxin signaled via two distinct pathways (CCR1 and CCR3, respectively) in murine eosinophils purified from spleen of IL-5 transgenic mice (36, 42). These results suggest that eotaxin appears to activate the mCCR3 receptor, and MIP-1 α the mCCR1 receptor. Moreover, our results suggest that there is receptor cross-desensitization that might occur through distinct receptors as reported for chemoattractant receptors on human neutrophils (43). Further studies using receptor-specific tools are needed to clarify and evaluate the intracellular mechanisms underlying CCR1 and CCR3 receptor cross-desensitization in mouse eosinophils.

Because exogenous MIP-1 α and eotaxin induced effective recruitment of ¹¹¹In-eosinophils, we evaluated whether blockade of the action of the endogenous chemokines would modulate the recruitment of ¹¹¹In-eosinophils in sites of allergic inflammation in mouse skin. Intradermal administration of an anti-MIP-1 α polyclonal antibody completely inhibited recruitment of ¹¹¹In-eosinophils induced by MIP-1 α , but failed to modulate the recruitment of these cells in the 4–8-h ACA reaction. These results contrast with previous studies demonstrating an important role for MIP-1 α in mediating eosinophil recruitment in the lung of *Schistosoma mansoni* egg antigen-sensitized and -challenged animals (20). In the latter studies, however, the anti-MIP-1 α antibody was given before antigen challenge, and could thus modulate migration of mononuclear cells into the lung, or reduce their activation before eosinophil recruitment. In agreement with this hypothesis, MIP-1 α has been shown to play an important role in directing the chemotaxis of mononuclear inflammatory cells in the T-cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (44). Alternatively, there could be a differential role for MIP-1 α in mediating eosinophil recruitment in the lung (20) and skin (this study) of mice. In this respect, we have observed no effect of a polyclonal anti-MIP-1 α on eosinophil recruitment induced by intradermal injection of *Schistosoma* egg antigen in sensitized mice (M.M. Teixeira and P.G. Hellewell, unpublished observations).

In contrast to the lack of effect of the anti-MIP-1 α antibody, an antiserum raised against mEotaxin suppressed ¹¹¹In-eosinophil recruitment into sites of 4–8 h ACA reaction by 55%; the first study to demonstrate a role for endogenous eotaxin in mediating eosinophil recruitment into cutaneous sites of allergic inflammation. Moreover, the results are in agreement with previous studies assessing the role of endogenous eotaxin in mediating recruitment of eosinophils into the lung of allergen-sensitized mice (18, 21). Thus, blockade of eotaxin with a polyclonal antibody (18) or by target disruption of the eotaxin gene (21) showed inhibition of 56 and 70% of the number of eosinophils in the bronchoalveolar lavage fluid of mice. Similarly, there was a 50% inhibition of eosinophil recruitment into the eyes of eotaxin-deficient mice challenged with antigens of the parasite *Onchocerca volvulus* (21). Together, these studies provide strong evidence to suggest an important role for eotaxin in inducing eosinophil recruitment into sites of allergic inflammation in different tissues.

We have previously shown that blocking the eotaxin receptor with the human chemokine RANTES blocked eosinophil

recruitment in response to eotaxin in guinea pig skin (35). To evaluate whether intervention at the level of the eotaxin (CCR3) receptor would also modulate eosinophil recruitment into sites of allergic inflammation in mouse skin, two strategies were used; blockade of the receptor with metRANTES, and desensitization of the receptor with mEotaxin. Extension of hRANTES by the retention of the initiating methionine produces a protein that is a potent antagonist at the human CCR1 receptor (29). The demonstration that metRANTES also blocks the effects of mEotaxin on murine eosinophils both in vitro and in vivo demonstrates that metRANTES also acts on the mEotaxin receptor CCR3. These results are consistent with the capacity of hRANTES to act as antagonist of eotaxin-induced elevation in intracellular calcium in murine (data not shown) and guinea pig eosinophils (35). When administered systemically, metRANTES inhibited eosinophil recruitment into sites of allergic inflammation in mouse skin by 68%. Similarly, pretreatment of eosinophils with eotaxin at a concentration that desensitized eosinophils to further stimulation by eotaxin and MIP-1 α , inhibited eosinophil recruitment in sites of allergic inflammation by 65%. Interestingly, eosinophil recruitment induced by exogenous eotaxin was less inhibited by these two strategies than were responses in sites of ACA reactions. In contrast to the ACA reaction where mediators are likely to be released continuously over a protracted period, exogenous administration of eotaxin achieves a high local concentration (2×10^{-7} M) that declines. The latter may be more difficult to inhibit, and indeed the ability of eotaxin to desensitize itself has been reported to be dose-dependent, and not observed with high concentrations of the chemokine (36). Future studies with better CCR3 receptor antagonists should clarify the reasons underlying the lesser inhibition of exogenous eotaxin-induced eosinophil recruitment.

Recently, Heath et al. (45) reported that an antibody that recognizes the human CCR3 receptor effectively blocked the action of different eosinophil-active chemokines on human eosinophils in vitro. Taken together with our in vivo observations, these results suggest that blockade of the CCR3 receptor is a valid strategy to inhibit eosinophil migration in vivo, and that development of drugs that block the human CCR3 is a feasible strategy for treatment of allergic diseases in man.

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