C-C Chemokines in Allergen-Induced Late-Phase Cutaneous Responses in Atopic Subjects: Association of Eotaxin with Early 6-Hour Eosinophils, and of Eotaxin-2 and Monocyte Chemoattractant Protein-4 with the Later 24-Hour Tissue Eosinophilia, and Relationship to Basophils and Other C-C Chemokines (Monocyte Chemoattractant Protein-3 and RANTES)

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Sun Ying,* Douglas S. Robinson,* Qiu Meng,* Luis T. Barata,* Alan R. McEuen,† Mark G. Buckley,‡ Andrew F. Walls,‡ Philip W. Askenase,* and A. Barry Kay$^{2,*}$

The relationship of expression of the C-C chemokines eotaxin, eotaxin 2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4 to the kinetics of infiltrating eosinophils, basophils, and other inflammatory cells was examined in allergen-induced, late-phase allergic reactions in the skin of human atopic subjects. EG2\textsuperscript{+} eosinophils peaked at 6 h and correlated significantly with eotaxin mRNA and protein, whereas declining eosinophils at 24 h correlated significantly with eotaxin-2 and MCP-4 mRNA. In contrast, no significant correlations were observed between BB1\textsuperscript{+} basophil infiltrates, which peaked at 24 h, and expression of eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 or elastase\textsuperscript{+} neutrophils (6-h peak), CD3\textsuperscript{+} and CD4\textsuperscript{+} T cells (24 h), and CD68\textsuperscript{+} macrophages (72 h). Furthermore, 83\% of eosinophils, 40\% of basophils, and 1\% of CD3\textsuperscript{+} cells expressed the eotaxin receptor CCR3, while eosinopin protein was expressed by 43\% of macrophages, 81\% of endothelial cells, and 6\% of T cells (6%). These data suggest that 1) eotaxin has a role in the early 6-h recruitment of eosinophils, while eotaxin-2 and MCP-4 appear to be involved in later 24-h infiltration of these CCR3\textsuperscript{+} cells; 2) different mechanisms may guide the early vs late eosinophilia; and 3) other chemokines and receptors may be involved in basophil accumulation of allergic tissue reactions in human skin. The Journal of Immunology, 1999, 163: 3976–3984.

Injection of allergen into the dermis of sensitized atopic subjects causes within minutes a weal and flare reaction, followed within hours by an edematous, red, and slightly indurated late-phase reaction (LPR)\textsuperscript{1} (1, 2), containing infiltrates of eosinophils, basophils, neutrophils, T cells, and macrophages (1–3). Eosinophils have attracted particular attention as proinflammatory cells in allergic tissue reactions through the release of basic proteins, lipid mediators, cytokines, and chemokines (4). The precise mechanisms by which eosinophils are recruited from blood vessels into local allergen-injected sites remains to be elucidated, but is believed to involve selective cell adhesion, chemotaxis, and survival due to the local release of cytokines that delay apoptosis (5–8). Basophils are also believed important because of their high content of histamine and their capacity to produce lipid mediators, including LTC\textsubscript{4}, and various cytokines (9).

During the past 10 years a number of small molecular polypeptides, termed chemokines, have been discovered (10–12). Based on whether the first two cysteines were adjacent or separated by a single amino acid, chemokines are generally divided into C-C and C-X-C subgroups (10–12). Compared with C-X-C chemokines, the C-C chemokines, particularly eotaxin (13), eotaxin-2 (14), monocyte chemotactic protein-4 (MCP-4) (15), MCP-3 (16), and RANTES (17), have more selective functions. Thus, eotaxin and eotaxin-2 are active on eosinophils and basophils preferentially, whereas MCP-3, MCP-4, and RANTES chemoattract eosinophils, basophils, T cells, and monocytes, but not neutrophils. In vitro, these C-C chemokines attract and also activate eosinophils and basophils for enhanced oxidative metabolism and mediator release (18–22). Recently, an eotaxin receptor designated CCR3 has been identified (23–25). This G-protein coupled, seven-transmembrane domain receptor is highly expressed on human eosinophils and basophils (26), and although the C-C chemokines generally act on several receptors, eotaxin and eotaxin-2 act uniquely through CCR3 (27). Other C-C chemokines, MCP-3, RANTES, and MCP-4, also bind to CCR3, but with lower affinity (21, 22, 28); all stimulate eosinophils and basophils with varying potency (13–15, 29), but are nonspecific, as they stimulate other cell types, such as monocytes and T cells (30).

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\textsuperscript{3} Abbreviations used in this paper: LPR, late-phase reaction; BAL, bronchoalveolar lavage; EG2, cleaved form of eosinophil cationic protein; HIC, immunohistochemistry; APAAP, alkaline phosphatase anti-alkaline phosphatase; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein.
A number of studies suggest that elevated expression of these C-C chemokines may play a critical role in the recruitment of inflammatory cells into local tissue in allergic inflammation (31–38). Although widely studied in vitro and in animal models, the kinetics of expression of these chemokines in provoked human allergic tissue reactions and their relationship to infiltration of eosinophils, basophils, and other inflammatory cells has not been determined. In a previous study using the cutaneous LPR, we demonstrated that the early 6-h peak of eosinophils was associated with MCP-3, whereas maximal infiltration of T cells was at 24 h and coincided with maximal RANTES expression (35). The recent availability of a basophil-specific mAb (39) and probes for eotaxin, eotaxin-2, and MCP-4 has enabled us to extend this study to determine more precisely the relationship between infiltration of eosinophils and basophils and CC chemokine expression in allergic inflammation. Our hypothesis was that the time course of infiltration of these cells with high CCR3 expression would be similar and related to the kinetics of expression of the C-C chemokines. We also anticipated that chemokines may act in a stepwise fashion, with, for example, eotaxin acting at an earlier time point as shown in eotaxin-deficient mice (40). We also studied the cell distribution of eotaxin expression, expecting, as previously shown in animals (41) as well as in baseline bronchial biopsies from asthmatics (29), that eotaxin can be expressed by several tissue cells, including endothelial cells and macrophages, which would establish chemotactic gradients for migration of CCR3+ cells into site of allergic tissue reactions.

Materials and Methods

Human subjects

Atopic subjects (n = 10) were recruited from the Allergy Clinic of the Royal Brompton Hospital and from the staff of the National Heart and Lung Institute (London, U.K.). Inclusion criteria were as follows: 1) age between 18–55 yr, 2) history of seasonal and/or perennial allergic rhinitis and/or asthma, 3) absence of any other illness, and 4) positive skin prick tests (weal diameter >5 mm) to timothy grass pollen (Phleum pratense; Soluprick; ALK, Horsholm, Denmark) in the presence of positive histamine and negative control vehicles. Patients taking oral medication were not included in this study. The median of total serum IgE concentrations was 183 IU/ml (range, 101–1780 IU/ml), and all patients had a positive radioallergosorbent test to Phleum pratense (median, 45.0 IU/ml; range, 6.3–172.0 IU/ml). All subjects gave informed consent, and the study was approved by the ethics committee of the Royal Brompton and Harefield National Health Service Trust (London, U.K.).

Study design and processing specimens

All injections were performed with a 29-gauge needle and a 0.5-ml plastic syringe. Using this method, 30 biological units of timothy grass pollen extract (0.05 ml) was injected intradermally into individual sites on the extensor aspect of the forearms of each subject. An additional site was injected with a similar volume of diluent. Macrophage responses were measured at 6, 24, 48, and 72 h by evaluating skin induration by resistance to the movement of a sharpened pencil point with which the reaction was outlined. Permanent sticky tape records of the outlines of the responses at all time points were then made. A 4-mm disposable biopsy punch was used to take a biopsy from the center of each reaction after using 1% plain lidocaine for local anesthesia. The control site injected with diluent was biopsied at 24 h. In this way, each patient served as his/her own control. Tissue biopsies were immediately fixed in 4% paraformaldehyde (BDH, Dagenham, U.K.) in 0.1 M PBS, pH 7.4, and washed in 15% PBS-SSC to minimize cross-reaction. Negative controls employed hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, U.K.) before hybridization with the antisense probe for autoradiography. Slides were dipped into K-5 emulsion (Ilford, Basildon, U.K.) and exposed at 4°C for 2 wk in absolute darkness in a desiccated environment. The slides were developed (D-19 developing solution, Eastman Kodak, Rochester, NY), rinsed, and counterstained with Harris hematoxylin. Dense deposits of silver grains on autoradiographs were present over cells expressing chemokine mRNA. Slides were counted in duplicate, blind to the patient’s clinical status, using an eyepiece graticule as previously described (31, 34, 35). The results were expressed as the total number of positive cells per square millimeter of biopsies. The coefficient of variability of the duplicate counts obtained from all slides was <5%.

Single immunohistochemistry (IHC)

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used to enumerate cells binding to mAbs against human activated eosinophils (EG2, Pharmacia, Uppsala, Sweden), macrophages (CD68, Dako, Kyoto, Japan), high Wycombe (U.K.), mast cells (anti-trypstatin, Chemocloud, CA), total T cells, and subtypes of T cells (CD3, CD4, and CD8, Becton Dickinson, Cowley, U.K.). Other mAbs against human neutrophils (anti-neutrophil elastase), and endothelial cells (CD31) were purchased from Dakoppats. BB1, a novel mAb recognizing a human basophil granular protein, was prepared as previously described (39). This mAb did not react with lymphocytes, monocytes, platelets, neutrophils, eosinophils, mast cells, or any other cell type or tissue structure (39). Anti-human eotaxin mAb (2G6) and anti-human CCR3 mAb (7B11) were provided by Drs. C. Mackay, P. Ponath, and W. Newman (LeukoSite, Cambridge, MA) (23, 31). The APAAP technique was performed as described previously (1, 2, 31). The optimal concentrations of all Abs used were determined in pilot experiments. Briefly, the sections were incubated with the mAbs against positive markers for 30 min in 0.1 M PBS containing 1% NaN3. After washing in 50 mM NaCl, then incubated with rabbit anti-mouse Ig (Dako, Carpenteria, CA) for 30 min. After washing in 50 mM Tris-HCl and 150 mM NaCl, the sections were incubated with soluble complexes of alkaline phosphatase and mouse APAAP (Dako) for an additional 30 min, and developed with Fast Red (Sigma) as chromogen for signal visualization. For immunostaining of BB1+ basophils, the slides were pretreated with 0.1% saponin (Sigma)/PBS for 30 min, incubated with BB1 Ab (1/100) for 30 min, washed as before and APAAP (Dako) was used then processed as described above. For immunostaining of eotaxin, the sections were incubated with 20% horse serum in PBS for 20 min and incubated with anti-human eotaxin 2G6 (1/50) overnight at room temperature. After washing, the slides were treated with rabbit anti-mouse Ig (1/30, 30 min) and APAAP (1/30, 30 min), respectively, as described above.

Positive cells stained red after development with Fast Red. Omission or substitution of the primary Ab with an irrelevant Ab of the same species was used as a negative control. The sections were counted in duplicate,

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The results are expressed as the numbers of positive cells (mean ± SEM) per square millimeter of skin biopsy. The coefficient of variability of the duplicate counts obtained from all slides was <5%. No immunoreactivity was observed in sections stained with omission of the primary Ab or substitution of this Ab with an irrelevant Ab of the same species. For CCR3 immunostaining, immunomagnetic purified peripheral blood eosinophils were used as positive controls.

**Double immunohistochemistry**

To examine the phenotype of eotaxin+ cells, cryostat sections were studied by double IHC as previously described (43) with some modifications. Briefly, after blocking endogenous peroxidase in 0.3% H2O2 and 0.1% NaN3, the mAbs for specific cell markers (CD68, CD31, CD3, EG2, tryptase, BB1, and elastase) together with rabbit polyclonal Ab against human eotaxin (gift from Dr. P. J. Jose, Leukocyte Biology, Imperial College School of Medicine, London, U.K.) were used simultaneously for the detection of eotaxin+ cells. For CCR3 immunostaining, immunomagnetic purified peripheral blood eosinophils were used as positive controls.

The time course of increases in EG2+ eosinophils, elastase+ neutrophils (A), BB1+ basophils (B), T lymphocytes (CD3, CD4, and CD8; C), and CD68+ macrophages (D) into allergen-challenged skin sites in atopic subjects. Diluent-challenged sites (Dil) at 24 h were used as controls. The results are expressed as the numbers of positive cells (mean ± SEM) per square millimeter of skin biopsy. The coefficient of variability of the duplicate counts obtained from all slides was <5%. No immunoreactivity was observed in sections stained with omission of the primary Ab or substitution of this Ab with an irrelevant Ab of the same species. For CCR3 immunostaining, immunomagnetic purified peripheral blood eosinophils were used as positive controls.

**Results**

**Cutaneous LPR**

All subjects exhibited an allergen-induced, but not diluent-induced, cutaneous LPR, with mean diameters (millimeters ± SEM; n = 10) of 65.2 ± 3.7 (6 h), 74.3 ± 9.8 (24 h), and 56.5 ± 11.9 (48 h). At 72 h and 7 days (n = 3), the LPR was virtually absent.

**Infiltration of inflammatory cells**

At the diluent challenge sites, there were very few EG2+ eosinophils or elastase+ neutrophils. After allergen challenge, infiltrating EG2+ eosinophils and elastase+ neutrophils were observed throughout the dermis and were elevated significantly at all time points compared with diluent challenge (p < 0.001; Fig. 1A). Infiltration of eosinophils and neutrophils was maximal at 6 h and subsequently declined progressively. The number of eosinophils at 6 and 24 h was significantly higher than that at 48 h (p < 0.01). After allergen, but not diluent, challenge, the numbers of BB1+ basophils were also significantly increased at 6 h, peaked at 24 h, and were lower at 48 h (p < 0.001), and then gradually declined thereafter, but were still elevated at 72 h and 7 days compared with those in diluent controls (Fig. 1B). The number of basophils at 24 h was significantly higher than that at 6 or 48 h (p < 0.05). Basophils were distributed mainly in the deep dermis and around blood vessels, and this pattern did not change substantially throughout the LPR. The total numbers of basophils were about one-third that of these eosinophils (Fig. 1, A vs B). There were also significant increases in CD3+ T cells, CD4+ T cells (Fig. 1C), and CD68+ macrophages (Fig. 1D) at allergen sites at all time points compared with values with diluent (p < 0.05). The numbers of CD3+ and CD4+ cells were maximal 24 h after allergen challenge.

**Statistical analysis**

Data were analyzed using a statistical package (Minitab Release 7, Minitab, State College, PA). Variability of the parameters studied was analyzed with Friedman’s test, followed by two-by-two comparisons between time points using the Wilcoxon signed rank test. Correlation coefficients were obtained by Spearman’s method with correction for tied values. For all tests, p < 0.05 was considered significant.

**FIGURE 1.** A–D. The time course of increases in EG2+ eosinophils, elastase+ neutrophils (A), BB1+ basophils (B), T lymphocytes (CD3, CD4, and CD8; C), and CD68+ macrophages (D) into allergen-challenged skin sites in atopic subjects. Diluent-challenged sites (Dil) at 24 h were used as controls. The results are expressed as the numbers of positive cells (mean ± SEM) per square millimeter of skin biopsy. The coefficient of variability of the duplicate counts obtained from all slides was <5%. No immunoreactivity was observed in sections stained with omission of the primary Ab or substitution of this Ab with an irrelevant Ab of the same species. For CCR3 immunostaining, immunomagnetic purified peripheral blood eosinophils were used as positive controls.

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and were slightly reduced at 48 h. The numbers of CD3\(^+\) and CD4\(^+\) cells at 24 and 48 h were significantly higher than those at 6 h (\(p < 0.05\)). CD68\(^+\) macrophages increased up to 72 h, and the 48-h point was significantly higher than that at 6 h (\(p < 0.05\)). All infiltrating inflammatory cells persisted for up to 7 days (Fig. 1). In contrast, the number of tryptase\(^+\) mast cells was reduced after allergen challenge compared with that in diluent controls, suggesting mast cell degranulation (data not shown), consistent with our previous findings (43).

**CC chemokines**

In diluent-challenged sites, very few cells expressed mRNA for eotaxin, eotaxin-2, MCP-4, MCP-3, or RANTES. At allergen-challenged sites, CC chemokine\(^+\) cells were mainly located within areas of inflammatory infiltrate in the upper and deep dermis, and there were significant increases in mRNA\(^+\) cells for all these chemokines at 6, 24, and 48 h compared with those at control sites (\(p < 0.01\); Fig. 2). Peak expression of eotaxin and MCP-3 mRNA occurred at 6 h and returned to baseline by 7 days (Fig. 2A), while the numbers of eotaxin-2, MCP-4, and RANTES mRNA\(^+\) cells were maximal at 24 h and decreased at 48 h (Fig. 2, B and C). At 72 h, but not at 7 days, all chemokine mRNA\(^+\) cells were still increased compared with diluent values (Fig. 2). When the difference in the numbers of mRNA\(^+\) cells between time points was analyzed by the Wilcoxon signed rank test, eotaxin at the 6-h point was significantly higher than at 24 and 48 h (\(p < 0.01\); Fig. 2). Peak expression of eotaxin and MCP-3 mRNA occurred at 6 h and returned to baseline by 7 days (Fig. 2A), while the numbers of eotaxin-2, MCP-4, and RANTES mRNA\(^+\) cells were maximal at 24 h and decreased at 48 h (Fig. 2, B and C). At 72 h, but not at 7 days, all chemokine mRNA\(^+\) cells were still increased compared with diluent values (Fig. 2). When the difference in the numbers of mRNA\(^+\) cells between time points was analyzed by the Wilcoxon signed rank test, eotaxin at the 6-h point was significantly higher than at 24 and 48 h (\(p < 0.01\)); eotaxin at 24 h was significantly higher than at 48 h (\(p < 0.01\)), eotaxin-2 at 6 and 24 h was significantly higher than at 48 h (\(p < 0.05\)), and MCP-4 at 24 h was significantly higher than at 6 and 48 h (\(p < 0.05\)).

Using specific mAb against human eotaxin, the time course of immunoreactive eotaxin expression (Fig. 3A) was similar to that

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**FIGURE 2.** The time course of increases in mRNA\(^+\) cells for eotaxin, MCP-3 (A), eotaxin-2 (B), MCP-4, and RANTES (C) into allergen-challenged skin sites in atopic subjects. Diluent-challenged sites (Dil; 24 h) were used as controls. The results are expressed as the numbers of positive cells (mean ± SEM) per square millimeter of skin biopsy. Significant differences were observed in the cells expressing all these chemokines (diluent and 6-, 24-, and 48-h allergen challenge time points; by Friedman’s test, \(p < 0.01\)). The differences between time points were analyzed by Wilcoxon signed rank test (A: ***, \(p < 0.01\), eotaxin at 6 h vs 24 and 48 h; *, \(p < 0.01\), eotaxin at 24 h vs 48 h; B: *, \(p < 0.05\), eotaxin-2 at 6 and 24 h vs 48 h; C: *, \(p < 0.05\), MCP-4 at 24 h vs 6 and 48 h).

**FIGURE 3.** The time course of increases in eotaxin protein\(^+\) (2G6\(^+\)) cells (A) and CCR3 mRNA\(^+\) cells and CCR3 protein\(^+\) (7B11\(^+\)) cells (B) into allergen-challenged skin sites in atopic subjects. Diluent-challenged sites (Dil; 24 h) were used as controls. The results are expressed as the numbers of positive cells (mean ± SEM) per square millimeter of skin biopsies. Significant differences were observed in the cells expressing all these chemokines (diluent and 6-, 24-, and 48-h allergen challenge time points; by Friedman’s test, \(p < 0.01\)). The differences between time points were analyzed by Wilcoxon signed rank test (A: *, \(p < 0.05\), eotaxin protein at 6 and 24 h vs 48 h; ***, \(p < 0.05\), CCR3 mRNA and protein at 6 h vs 24 and 48 h; B: ***, \(p < 0.05\), CCR3 mRNA and protein at 6 h vs 24 and 48 h; *, \(p < 0.01\), CCR3 mRNA and protein at 24 vs 48 h).
observed for eotaxin mRNA (Fig. 2A). At 6 h, the numbers of eotaxin protein$^+$ cells correlated highly significantly with the numbers of eotaxin mRNA$^+$ cells ($r_s = 0.903; p = 0.0001$).

**CCR3**

The numbers of CCR3 mRNA$^+$ cells at the diluent site were negligible. After allergen challenge there were increases at all time points, which were significant at 6, 24, and 48 h ($p < 0.01$), with peak expression at 6 h (Fig. 3B), and CCR3 mRNA$^+$ cells correlated with CCR3 protein$^+$ cells at 6 h ($r_s = 0.806; p = 0.005$), but not at 24 h. There was also a significant correlation between the numbers of cells expressing eotaxin and CCR3 protein ($r_s = 0.770; p = 0.02$). At 6 h, EG2$^+$ cells correlated with CCR3 mRNA ($r_s = 0.661; p = 0.045$; Fig. 4A) and protein ($r_s = 0.672; p = 0.033$; Fig. 4B). Thus, expression of CCR3 correlated with the peak of eosinophil infiltration at 6 h.

**Relationship between infiltration of eosinophils, basophils, and other inflammatory cells and expression of CC chemokines and CCR3**

At 6 h the numbers of EG2$^+$ eosinophils correlated significantly with the numbers of cells expressing eotaxin mRNA ($r_s = 0.661; p = 0.038$; Fig. 5A) and protein ($r_s = 0.806; p = 0.005$; Fig. 5B). In contrast, at 24 h the numbers of EG2$^+$ eosinophils correlated with the numbers of cells expressing mRNA for MCP-4 ($r_s = 0.782; p = 0.008$; Fig. 5C) and mRNA for eotaxin-2 ($r_s = 0.766; p = 0.01$; Fig. 5D). Also, the numbers of eotaxin-2 and MCP-4 mRNA$^+$ cells correlated significantly with the numbers of CCR3 mRNA$^+$ cells ($r_s = 0.661; p = 0.045$ and $r_s = 0.673; p = 0.033$, respectively). There were no other significant correlations between the C-C chemokines assayed or the numbers of basophils nor other cell types at any time point, although there was a trend, at 6 h only, for basophils to be associated with eotaxin$^-$ cells ($r_s = 0.591; p = 0.072$) and with MCP-4 mRNA$^+$ cells ($r_s = 0.588; p = 0.073$). Thus, eotaxin was associated with 6-h peaking eosinophils, while other C-C chemokines (eotaxin-2 and MCP-4) were associated with declining, but still present, 24-h eosinophils, but none of the measured C-C chemokines was associated with the 24-h peaking of basophils.

**Phenotypes of cells expressing eotaxin and CCR3 protein**

Using a polyclonal rabbit anti-human eotaxin and anti-human CCR3, cells expressing eotaxin and CCR3 were studied in 6-h allergen-challenged sites by double Ab IHC ($n = 6$). CD68$^+$ macrophages and CD31$^+$ endothelial cells accounted for 45 and 30% of the total cells expressing eotaxin, respectively, whereas only 6% were CD3$^+$ T cells, and there were negligible contributions from...
CD31+ endothelial cells 30 ± 2 81 ± 5
EG2+ eosinophils 3 ± 0 3 ± 0
Tryptase+ mast cells 1 ± 0 1 ± 1
BB1+ basophils 1 ± 0 1 ± 0
Elastase+ neutrophils 1 ± 0 1 ± 0
Others 13 N/A

Table II. Distribution of eotaxin protein+ cells and percentage of cells expressing eotaxin protein in allergen-induced LPR on skin (n = 6) a

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>% of Eotaxin+ Cells</th>
<th>% of Each Cell Type Coexpressing Eotaxin</th>
</tr>
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<tr>
<td>CD68+ macrophages</td>
<td>45 ± 4</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>CD31+ endothelial cells</td>
<td>30 ± 2</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
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<tr>
<td>BB1+ basophils</td>
<td>10 ± 2</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>CD68+ macrophages</td>
<td>3 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>CD3+ T cells</td>
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<tr>
<td>Elastase+ neutrophils</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>CD31+ endothelial cells</td>
<td>1 ± 1</td>
<td>3 ± 2</td>
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</table>

a Double IHC (employing a polyclonal rabbit anti-human eotaxin and mAb against several phenotype markers as indicated) was performed in skin LPR at 6 h after allergen challenge.

EG2+ eosinophils, tryptase+ mast cells, BB1+ basophils, and elastase+ neutrophils (Table I). Overall, 43% of CD68+ macrophages and 81% of CD31+ endothelial cells were eotaxin+ (Table I).

Colocalization for CCR3 also was performed on the 6-h biopsies. EG2+ eosinophils accounted for 83% of the CCR3-bearing cells, and 83% of the eosinophils were positive. The remaining CCR3+ cells were mainly BB1+ basophils, and 40% of the basophils in 6-h LPR expressed CCR3 (Table II).

Discussion

In this comprehensive study over a prolonged time course of provoked human allergic tissue reactions in the skin, we attempted to relate the numbers of infiltrating eosinophils and basophils to expression of associated C-C chemokines. We have demonstrated a clear relationship between early 6-h infiltration of eosinophils and eotaxin expression and later 24-h eosinophil infiltration to eotaxin-2 and MCP-4 in allergen-induced LPR. In contrast, none of the five C-C chemokines tested could be related to the 24-h peak infiltration of basophils that also express CCR3. We have shown that CCR3 immunoreactivity was predominantly associated with eosinophils, less so with basophils, and minimally on T cells, and that eotaxin is expressed by several local tissue cell types, such as macrophages and endothelial cells, during the cutaneous allergic reaction.

Chemokines are involved in both firm adhesion of leukocytes to vascular endothelial surfaces and migration of cells into the tissues. They are produced by a variety of tissue cell types and attract and activate leukocytes that express multiple chemokine receptors. Thus, the migration of cells from blood vessels into the tissues involves cell adhesion molecules, chemokines, and chemokine receptors, participating in sequence. It has been suggested that these elements may combine to produce a combinatorial address code that determines the egress and infiltration of different cells at particular tissue sites (44, 45). Thus, the identification of a limited number of chemokines and chemokine receptors in tissues at a given time may offer an incomplete picture of events surrounding diapedesis and the positioning of cells within the tissues, especially since, in addition, there will be modulation of receptor expression consequent to ligation. Nevertheless, it was of interest that the kinetics of mRNA expression paralleled immunostaining of eotaxin and CCR3 at all time points and significantly correlated with numbers of eotaxin and CCR3 mRNA- and protein-expressing cells at 6 h after allergen challenge. The precise mechanisms surrounding these events are uncertain, but it seems unlikely that T cells are responsible, because few had arrived by 6 h. Thus, it is more likely that early eotaxin expression and recruitment of CCR3+ eosinophils may have been due to an early phase allergen-induced IgE-dependent mast cell activation.

Although it is well documented that eosinophils, neutrophils, basophils, T cells, and macrophages infiltrate the site of allergen-induced LPR in the skin (1–3), the kinetics and mechanisms of cell accumulation in vivo in man were previously unclear, because most studies were performed either in vitro or in animal models (10–12). It has been shown in vitro that eotaxin is the most potent and specific chemoattractant for eosinophils (10–13). Intradermal injection of recombinant mouse eotaxin (46) and human eotaxin (47) into animals induced accumulation of eosinophils in local skin tissue. Eotaxin-induced eosinophil accumulation appears to be IL-5 dependent (48, 49). Furthermore, increased IL-5 mRNA+ cells in allergen-induced LPR of skin was observed as early as 1–3 h, with a peak at 6–24 h (50). Thus, IL-5 appears to act together with CC chemokines in eosinophil accumulation to mobilize and release eosinophils from the bone marrow. Eotaxin-2, a more recently discovered C-C chemokine, also showed specific chemoattractant activity for eosinophils and basophils through binding to CCR3, although the homology between eotaxin-2 and eotaxin is only 39% (14). In addition to eotaxin and eotaxin-2, the C-C chemokines MCP-4, MCP-3, and RANTES have chemoattract activity for eosinophils and basophils (10–12), and expression of eotaxin, MCP-3, and RANTES occurs at baseline in human asthmatic lung and bronchoalveolar lavage (BAL) (31–38), suggesting involvement in eosinophil recruitment to airways. Our time-course studies in human skin LPR show that peak expressions of eotaxin and MCP-3 were earlier than those of eotaxin-2, MCP-4, and RANTES (Fig. 2), and this correlated with the early 6-h peak of eosinophils, suggesting that eotaxin and MCP-3 are involved in early eosinophil mobilization in man, and eotaxin-2 and MCP-4, on the other hand, may be more involved in the later stages of eosinophil infiltration, as shown by correlation between eosinophils and expression of eotaxin-2 and MCP-4, respectively. Animal experiments support this view, because eotaxin-deficient mice had reduced eosinophils in BAL fluid early, but not late, after allergen challenge (40). However, the numbers of eotaxin and eotaxin-2 mRNA+ cells were similar at the 6-h point (Fig. 2). Furthermore, there were no significant differences in the expression of eotaxin-2 and RANTES between 6 and 24 h after allergen challenge (Fig. 2).

Thus, although eotaxin-2 and RANTES may also contribute to the early eosinophil recruitment, there were no significant correlations between expression of the transcripts and eosinophil numbers. Nevertheless, these findings suggest that 24-h late-phase influx of eosinophils into local allergic tissue responses may be dependent on other C-C chemokines besides eotaxin (i.e., eotaxin-2 and MCP-4) that may be generated by early IgE activation of mast cells or possibly are induced via later recruited Th2 T cells that are known to be increased, activated, and express Th2 cytokines in human skin LPR (51). After arrival and local activation, probably...
by allergen peptides on APC, the Th2 cells may then activate local tissue cells for production of these later acting chemokines.

The infiltration of neutrophils was also observed in allergen-challenged sites, consistent with our previous findings (1, 7, 8). However, there were no significant correlations between the infiltration of neutrophils and expression of the C-C chemokines studied. This observation would be expected, because C-C chemokines lack neutrophil chemotactic activity in vitro. It is well known that IL-8 is a potent chemoattractant for neutrophils in vitro. In the presence of histamine, intradermal injection of IL-8 (3 h after injection) provoked a greater neutrophil infiltration, but not lymphocyte or eosinophil infiltration, into local tissue of man (52). Compared with diluent controls, significant increases in the levels of IL-8 and mediators derived from mast cells (such as histamine) were observed as early as 2 h after allergen challenge in human skin chamber fluid (53). These studies suggest that IL-8 and mediators derived from mast cells contribute to neutrophil accumulation after allergen exposure.

There is widespread constitutive expression of eotaxin in various tissues, especially heart, gut, lung, and kidney (54, 55). Thus, eotaxin expression and eosinophil infiltrates were detectable in lamina propria of the jejunum from normal wild-type mice, while eosinophils were reduced in the jejunum in eosinophil-deficient mice (56). In a previous study of bronchial biopsies and BAL obtained from normal subjects there was some baseline expression of eotaxin mRNA and protein, and small numbers of eosinophils also were observed (31-33). In contrast, in normal skin dermis (e.g., the diluent injection sites) that, presumably, are not in daily contact with environmental allergens, there was little eotaxin expression (mRNA, 2.4 ± 0.7 mm2; protein, 4.3 ± 0.6 mm2) and few eosinophils (0.7 ± 0.3 mm2; Figs. 1–3). Thus, baseline levels of C-C chemokines, especially eotaxin, may be required for baseline numbers of tissue eosinophils. Because the numbers of eosinophil cells were too small and the intensity of the staining was quite weak in diluent-challenged sites, it is difficult to identify the cell sources of eotaxin by double staining.

The presence of basophil infiltrates in atopic allergic inflammation in human skin was recently established using basophil granule-specific mAbs (3). We have used BB1, a similar basophil granule-specific mAb that recognizes a protein of 124 ± 11 kDa and does not cross-react with mast cells, eosinophils, neutrophils, lymphocytes, or macrophages (39, 57). We extended the observations of Irani et al. (3) to show that basrophils were clearly present at 6 h, when eosinophils were maximal. However, basophils peaked at 24 h, when eosinophils were declining. It has been previously observed that eosinophils highly express CCR3 and weakly express CCR1 (58). Unlike eosinophils, basophils express CCR1, CCR2, CCR3, and CCR4 (58). Although it remains to be confirmed whether all these receptors are involved in the migration of basophils, the CC chemokines eotaxin, eotaxin-2, MCP-4, MCP-3, and RANTES are potent chemoattractants for basophils in vitro (13–21). Additionally, MCP-1, MCP-2, and MIP-1α have chemoattractant capacity for basophils in vitro (30). In the present study we found no significant correlations between the expression of the five CC chemokines tested and infiltration of BB1+ basophils, although a trend was noted for eosinoph and MCP-4 (that conceivably might act in concert on CCR3 and CCR2). Thus, compared with eosinophils, which are attracted via CC chemokines acting on CCR3, basophil chemotaxis in allergen LPR of the skin may require a combination of other CC chemokines, possibly unknown chemokines, and CC chemokine receptors.

Since our data indicated a key role for eotaxin in early 6-h eosinophil infiltration, we attempted to identify cell sources to explain the egress of eosinophils into the allergic tissues. In general, CC chemokines are widely expressed by various tissue cell types, including epithelial cells, endothelial cells, macrophages, fibroblasts, and eosinophils themselves (10–38). Using a double-Ab IHC technique, we demonstrated that CD68+ macrophages and CD31+ endothelial cells were major cell sources for eotaxin protein at 6 h (Table II), while skin epithelial cells (keratinocytes) express little if any eotaxin mRNA or protein, in contrast to findings in the lung (31–38). Thus, recruited eosinophils, emerging from vascular activation interactions, on entry into different allergic tissues may experience gradients of eotaxin provided by different local environmental tissue cells (59). Although the polyclonal Ab against eotaxin used in the present study may also recognize MCP-2 and MCP-3, it was previously shown that epithelial cells and fibroblasts were the major source of MCP-2 and MCP-3 (60, 61). Although monocytes and endothelial cells also expressed MCP-3 transcripts in vitro after stimulation with LPS, IL-1, and TNF, this was relatively weak (62).

The mechanisms of multi-CC chemokine gene expression in allergen-induced skin LPR is incompletely understood. Because the patients had elevated specific IgE to common allergens and uniformly expressed strong immediate wheal and flare responses that preceded the 6-h aspect of the LPR, it may be that IgE activation of mast cells contributed to the early 6-h findings, including the eosinophil peak. On the other hand, IgE/mast cell late-phase-released mediators, including cytokines, may contribute to the late 24- to 48-h aspects together with mediators produced by infiltrating allergen-specific Th2 cells that began to infiltrate at about 6 h and were numerous at the later time points (Fig. 1C). A number of proinflammatory cytokines and mediators are likely to be involved in either or both phases. For example, IL-1 and TNF-α, known to be released by mast cells via IgE activation, could up-regulate the expression of eotaxin (54, 63), MCP-4 (28), and other CC chemokines in epithelial and endothelial cells in the early phase of LPR. On the other hand, late-recruited Th2 cell-derived cytokines probably contribute to eotaxin-mediated tissue eosinophilia, because adoptive transfer of Th2 cells into mice induced Ag-dependent lung eotaxin expression and eosinophilia (64). Also, IL-4, the prototypic Th2 cytokine, enhanced eotaxin expression by epithelial, endothelial cells and dermal fibroblasts in vitro (54, 65), and injection of IL-4 into rats induced eotaxin accumulation in skin that was partially mediated by endogenous production of eotaxin (47). In addition, the Th2 cytokines IL-4 and IL-13 both induce up-regulation of VCAM-1 on endothelium, which is probably involved in eotaxin-induced eosinophil accumulation. Finally, peptidolipid mediators such as leukotrienes C4, D4, and E4, as well as histamine, derived early from mast cells and recruited eosinophils and later from basophils and macrophages, may also regulate the expression of CC chemokines. We recently observed that these agents can increase eotaxin expression on human endothelial cells in vitro (66), indicating that these mediators may contribute to the early eosinophil influx by up-regulating eotaxin.

The kinetics of CCR3 expression paralleled the infiltration of EG2+ eosinophils (Figs. 1 and 3), and there were significant correlations between CCR3+ cells (both mRNA and protein) and the numbers of EG2+ eosinophils (Fig. 4B). Double-Ab IHC indicated that CCR3 predominantly colocalized to EG2+ eosinophils (Table II). Because eotaxin, eosinophil-2, MCP-4, MCP-3, and RANTES all stimulate eosinophils via CCR3 (10–12), this highlights CCR3 as a prime target for therapeutic intervention in diseases featuring eosinophil-mediated tissue damage. Because few basophils expressed CCR3 in the LPR, and the five C-C chemokines studied did not correlate with basophil infiltrates, we postulate that additional chemotactic influences may apply to basophil recruitment into allergic.
tissues. A previous study showed that Th2-type T cell lines expressed CCR3 (67). However, in the present study only 2% of the CCR3+ cells were CD3+ (Table II), and only 1% of CD3+ cells were CCR3+, suggesting little expression of this receptor on the bulk of infiltrating Th2 cells. Further studies will be needed to determine whether these might be the few allergen-specific Th2 cells activated by local APC, possibly responsible for the late eosinophil and basophil recruitment in the skin LPR, because, as previously shown, very few Ag-specific T cells can mediate a complete late cutaneous hypersensitivity reaction (68).

In summary, the data are compatible with the conclusion that eotaxin plays a major role in the early (6 h) and that eotaxin-2/previously shown, very few Ag-specific T cells can mediate a complete late cutaneous hypersensitivity reaction (68).

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References


