Evidence for interleukin-5 in nasal polyps in aspirin-induced asthma

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ABSTRACT
Aspirin-induced asthma is often accompanied by nasal polyps, in which tissue eosinophils are abundant and activated. However, the mechanism of eosinophil infiltration remains unknown. We encountered two aspirin-induced asthma patients with nasal polyps and investigated eosinophil infiltration into nasal polyp tissue. Eosinophil chemotactic activity of extracts from the nasal polyp was elevated and could be inhibited by 40% with anti-interleukin (IL)-5 antibody. Interleukin-5 was detectable in the extract. The chemotactic activity of peripheral blood eosinophils to recombinant human (rh) IL-5 was increased compared with normal volunteers. Messenger RNA expression for IL-5 in CD3+ lymphocytes for polyp tissue was detected using the reverse transcription-polymerase chain reaction. These results suggest that IL-5 from local T lymphocytes may be one of the candidates for recruitment of eosinophils into nasal polyps in aspirin-induced asthma.

Key words: aspirin-induced asthma, chemotaxis, eosinophil, interleukin-5, nasal polyp.

INTRODUCTION
Aspirin-induced asthma is a variant of an intrinsic asthma associated with intolerance of aspirin and/or non-steroidal anti-inflammatory drugs (NSAID). Nasal polyposis, with abundant tissue eosinophils, is a frequent finding in aspirin-induced asthma. A recent immunocytochemical study has demonstrated that eosinophils infiltrating nasal polyps are activated (EG2+). However, the mechanism of eosinophil infiltration into nasal polyps is unclear.

In the present report, we examine the eosinophil chemotactic activity of extracts from nasal polyps in a patient with aspirin-induced asthma. We focus upon interleukin (IL)-5 as a chemoattractant because IL-5 is one of the key cytokines for eosinophil recruitment and activation.

CLINICAL SUMMARY
Case 1
A 45-year-old woman had suffered from chronic sinusitis for 6 years. After a right Caldwell-Luc operation 5 years ago, she developed bronchial asthma. Asthma attacks were worse when she took NSAID. The diagnosis of aspirin-induced asthma was made on the basis of hypersensitivity to acetylsalicylic acid (aspirin) at the Department of Respiratory Medicine, Kumamoto University (Kumamoto, Japan). The patient was referred to our clinic for further examination. Rhinoscopy showed slightly hypertrophic middle turbinates and left nasal polyps. The patient’s characteristics are shown in Table 1. Because of nasal blockage and watery rhinorrhea, she underwent endonasal sinus surgery (ESS). She had not received glucocorticoestroids for 3 months prior to ESS. Polyps were removed, washed, homogenized with Polytron® (Kinematica, Switzerland) and were centrifuged at 20,000 g for 10 min. The supernatant was collected, filtered through a 0.45 μm Millipore filter and stored at −80°C until use. Her nasal blockage improved after ESS.
Table 1. Patient characteristics

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<thead>
<tr>
<th>Case</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>RAST scores*</td>
<td>All negative</td>
<td>All negative</td>
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<tr>
<td>IgE (IU/mL)</td>
<td>57</td>
<td>364</td>
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<td>Blood eosinophils (%)</td>
<td>14.3</td>
<td>14.7</td>
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* IgE antibodies against house dust mite, Japanese cedar pollen, grass pollen, molds.

Case 2

A 63-year-old woman had suffered from chronic sinusitis and nasal polyps for 10 years. Although the onset of bronchial asthma had occurred at the age of 62, the patient’s asthma condition was stable with the use of a topical corticosteroid and an oral bronchodilator. Aspirin-induced asthma was revealed after the patient took NSAID when polypotomy was performed. The patient’s characteristics are shown in Table 1. As the patient complained of severe nasal blockage, another polypotomy was performed while she was free of asthma. The patient had not received glucocorticosteroids for 1 month prior to polypotomy.

METHODS

Peripheral blood eosinophils were isolated by discontinuous Percoll density gradient centrifugation followed by negative selection with immunomagnetic beads, as previously described. Briefly, heparinized blood was separated by sedimentation of erythrocytes with dextran for 30 min. The granulocyte rich fraction was centrifuged for 30 min through Percoll to reduce the number of neutrophils. The eosinophil-rich fraction was collected and labeled with anti-CD16 monoclonal antibody (mAb) for 45 min at 4°C. Subsequently, cells were washed twice with Hanks’ balanced salt solution containing 2% fetal calf serum and were incubated with sheep anti-mouse immunomagnetic dynabeads (Dynal, Great Neck, NY, USA) for 60 min at 4°C. Neutrophils were subsequently removed using a magnetic particle concentrator (MPC™-1; Dynal). Eosinophil purity exceeded 95% and showed a viability greater than 98% as judged by trypan blue exclusion.

Chemotactic activity was assessed by a modified Boyden chamber assay using 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD, USA) as previously described. In brief, cells were adjusted to 1×10⁶ cells/mL in RPMI-1640 containing 10% fetal calf serum (FCS) and 50 µL was placed in the upper well, which was separated from 29 µL chemoattractant in the lower well by a polystyrene-free nucleopore filter (pore size 5 µm). The chambers were incubated at 37°C in 5% CO₂ air for 90 min. Following incubation, the filter was fixed with methanol and stained with Diff-Quick, (Kokusai Shiyaku, Kobe, Japan). Cells that passed through the filter to the lower surface were counted using a 10 mm eyepiece graticule in a light microscope using 400× magnification.

Tissue extracts were prepared from nasal polyps of case 1 and were examined for the chemotactic response of the patient’s peripheral blood eosinophils against these extracts. To determine whether IL-5 contributed to this chemotactic response, 30 µg/mL mAb against IL-5 (Genzyme, Boston, MA, USA) was added to the lower chamber. We used recombinant human (rh)IL-3 and rhIL-5 in the eosinophil chemotaxis assay. A pellet of the extract was resuspended in RPMI-1640 supplemented with 2% FCS, washed and centrifuged for 30 min through Percol. The lymphocyte-rich fraction was collected and was labeled with magnetic cell sorter (MACS) CD3 Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 4°C. Subsequently, cells were passed through the MACS (Miltenyi Biotech), and positive selection was performed according to the manufacturer’s instructions. Purity of CD3+ cells exceeded 95%.

Serum and extract concentrations of IL-5 were determined by an enzyme-linked immunosorbent assay (ELISA) according to procedures as described previously. ELISA plates (96 wells, Immulon 4; Dynatech Laboratories Inc., VA, USA) were coated with IL-5-specific mAb (clone NC17, 2 µg/mL in phosphate-buffered saline (PBS), 100 µL/well). After incubation overnight at 4°C, wells were washed three times with PBS/Tween, after which 100 µL aliquots of sample or standards were added to each well. After another overnight incubation at 4°C, wells were washed three times and 100 µL rabbit anti-human IL-5 serum was added to each well. After incubation for 4 h at room temperature, wells were washed three times with PBS/Tween, after which 100 µL horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:3000; Bio-Rad, Hemel Hempstead, England) was added to each well. After incubation for 2 h at room temperature and washing, the chromogen was developed by adding the substrate O-phenylenediamine in the presence of 0.015% hydrogen peroxide for 20 min. Reactions were stopped by adding 50 µL of 2 mol/L sulfuric acid and the optical densities (OD) were read at 490 nm with an...
automated plate reader. The lower and upper limits of sensitivity of the assay were 100 and 40,000 pg/mL, respectively. No cross-reactivity was detected with IL-2, IL-3, IL-4, IL-6, or granulocyte-macrophage colony stimulating factor (GM-CSF). Standards were prepared from rhIL-5 purified on NC17-coupled beads from culture supernatants of Chinese hamster ovary cells transfected with human IL-5 cDNA.

Total cellular RNA was extracted from T lymphocytes with Isogen, according to the protocol of Nippon Gene (Tokyo, Japan), a modified guanidium thiocyanate single-step procedure. After isolation, the RNA was reverse transcribed in a total of 20 µL reaction mixture containing 50 ng random hexamer (Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA), 0.5 mmol/L of each dNTP and 100 U Superscript reverse transcriptase (Gibco BRL) in 20 mmol/L Tris.HCl (pH 8.4), 50 mmol/L KCl, 10 mmol/L dithithreitol and 2.5 mmol/L MgCl₂. The reaction was incubated at 42°C for 50 min, then at 95°C for 5 min and then chilled on ice. cDNA was amplified in a 50 µL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.8), 1.5 mmol/L MgCl₂, 2.5 nmol of each dNTP, 25 pmol of each primer and 1.0 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CA, USA). Nucleotide sequences for oligonucleotide 5'- and 3'-primers, respectively, were as follows: β-actin, CTACAATGAGCTGCGTGTGG and CGGTGAGGATCTTCATGAGG; IL-5, GCTTCTGCATT TGAGTTTGCTAGCT and TGGCCGTCAATGTATTTCTTT ATTAAG.8 The PCR was performed for 35 cycles (1 min at 94°C, 2 min at 60°C, 3 min at 72°C). The PCR product was electrophoresed in a 2% agarose gel in the presence of 0.5 mg/mL ethidium bromide. The PCR product was transferred to a nylon membrane filter after degeneration with alkaline solution. Transferred DNA was fixed by UV-irradiation and hybridization was performed at 55°C for 12 h using a 32P-labeled internal probe (GGCAGTCTTCTTACTCATCG).8 Peripheral blood lymphocytes from Kimura’s disease and sterilized water were used as positive and negative controls for IL-5 mRNA expression, respectively.

RESULTS

Peripheral blood eosinophils obtained from a patient (case 1) with aspirin-induced asthma showed an increased chemotactic response against tissue extracts in a dose-dependent pattern (Fig. 1). This chemotactic response was inhibited by 40% when an antibody against IL-5 was added (Fig. 2). As shown in Table 2, IL-5 was
Fig. 3 Chemotactic response of blood eosinophils to recombinant human interleukin (rhlL)-3 and rhlL-5. Chemotactic responses of eosinophils from an aspirin-induced asthma case 1 (△), case 2 (□) and a healthy donor (■) to phosphate-buffered saline, IL-3, and IL-5 were assessed. Interleukin-3 and IL-5 were used at a concentration of $10^{-9}$ mol/L. Data represent the mean number of migrated cells/5 high power fields (h.p.f.) in duplicate.

Table 2. Concentration of interleukin-5

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<th>Case 1</th>
<th>Case 2</th>
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<tr>
<td>Nasal polyp (ng/g tissue)</td>
<td>10.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Serum (ng/mL)</td>
<td>7.0</td>
<td>&lt;0.1</td>
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</table>

detected in polyp extracts from both case 1 and case 2. Although serum concentrations of IL-5 in three normal volunteers were undetectable (data not shown), one patient (case 1) had a serum concentration of IL-5 above the lower limit of the ELISA. Furthermore, IL-5 was not detected in polyp extracts nor in sera from two patients with chronic sinusitis that was not associated with aspirin-induced asthma or allergic diseases (data not shown).

Figure 3 shows the chemotactic response of eosinophils isolated from the peripheral blood of patients and from a healthy individual to rhIL-3 ($10^{-9}$mol/L) and rhIL-5 ($10^{-9}$mol/L). Eosinophils isolated from these patients showed an increased chemotactic response to rhIL-5 compared with eosinophils from a normal subject. In contrast, chemotactic responses to rhIL-3 were increased both in asthmatic and in normal eosinophils.

Fig. 4 Southern blot analysis of RT-PCR from peripheral blood CD3+ cells (lane 1) and nasal polyp CD3+ cells (lane 2). Peripheral blood mononuclear cells from Kimura's disease were used as a positive control (lane 3) and sterilized water was used as a negative control (lane 4).

Messenger RNA for IL-5 was detectable in CD3+ T lymphocytes from nasal polyps but not from peripheral blood, as shown in Fig. 4.

**DISCUSSION**

Nasal polyps in aspirin-induced asthma are characterized by infiltration of inflammatory cells, especially eosinophils. In addition, immunohistochemical studies have demonstrated that most of these eosinophils are activated (EG2+ cells). Picado et al. have found a significant increase in peptide leukotrienes in aspirin-induced asthma patients 60 min after nasal challenge. These findings suggest that activated eosinophils may be involved in nasal symptoms and polyp formation because eosinophils can produce peptide leukotriene. However, the mechanism of eosinophil recruitment into nasal polyp tissue has not been clarified.

Recent studies have suggested that T lymphocytes play an important role in allergic inflammation, especially for recruitment and activation of eosinophils. In situ hybridization studies of biopsies of human skin and nose have revealed significant increases in cells expressing mRNA for IL-4, IL-5 and GM-CSF in late-phase reactions. In addition, a significant correlation has been found between the number of EG2+ cells and IL-5 mRNA+ cells in late nasal responses. Accordingly, we examined IL-5 in nasal polyp tissue.
We detected eosinophil chemotactic activity in extracts of nasal polyps in case 1 and found that this activity was inhibited by the antibody against IL-5. Interleukin-5 was also detectable in nasal polyp extracts in these cases. Furthermore, rhIL-5 had a higher chemotactic activity against the patients’ peripheral blood eosinophils compared with normal eosinophils. By using RT-PCR, we observed the expression of mRNA for IL-5 in CD3+ T lymphocytes from polyp tissue but not from peripheral blood.

Interleukin-5 is considered to be a weak chemotactant for normal eosinophils; however, an IL-5 response was not found for peripheral blood eosinophils from bronchial asthma patients. Conversely, peripheral blood eosinophils of patients with allergic rhinitis exhibit a higher locomotory response towards IL-5 than do normal subjects. We have also found a higher chemotactic activity of eosinophils in patients with Kimura’s disease towards IL-5. These findings suggest that there may be heterogeneity in the eosinophil response to IL-5, but this has not been confirmed.

We were not able to completely inhibit the chemotactic response of peripheral blood eosinophils against tissue extracts with anti-IL-5 antibody. Recent studies have shown that the synergism between IL-5 and eotaxin will induce the response of peripheral blood eosinophils from bronchial asthma patients. Conversely, peripheral blood eosinophils of patients with allergic rhinitis exhibit a higher locomotory response towards IL-5 than do normal subjects. We have also found a higher chemotactic activity of eosinophils in patients with Kimura’s disease towards IL-5. These findings suggest that there may be heterogeneity in the eosinophil response to IL-5, but this has not been confirmed.

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REFERENCES