

Eosinophils are activated in middle ear mucosa and middle ear effusion of patients with intractable otitis media associated with bronchial asthma

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Summary

Background Although patients with intractable otitis media associated with bronchial asthma have extensive accumulation of eosinophils in the middle ear mucosa and middle ear effusion, systematic histological and immunohistochemical studies have not been performed.

Objectives To clarify the pathogenesis of middle ear diseases, we carried out immunohistochemical studies on middle ear specimens, particularly focusing on the characteristics of accumulated eosinophils.

Methods Middle ear specimens obtained from eight adult patients and from 17 controls were immunohistochemically stained using monoclonal antibodies against EG1, EG2, mast cell tryptase, IgA and IgE. The concentration of eosinophil cationic protein (ECP) in middle ear effusion samples was also measured.

Results In the asthmatic patients, severe round-cell infiltration was observed in the submucosa and most of the EG1-positive cells were also EG2-positive. In the control patients, the mucosa showed a fibrotic change with a few inflammatory cells, and EG1- or EG2-positive cells were quite few. The expression of IgE was found not only on the surface of mast cells but also within the plasma cells in the asthmatic patients, and the number of IgE-positive cells was about twice as high as that of mast cells. A significantly higher concentration of ECP was noted in middle ear effusion obtained from the asthmatic patients than that from the control patients.

Conclusion Most of the eosinophils in the middle ear mucosa and middle ear effusion were activated, resulting in degranulation and release of ECP, and local IgE production occurs in the middle ear mucosa, indicating that the intractable inflammation is closely associated with IgE-mediated late phase response with eosinophil accumulation.

Keywords: otitis media with effusion, chronic otitis media, bronchial asthma, eosinophils, IgE-mediated allergy.

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Introduction

Recently, there have been some reports of patients with intractable middle ear diseases, such as otitis media with

effusion (OME) and chronic otitis media, associated with bronchial asthma [1,2]. The reported patients exhibited generally the same characteristics of the clinical course and symptoms. Derlacki [3] summarized the markedly different clinical characteristics of intractable otitis media, compared with those of the common type of OME or chronic otitis media, as follows: (i) a secretion having a markedly viscous

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or gelatinous consistency with eosinophils; (ii) a middle ear mucous membrane which is fairly thickened with numerous eosinophils; (iii) a protracted healing time; (iv) occurrence in allergic disposition such as allergic rhinitis; and (v) a likely good response to anti-allergy treatment. He suggested that the condition resulted from an allergic aetiology.

Type I allergy, that is, IgE-mediated hypersensitivity to some antigens, is one of the important aetiologies of bronchial asthma and nasal allergy. However, antigens are not always detected by skin testing or radioallergosorbent testing (RAST), and the condition has been called non-atopic type of bronchial asthma. Although marked infiltration of eosinophils to middle ear effusions and middle ear mucosa is a common feature in patients with the so-called 'allergic otitis media', some of them have non-atopic type of bronchial asthma. Therefore, Tomioka *et al.* [4] designated a new name to this specific type of intractable otitis media: eosinophilic otitis media.

Accumulation and activation of eosinophils in the airway mucosa is an essential process in respiratory inflammations such as bronchial asthma, allergic rhinitis and nasal polyps. Eosinophil cationic protein (ECP) is one of the eosinophil-derived proteins which are released from activated eosinophils. Because ECP has been reported to have various biological activities, including killing of parasites, causing injury to respiratory epithelial cells and acceleration of mucus production from the respiratory epithelial cells, it plays an important role in the pathogenesis of chronic respiratory inflammation.

Intractable OME and chronic otitis media associated with bronchial asthma do not appear to be rare conditions. However, to the best of our knowledge, there have been no systematic studies of the pathogenesis of the disease using histological and immunohistochemical techniques, although there have been a few case reports of the condition. In the present study, we carried out histological and immunohistochemical analyses of middle ear specimens to clarify the pathogenesis of intractable middle ear conditions. In particular, we focused on the characteristics of accumulated eosinophils and on whether the inflammatory condition was related to type I allergy or not. In addition, we measured the concentration of ECP in the middle ear effusion to determine the relationship between eosinophils and the inflammatory process. We found that most eosinophils in the middle ear mucosa were activated with degranulation, and ECP at high concentration was present in the middle ear effusion.

Patients and methods

Study subjects

Middle ear specimens were obtained from eight patients

(six females and two males, aged between 35 and 57 years) who participated in this study. All the patients had a long history of bronchial asthma and were under medical treatment which involved systemic and topical steroids. One patient was diagnosed as having aspirin-intolerance asthma. Six patients were diagnosed as having non-atopic asthma based on the low score of RAST for inhaled antigens. The remaining two patients had atopic asthma. All the patients exhibited a high titre of serum IgE. Some had also been treated for OME by otolaryngologists for a long period, including medication, catheter inflation, myringotomy and insertion of tympanostomy tubes. Bilateral or unilateral perforation of the eardrum was present in five patients. The perforation was either a spontaneous rupture or persistent perforation after extrusion of the tympanostomy tubes. Nasal polyposis was noted in all patients and some of them underwent polypectomy or endoscopic sinus surgery.

The granulated middle ear mucosa samples were obtained through the perforation in three patients and at the time of ear surgery in two patients, and used for histological and immunohistochemical analyses. Middle ear effusion samples were collected through the perforation or by myringotomy in six patients and used for the measurement of ECP. Histological and immunohistochemical analyses were also carried out for some of the middle ear effusion samples.

As controls, samples of the granulated or hypertrophic middle ear mucosa were obtained from 10 adult patients during middle ear surgery. The patients were diagnosed as having chronic perforative otitis media without cholesteatoma, and none had bronchial asthma or nasal polyposis. For the measurement of ECP, the middle ear effusion samples were also obtained through the perforation or by myringotomy in seven other adult patients; three patients had chronic perforative otitis media and four patients had OME without bronchial asthma or other allergic diseases.

Tissue preparation

The middle ear mucosa or effusion was immediately fixed in 10% formaldehyde solution and paraffin-embedded sections were prepared using a conventional method.

Immunohistochemical reagents

The mouse monoclonal antibodies used were the following: EG1 (Kabi Pharmacia, Uppsala, Sweden), which recognizes both resting and activated eosinophils; EG2 (Kabi Pharmacia), which recognizes only activated eosinophils; IgA (DAKO A/S, Glostrup, Denmark); IgE (DAKO A/S); and human mast cell tryptase (DAKO A/S). The antibodies were appropriately diluted in 0.01 mol/L PBS (pH 7.4)

containing BSA and used for immunohistochemical staining.

Immunohistochemistry

To examine the distribution of cells positive for the above-mentioned antibodies in the middle ear specimens, an immunohistochemical method using a labelled streptavidin biotin kit (LSAB kit; DAKO A/S) was used. Briefly, after blocking of endogenous peroxidases in 3% hydrogen peroxide and blocking with normal bovine IgG, the sections were treated with one of the primary monoclonal antibodies. Each of these sections was incubated with a secondary antibody, biotinylated goat anti-mouse IgG, then with a HRP-labelled streptavidin complex and stained with 3,3-diaminobenzidine tetrahydrochloride.

Evaluation

The sections were coded and evaluated independently by two individuals using a conventional light microscope at lower and higher magnifications and the immunohistochemically stained cells in the submucosal layer were observed. We counted immunopositive cells at $400\times$ magnification in five fields where many inflammatory cells were observed. Simultaneously, the sections were stained with haematoxylin and eosin.

Measurement of ECP

The middle ear effusion samples were collected using a Jun-Tym-Tap middle ear fluid aspirator/collector (Xomed, Jacksonville, FL, USA). Fifty to 100 mg of the effusion samples were suspended in 1 mL of saline until the fluid became homogeneous by pipetting and vigorous vortexing at room temperature. When the effusion was too viscous to be homogenized, dithioerythritol (DTE, Fluka, Gillingham, Dorset, UK, final concentration: 0.01 M) solution was added to dissolve the effusion according to the method reported by Louis et al. [5]. The fluid samples were allowed to stand for 60–120 min and then centrifuged at 1000–1350 g for 10 min. The supernatant was collected and stored at -80°C until the measurement of ECP concentration. The ECP concentration in the samples was measured using a Pharmacia RIA kit (Pharmacia AB, Uppsala, Sweden). Prior to the study, a standard curve of ECP concentration was generated using the pooled effusion samples from the patients without allergic predisposition to ensure that ECP recovery was adequate in the samples treated with and without DTE. The recovery of ECP was satisfactory in the effusion samples both with and without DTE treatment.

Statistics

Statistical analysis was carried out using an unpaired Student's *t*-test or Mann–Whitney *U*-test. Two-tailed *P*-values of less than 0.05 were considered significant.

Results

Histopathology of middle ear mucosa

The middle ear mucosa samples obtained through the perforation or at the time of ear surgery exhibited marked round-cell infiltration in the submucosal area. The infiltrating cells consisted mainly of lymphocytes and plasma cells, as well as various amounts of eosinophils and neutrophils (Fig. 1a). In the middle ear mucosa samples obtained from the control patients, minimal infiltration of inflammatory cells, such as lymphocytes, neutrophils as well as fibroblasts and fibrocytes, was observed (Fig. 1b). Fibrosis and a scar tissue rather than active inflammation, were observed in the submucosal layer of most of the control patients.

Immunohistochemically stained cells in middle ear mucosa

Numerous eosinophils labelled with antibodies against EG1 and EG2 were observed in the submucosal layer in the middle ear mucosa of patients with bronchial asthma. Most of the EG1-positive cells were also EG2-positive in these patients (Figs 2a and b). On the other hand, in the control patients, the number of EG1-positive cells was low and few EG2-positive cells were distributed in the submucosal area. The number of EG1- and EG2-positive cells was significantly higher in the patients with asthma than in the control patients (Table 1).

Both asthmatic and control patients had many tryptase-containing mast cells within the mucosa and in the submucosa (Fig. 3). There was no difference between the number of mast cells in the asthmatic patients and that in the control patients (Table 1).

Positive staining for IgA was observed in the cytoplasm of plasma cells, and for IgE was observed on the surface of the mast cell-like cells in the submucosa, as well as within the cytoplasm of plasma cells (Fig. 4). The latter cells were mostly found in the asthmatic group. Although the number of IgA- and IgE-positive cells was higher in the patients with asthma than in the control patients, only the difference in the IgA parameter between the two groups was statistically significant. About half the number of the mast cells were IgE-positive in the control patients. In contrast, the number of IgE-positive cells was much higher than that of tryptase-positive mast cells in all the asthmatic patients, because many plasma cells were also IgE-positive.

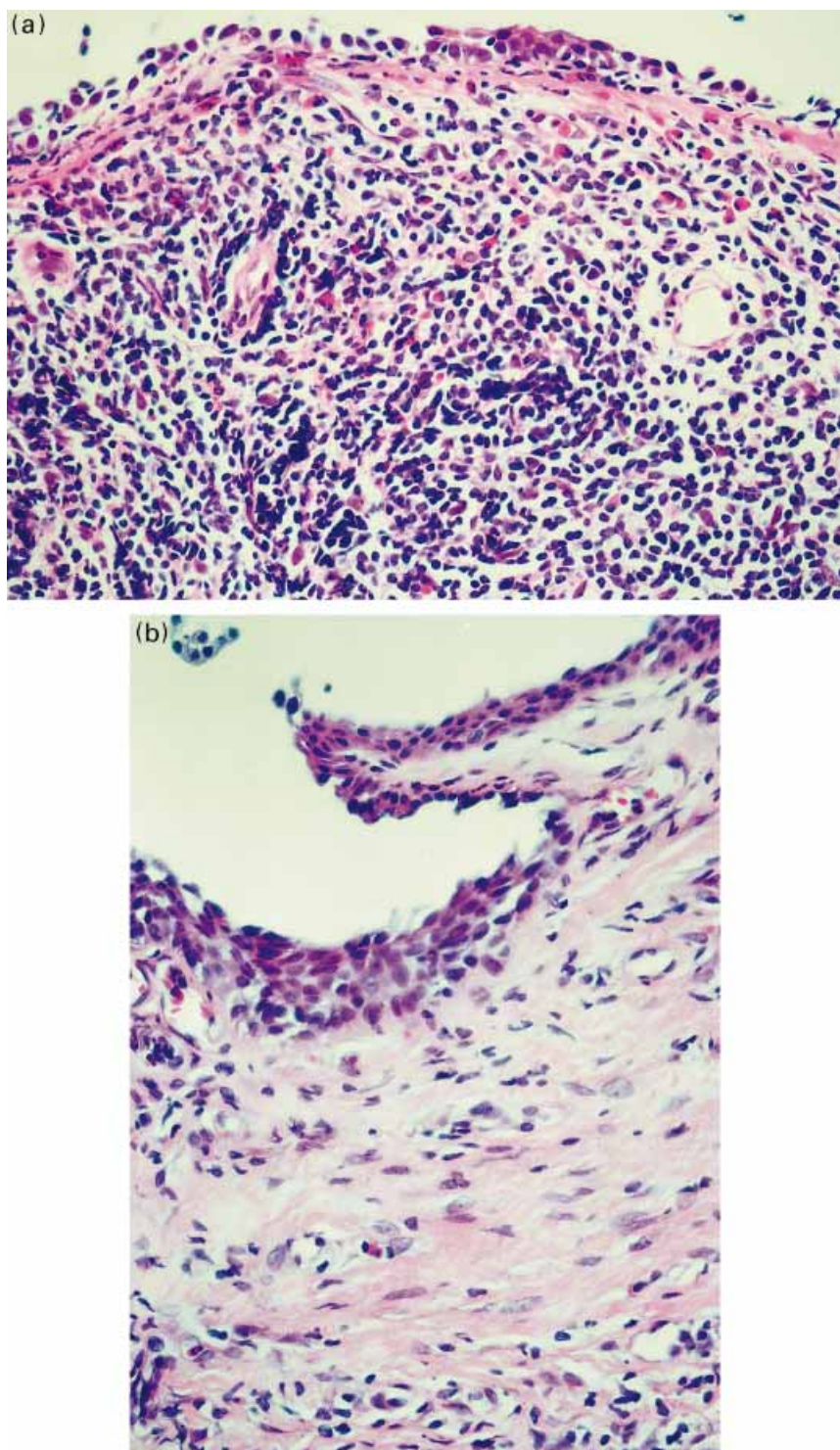


Fig. 1. (a) A histological section of the middle ear mucosa from a patient with intractable otitis media associated with bronchial asthma. Severe infiltration of round cells containing numerous eosinophils was observed in the submucosa (HE staining, $\times 400$). (b) A histological section of the middle ear mucosa from a control patient with chronic otitis media with central eardrum perforation. The infiltrating cells were mainly fibroblasts and fibrocytes with a few inflammatory cells. (HE staining, $\times 400$).

Histology and immunohistochemistry of middle ear effusion

Analysis of formalin-fixed, paraffin-embedded sections of the middle ear effusion from patients with asthma revealed accumulation of eosinophils, neutrophils and lymphocytes

(Fig. 5). The eosinophils were mostly degranulated and some of them were without nucleus. The effusion also contained granules probably released from eosinophils. Immunohistochemical staining for EG2 showed that most of the eosinophils were EG2-positive.

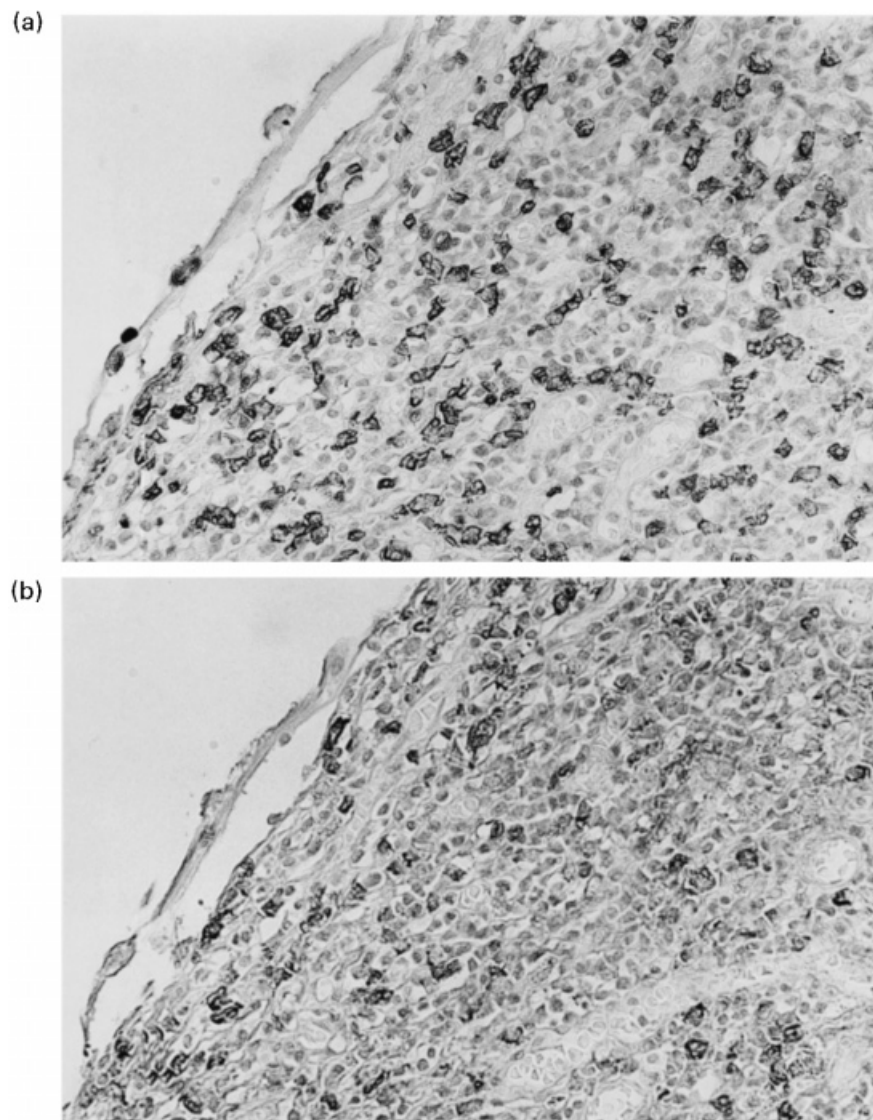


Fig. 2. (a) Immunohistochemical staining of the middle ear mucosa from a patient with intractable otitis media associated with bronchial asthma, using an antibody against EG-1. Many EG1-positive cells were observed in the submucosa ($\times 400$). (b) Immunohistochemical staining of the middle ear mucosa from a patient with intractable otitis media associated with bronchial asthma, using an antibody against EG2. Most of the EG1-positive cells were also EG2-positive ($\times 400$).

Table 1. The number of immuno-positive cells in the middle ear mucosa from the patients with asthma and the control patients ($\times 400$, five fields)

	Patients with asthma (<i>n</i> = 5)	Controls (<i>n</i> = 10)	<i>P</i>
EG1	128 $\pm$ 113	15 $\pm$ 12	< 0.01
EG2	106 $\pm$ 82	9 $\pm$ 9	< 0.01
Mast cell tryptase	40 $\pm$ 38	30 $\pm$ 14	n.s.
IgE	63 $\pm$ 52	17 $\pm$ 18	n.s.
IgA	168 $\pm$ 201	19 $\pm$ 20	< 0.05

n.s. = not statistically significant.

ECP concentration in middle ear effusion

A higher concentration of ECP was noted in the middle ear effusion samples obtained from six patients with asthma than in those from the control patients, as shown in Fig. 6. The difference was statistically significant between the two groups.

Discussion

It has been reported that in allergic otitis media or eosinophilic otitis media, one of the most striking features that is clearly different from the common type of otitis media is the marked accumulation of eosinophils in the middle ear mucosa and middle ear effusion [1–4]. However, the characteristics of eosinophils in cases of

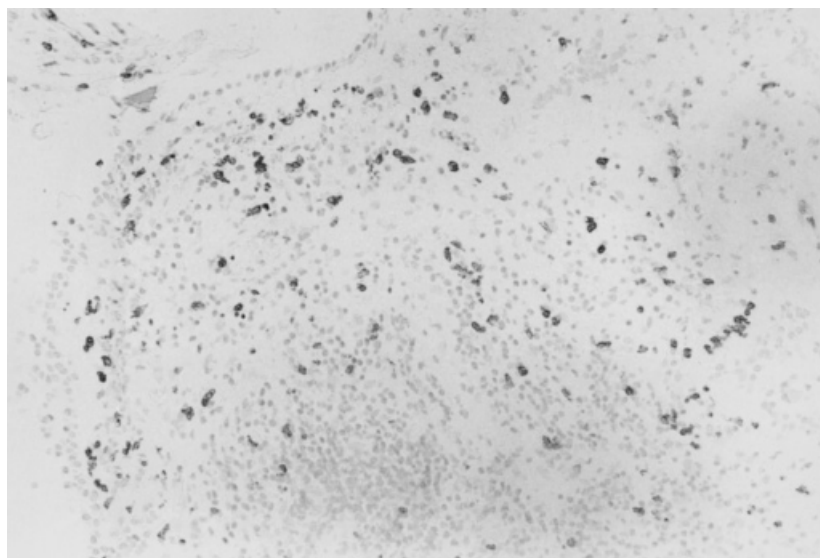


Fig. 3. Immunohistochemical staining of the middle ear mucosa from a patient with intractable otitis media associated with bronchial asthma, using an antibody against mast cell tryptase. Many tryptase-positive mast cells were observed in the middle ear mucosa ($\times 200$).

intractable otitis media have never been reported. In this study, we compared the middle ear specimens from asthmatic patients with those from control patients who were diagnosed as having a common type of otitis media such as chronic perforative otitis media or OME without bronchial asthma. The latter generally responded very well to the conventional treatment. As a result, a few eosinophils were found, and very few EG2-positive activated eosinophils were found, in the middle ear mucosa of the patients with the common type of otitis media. On the other hand, eosinophils in the middle ear mucosa and middle ear effusion in the asthmatic patients appeared highly activated and degranulated. These activated eosinophils may participate in the generation of granulated tissues or papillomatous

growth of the middle ear mucosa. That is, ECP released from the activated eosinophils has a potential of causing epithelial toxicity and damage to the middle ear epithelium, resulting in granulomatous tissue formation. In addition, activated eosinophils can release various cytokines and chemokines, such as IL-3, GM-CSF, IL-5, transforming growth factors and RANTES, which may perpetuate the inflammatory process, including the increased fibroblast function. These cytokines and chemokines play an important role in autocrine activation and survival of eosinophils.

Eosinophilic infiltration in the submucosal tissue is frequently found in nasal polyp tissues. Settupane [6] reported the incidence of the association between nasal

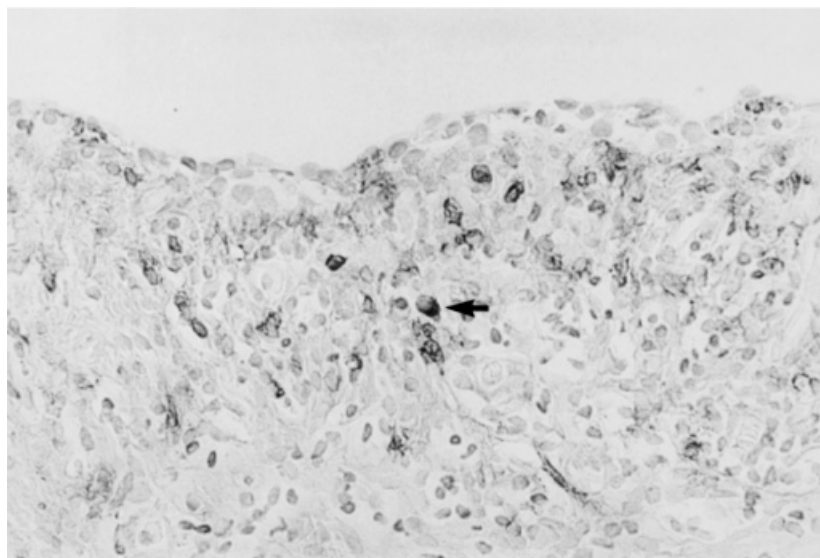
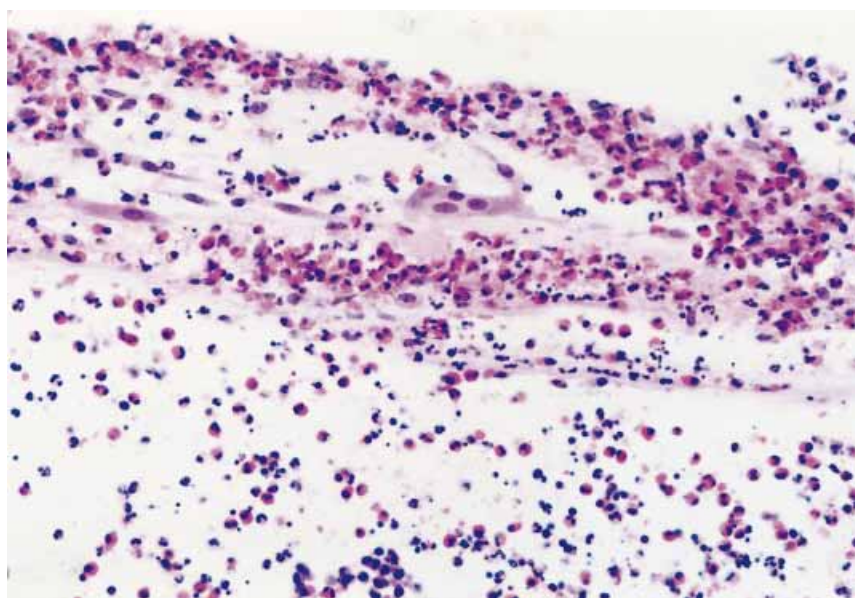


Fig. 4. Immunohistochemical staining of the middle ear mucosa from a patient with intractable otitis media associated with bronchial asthma, using an antibody against IgE. Many IgE-positive cells were observed in the submucosa. Positive staining was noted on the surface of mast cells as well as within plasma cells (indicated by an arrow).

Fig. 5. A histological section of the middle ear effusion from a patient with intractable otitis media associated with bronchial asthma. The middle ear effusion contained numerous neutrophils and eosinophils. Some of the eosinophils were degranulated and cytolytic. Eosinophils lacking the nucleus were also noted ($\times 400$).



polyposis and asthma, the rate being 5% in patients with atopic asthma, 13% in those with non-atopic asthma and 36–72% in patients with aspirin-intolerance asthma. All asthmatic patients in this study had nasal polyposis in addition to intractable otitis media. In the studies of nasal polyps from allergic subjects, numerous EG2-positive eosinophils were found in both the stroma and epithelium, suggesting excessive response of activated eosinophils in the polyps [7]. Bronchial biopsy and lavage studies on asthmatic patients also showed that many EG2-positive cells were found in the bronchial mucosa and a high amount of ECP was recovered [8,9]. The present study showed that the accumulated eosinophils in the middle ear

mucosa and effusions are highly activated and degranulated. It is suggested that the same intrinsic factor could occur in the entire respiratory mucosa, including the mucosa of the ear, nose and bronchus.

There are at least two degranulation events in eosinophils: piecemeal degranulation, in which the granular content is released from intracellular granules leaving more or less empty granules in intact cells, and eosinophil cytolysis, in which the cell membrane ruptures, causing the release of clusters of free eosinophil granules. A recent study showed that eosinophil cytolysis occurs in the human airway mucosa, such as nasal polyps, and that it is a major mechanism for eosinophil activation, together with piecemeal degranulation [10]. In the present histological and immunohistochemical study on middle ear effusion, we found that EG2-positive eosinophils, as well as cytolytic eosinophils, released cytotoxic eosinophil granules, suggesting that activation of eosinophils occurs in the middle ear.

Sensitive immunoassays for ECP serve as an indicator of eosinophil activation in asthma and allergic diseases [8,11,12]. Hurst et al. [13,14] observed high levels of ECP in persistent middle ear effusions in children positive for skin-test allergens, while low ECP levels were observed in middle ear effusions from adult controls. In the middle ear effusion in this study, significantly high ECP levels were observed in the asthmatic adult patients as compared with the control patients. This indicates an intrinsic immune-mediated process involving eosinophils activated within the middle ear itself.

Mast cells are believed to be the key cells involved in allergic inflammation, with an ability to release chemical mediators. The most widely recognized cause of mast cell

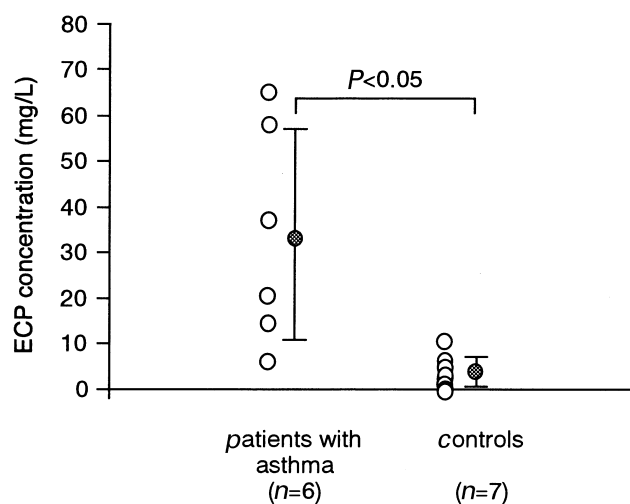


Fig. 6. ECP concentration in middle ear effusion from the patients with asthma and the control patients.

degranulation is allergic reaction, which is mediated mainly by IgE attached to the mast cells. A number of mast cells are also found in connective tissues, suggesting that they may play an important role in connective tissue repair. A recent enzyme-histochemical study showed that there are two kinds of mast cells; one contains only tryptase and the other contains both tryptase and chymase [15]. The tryptase-chymase double-positive mast cells are usually found at sites of allergic inflammation and of connective tissue repair, sometimes expressing an eotaxin receptor (CCR3), and are attracted to CCR3-binding chemokines, eotaxin and RANTES [16]. We showed that the number of mast cells in the middle ear mucosa of the asthmatic patients was not significantly different from that of the control patients with chronic perforative otitis media; however, these cells were distributed equally in the submucosa and close to the surface of the epithelium. Those found in the control group were mostly spindle-shaped and found in the deep layer, suggesting they might play a role in connective tissue repair. Consistent with this notion, the histological findings on submucosa of the control patients showed prominent scar tissues with few inflammatory cells.

Hurst et al. [17] histochemically examined the middle ear mucosa from atopic children with OME and normal adult subjects, and found that mast cells were present in the ears of most of the atopic children with OME but absent in the ears of all normal patients. In the bronchial mucosa, the number of mast cells in asthmatic patients does not differ from that in normal subjects but the cells display features of continuous granule secretion [18]. Mast cells are usually present in the normal or inflamed skin and mucosa. However, in the middle ear mucosa, there are very few resident mast cells, and they are extravasated into the mucosa and become activated under various conditions such as allergic and bacterial inflammation.

In the present study, all the asthmatic patients showed high serum IgE levels, although six of the eight patients exhibited no significantly high level of RAST score against inhaled antigens tested. The number of IgE-positive cells tended to increase in the middle ear mucosa of asthmatic patients compared with that of the control patients. About half the number of mast cells were IgE-positive on the surface of the cells in the mucosa of the control patients. In the asthmatic patients, the number of IgE-positive cells was much higher than that of mast cells, which indicates that there are many IgE-producing cells, probably plasma cells, in the middle ear mucosa. Eosinophils represent a key cell of the allergen-induced IgE-mediated late-phase mucosal inflammation seen in rhinitis, asthma [19,20] and atopic dermatitis [21]. Therefore, the accumulation of eosinophils in the middle ear mucosa is probably a late-phase response in the asthmatic patients. We have already reported that the

pathological change in the middle ear mucosa was severe around the tympanic orifice of the eustachian tube and mesotympanum in patients without eardrum perforation, and markedly severe and papillomatous in patients whose middle ear mucosa was exposed due to eardrum perforation [22]. The inflammatory response of the middle ear mucosa was stronger in the asthmatic patients than in the control patients because a significantly high number of IgA-positive cells was observed in the former group. We suggest that a strong inflammatory response to some foreign materials in the middle ear may occur in patients with highly sensitive allergic predisposition.

It has also been reported that the distribution of T lymphocytes, macrophages and activated eosinophils in bronchial mucosa did not differ between atopic and non-atopic asthmatic patients. The data suggest that non-atopic asthma may involve T lymphocyte activation by an as yet unidentified antigen [23]. The patients in this study with negative response to common inhalant allergens in terms of serum-specific IgE level, still showed high total serum IgE level. In nasal polyps, patients designated as non-atopic by skin testing and serum-specific IgE level exhibited local specific IgE in nasal tissues [24]. Thus, local IgE production in the middle ear mucosa in response to some unidentified antigenic materials could contribute to intractable inflammation in these patients. The present results indicate that intractable middle ear inflammation is closely associated with IgE-mediated late-phase response with eosinophil accumulation.

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