

Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma

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Summary

There is increasing evidence for the role of basophils in the pathogenesis of bronchial asthma. To examine the presence of basophils in the airways of patients with fatal asthma by immunohistochemistry, we stained lung tissues from four post-mortem cases who had died from severe asthmatic attacks and four controls with a monoclonal antibody raised against tryptase (AA-1) and anti-IgE. Mast cells and basophils were identified in the bronchioles as AA-1- and anti-IgE-positive cells, and anti-IgE-positive cells, respectively. Airway mast cells were found beneath the basement membrane, near blood vessels in the submucosa, and adjacent to the submucosal glands, and scattered throughout the muscle bundles. There was a significant increase of mast cells in the asthma group compared with the control group ($203.5 \pm 84.6/\text{mm}^2$, mean $\pm$ s.d. vs $37.7 \pm 8.7/\text{mm}^2$, $P < 0.05$, $n = 4$). In contrast, basophils were observed in the airway lumen, in the bronchial epithelium and in the submucosa. The number of basophils in the bronchioles was $81.8 \pm 55.5/\text{mm}^2$ ($n = 4$); however, basophils were not found at all in the airways of the control group. Although eosinophils, B lymphocytes and macrophages bear low affinity IgE receptors and could react with anti-IgE, the location of these cells in the close sections did not correspond closely with basophils. The presence of basophils in lung tissues obtained from fatal asthma patients supports the view that basophils play a role in the pathogenesis of bronchial asthma.

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Introduction

Bronchial asthma has now been recognized as an inflammatory disease. The inflammatory cells identified both by bronchoalveolar lavage (BAL) and by bronchial biopsy include mast cells, eosinophils, T lymphocytes, and macrophages [1–5]. Chronic inflammation of the airways results in the characteristic airway hyperresponsiveness of asthma.

The role of mast cells in the immediate asthmatic response is well established [6]. A recent study demonstrated that among patients studied 24 hr after allergen challenge, those who had developed a late asthmatic response had an increased number of mast cells as compared with patients who had not developed a late

response [1]. This result indicates that mast cells as well as other types of cells such as eosinophils are also involved in the late asthmatic reaction.

Increased numbers of basophils have been observed in skin biopsy sites and in body fluids obtained from the sites of cutaneous late-phase reaction (LPR) [7,8]. Bascom *et al.* demonstrated that basophil influx occurred after nasal antigen challenge [9]. Iliopoulos *et al.* have also shown that histamine-containing cells accumulating after nasal antigen challenge have functional and phenotypic characteristics of basophils but not mast cells [10]. The pattern of mediator release in the nasal LPR, particularly the lack of a secondary increase in PGD₂ which is specific to mast cells, and the steroid sensitivity of this response indicate the role of basophils in this reaction [9,11].

These data suggest that basophils are involved in the cutaneous and nasal LPR after antigen challenge. However, the role of basophils in the pathogenesis of asthma

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remains to be elucidated.

The aim of the present study was to examine the presence of basophils in the airways of four asthmatic patients, who had died of severe asthmatic attacks, by means of immunohistochemistry using a monoclonal antibody raised against tryptase (AA-1) and anti-human Ig.

Materials and methods

Subjects

Lung tissues obtained from four cases of fatal asthma (Case 1, 49-year-old man; Case 2, 6-year-old boy; Case 3, 6-year-old girl; Case 4, 25-year-old woman) were included in this study. All patients had atopic type of asthma. Autopsy was performed within a few hours after death from a severe fatal attack. The control lung tissues were obtained from four patients who underwent surgical operation for lung cancer. Intact portions of tissue were used as controls.

Blocks of lung tissue were fixed in 10% buffered formalin at 4°C for 24 hr and subsequently embedded in paraffin. Three micron paraffin sections were cut using a microtome.

Immunohistochemistry

Slides were deparaffinized, dehydrated and stained by peroxidase-antiperoxidase method (PAP) with a panel of monoclonal and polyclonal antibodies, with diaminobenzidine as the substrate for peroxidase. Double staining for mast cells and basophils was performed by combining PAP and alkaline phosphatase-antialkaline phosphatase method (APAAP), using a Double Stain Kit (Dako Japan Ltd, Tokyo, Japan). The substrate for alkaline phosphatase was fast red.

Tryptase is a neutral protease present almost exclusively in the granules of human mast cells, and we have used it as a specific marker for mast cells. The mouse anti-human tryptase monoclonal antibody AA-1 was kindly provided by Dr Walls (Southampton General Hospital, Southampton, U.K.) [12]. Mast cells and basophils bear high affinity IgE receptors (FcεRI) on their cell surfaces [13]. The rabbit anti-human IgE antibody has been used here as a marker for these cells. The sections were initially treated with 0.1% trypsin (Difco Laboratories, Detroit, Michigan, U.S.A.) at 37°C for 30 min to expose the epitopes of mast cell tryptase. For double staining for mast cells and basophils, the sections were incubated with AA-1 and anti-IgE at optimal dilutions determined by preliminary experiments (AA-1 at 1/200 and anti-IgE at 1/200). AA-1 binding was subsequently detected by the presence of a red reaction product using APAAP, while

anti-IgE binding was detected by the presence of a brown reaction product using PAP.

Mouse monoclonal antibodies EG1 and EG2 raised against eosinophil cationic protein (ECP) were used to stain the ECP present in the eosinophil granules [14]. Primed T lymphocytes, which include memory T cells were identified using UCHL1 (DAKO-CD45RO, Dako Japan Ltd, Japan) [15]. B lymphocytes were detected with SL26 (Kyowa Medics, Tokyo, Japan) [16]. Macrophages were stained with KP1 (CD68, Dako Japan Ltd) which reacts in paraffin sections [17].

Other staining

Close sections were also stained with haematoxylin-eosin and toluidine-blue (pH 4.0) for morphological estimation of these cells.

Some sections were double-stained with EG2 and anti-IgE to examine whether eosinophils could react with anti-IgE.

Quantitation

All slides were randomly coded and analysed by a single observer without knowledge of the subjects. Light microscopy was performed with an Olympus microscope BH-2 (Olympus Corp., Tokyo, Japan) using an eyepiece graticule at a magnification $\times 400$ unless otherwise indicated. Numbers of cells staining positively for antibodies, which were shown as the means of counted cells/mm² in five separate fields of the section, were determined by counting cells in the epithelium and submucosa of the bronchioles where cellular infiltration was most prominent in airway tissues. The diameters of the bronchioles were 0.1–5 mm.

Preparation of human lung mast cells

Human lung mast cells were prepared using a slight modification of the method of Lowman *et al.* [18]. Briefly, human lung tissue was dissected free from pleura, bronchi and blood vessels. Normal lung parenchyma was cut into fragments with a chopper for enzymatic dispersion. Tissue fragments were washed and stored at 4°C overnight in RPMI 1640 (GIBCO Laboratories, Grand Island, NY, U.S.A.) containing 15% heat-inactivated FCS, 4 mM glutamine and antibiotic-antimycotic mixture. On the following day, fragments were washed and then incubated with 1 mg/ml of collagenase (Wako Pure Chemical Industries, Osaka, Japan), 0.5 mg/ml of hyaluronidase (type 1, Sigma Chemical Co., St Louis, MO, U.S.A.) and 10 µg/ml of deoxyribonuclease (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) in RPMI 1640 with

2% FCS for 60 min at 37°C. Dispersed cells were separated from undissociated tissue by filtration through nylon gauze (100 µm) and were washed twice with RPMI containing 2% FCS. The residual tissue was subjected to a second digestion and cell separation. Cells obtained from each digestion were pooled, and dispersed mast cells were passively sensitized with human IgE myeloma serum (donated by Dr H. Tomioka, Toho University School of Medicine, Chiba, Japan) in RPMI 1640 with 15% FCS by overnight incubation at 37°C. The cells were washed twice with Pipes A (2 mM Pipes, 119 mM NaCl, 5 mM KCl, 0.03% human serum albumin), and finally resuspended with Pipes A containing 2 mM Ca^{2+} and 0.5 mM Mg^{2+} .

Then human lung mast cells were stimulated with 1/100 of anti-IgE or 0.2 µg/ml of calcium ionophore A23187 for 60 min at 37°C. Supernatants of the reaction mixture were assayed for histamine [19]. The cells were cytospun on slide glasses and fixed with 10% buffered formalin at 4°C overnight. The slides were stained with AA-1 using the APAAP method.

Statistical analysis

The unpaired *t*-test was used to compare results between groups.

Results

Immunohistochemical double staining study with anti-tryptase antibody (AA-1) and anti-IgE demonstrated two types of cells; tryptase-positive (stained red) anti-IgE-positive (stained brown) cells with a larger nucleus, and tryptase-negative anti-IgE-positive (stained brown) cells with a smaller nucleus (Fig. 1a). The former were mast cells (Fig. 1a, small arrow), and the latter were basophils (Fig. 1a, large arrow). Both types of cells were stained with toluidine blue. Increased numbers of mast cells were observed in the airway epithelium, beneath the epithelial basement membrane, in the submucosa, adjacent to submucosal glands and in muscle bundles of the bronchioles (Fig. 1b).

Basophils were mostly found beneath the basement membrane and in the submucosa of the bronchioles, and some basophils were also found in the lumen of the bronchioles (Fig. 1c, arrow). These cells were not found in the submucosal glands nor in the muscle bundles.

Many types of cells including eosinophils, B lymphocytes, T lymphocytes and macrophages have low-affinity IgE receptors (FcεRII) on their surfaces [20]. These cells could be regarded as anti-IgE-positive cells and be mistaken as basophils.

Activated eosinophils, as detected by the monoclonal antibody EG2, were markedly increased around the

airways and in the airway lumen (Fig. 1d). Some eosinophils were stained with EG1 (data not shown).

B lymphocytes which were stained with SL26 showed focal accumulation in the submucosa far from the airway epithelium (Fig. 1e). T lymphocytes stained with UCHL-1 (CD45RO) were found in the airway lumen and around the airway. Macrophages identified by KP1 were only present in the airway lumen (Fig. 1f). Therefore, the location of all these cells did not correspond closely with the anti-IgE-positive tryptase negative cells (basophils). Finally, eosinophils were double-stained with EG2 and anti-IgE at the same concentration. However, eosinophils which reacted with EG2 were not stained with anti-IgE. This result confirmed that the cells that were stained with anti-IgE were different from eosinophils.

Mast cells and basophils in the bronchioles, as determined by double staining, were counted. The results are shown in Table 1. There was a significant increase of mast cells in the lung tissue obtained from the asthma group compared with the control group ($203.5 \pm 84.6/\text{mm}^2$ (mean $\pm$ s.d.) vs $37.7 \pm 8.7/\text{mm}^2$, $P < 0.05$, $n = 4$). The asthma group showed $81.8 \pm 55.5/\text{mm}^2$ basophils in the airways. In contrast, basophils were not found at all in the control group. In the asthma group, there were about three times more mast cells than basophils.

To rule out the possibility that completely degranulated mast cells released all their tryptase, lost their reactivity to AA-1 and were counted as basophils, human lung mast cells obtained from a patient with lung cancer were stimulated with an optimal concentration of anti-IgE or calcium ionophore A23187 and stained with AA-1. As shown in Fig. 2, mast cells still retained their reactivity with AA-1 after degranulation as evidenced by release of histamine (60% with anti-IgE and 90% with ionophore A23187).

Discussion

This study demonstrated the presence of basophils present in the airways of patients with fatal asthma. We employed histochemical techniques to detect basophils, because metachromatic cells such as mast cells and basophils are possibly under-counted by toluidine blue staining after formalin fixation [21], and the distinction between mast cells and basophils is difficult when this dye is used. Antibodies used in this study were chosen for their ability to stain antigens in formalin-fixed and paraffin-embedded tissue sections.

It has been demonstrated that extracts of basophil-enriched preparations contained 0.046 ± 0.013 pg of tryptase per basophil, compared with 12 pg of tryptase per human lung mast cell [22]. However, the antibody (AA-1) which we used in this study has been reported not

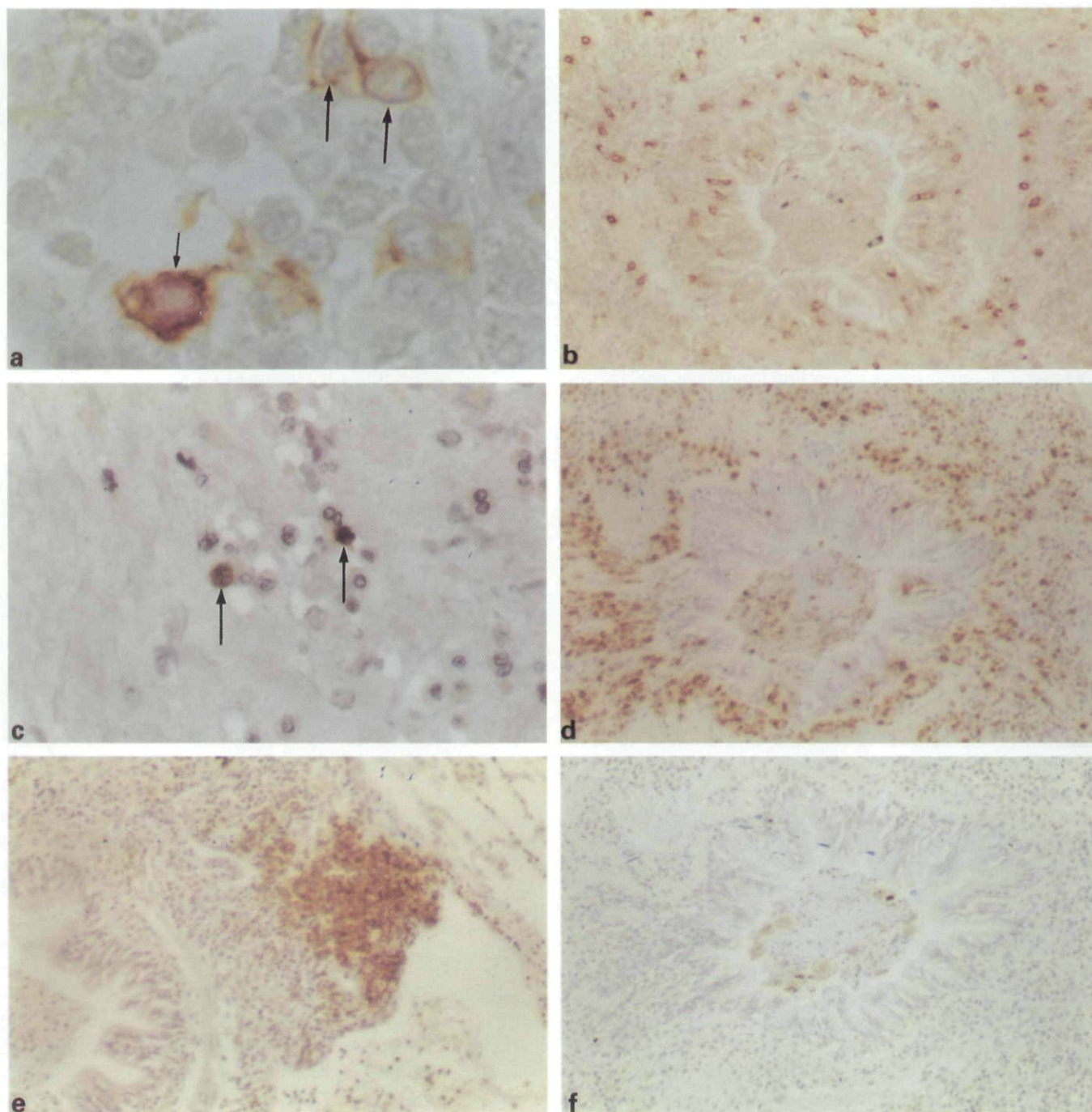


Fig. 1. Paraffin sections of a bronchiole from autopsy cases of asthma; (a), (b) and (c) show double staining with anti-tryptase antibody (AA-1) and anti-IgE antibody. (a) Mast cells show red staining of the cytoplasmic granules by APAAP reaction and simultaneous brown staining of the cell surfaces by PAP reaction. On the other hand, basophils show only brown staining of cell surfaces (magnification: $\times 1000$, Case 1). (b) Low magnification of the same section of Fig. 1(a) ($\times 200$). Mast cells are observed in the airway epithelium, beneath the basement membrane, and in the submucosa. Basophils are mostly found beneath the basement membrane and in the submucosa. The lumen is filled with a plug. (c) Two basophils are observed in the mucus plug in the airway lumen. Many inflammatory cells are also observed in this field (magnification: $\times 200$, Case 3). (d), (e) and (f) Immunohistochemistry of the close sections of Fig. 1(b) stained with EG2 antibody (d), SL-26 antibody (e) and KP1 antibody (f). The location of these cells does not correspond closely with basophils. (d) Increased numbers of EG2-positive activated eosinophils are observed in the airway lumen, beneath the epithelium and in the submucosa. (e) A focal localization of B lymphocytes is observed far from the airway epithelium. (f) KP1-positive macrophages are found only in the airway lumen (magnification: $\times 200$).

Table 1. Average numbers of mast cells and basophils in the airways of autopsy cases of fatal asthma and control

	Asthma	Control
Mast cell	203.5 ± 84.6	37.7 ± 8.7
Basophil	81.8 ± 55.5	0

The data are expressed as mean ± s.d./mm² (n = 4).

to react with basophils or other blood leucocytes [12]. Our preliminary study also showed AA-1 antibody did not bind to human peripheral basophils (data not shown). Therefore, we regarded the tryptase-positive anti-IgE-positive cells as mast cells and tryptase-negative anti-IgE-positive cells as basophils. It is possible that completely degranulated mast cells released all their tryptase, were not stained with AA-1, and were counted as basophils. However, when we stimulated human lung mast cells with anti-IgE or calcium ionophore A23187, mast cells retained their reactivity to AA-1. Thus, such a possibility is unlikely.

Eosinophils, T lymphocytes, B lymphocytes and macrophages/monocytes bear FcεRII on their surfaces [20]. Therefore, these cells could react with anti-IgE and be mistaken for basophils. However, eosinophils, which are identified as EG1- or EG2-positive cells, T lymphocytes detected by UCHL-1, B lymphocytes detected by SL26 and macrophages identified by KP1, did not correspond closely to anti-IgE-positive cells in close sections. Furthermore, a similar distribution of anti-IgE-positive cells and toluidine blue stained cells was observed. Thus, the possibility that FcεRII bearing cells were counted as basophils is unlikely.

Basophils were present in the lung tissue of patients who died of asthma. In contrast, they were not found at all in the control tissue. It is possible that the surgically removed tissue behaved differently than the post-mortem tissue due to factors such as the presence of neoplastic disease, prior therapeutic manipulation, and the different time course from tissue removal to fixation and staining. This difference could possibly account for the lack of basophils in control tissue. Thus, post-mortem lung tissue from normal individuals with accidental death would be best as a control. However, we were not able to obtain such tissue.

Although basophils have been implicated in LPR in the skin and nose, the participation of basophils in pulmonary reactions remains to be clarified [7–11]. However, recent data suggest a role of basophils in the pathogenesis of asthma. Liu *et al.* examined BAL fluids for cells 5 min and 19 hr following instillation of rag weed antigen

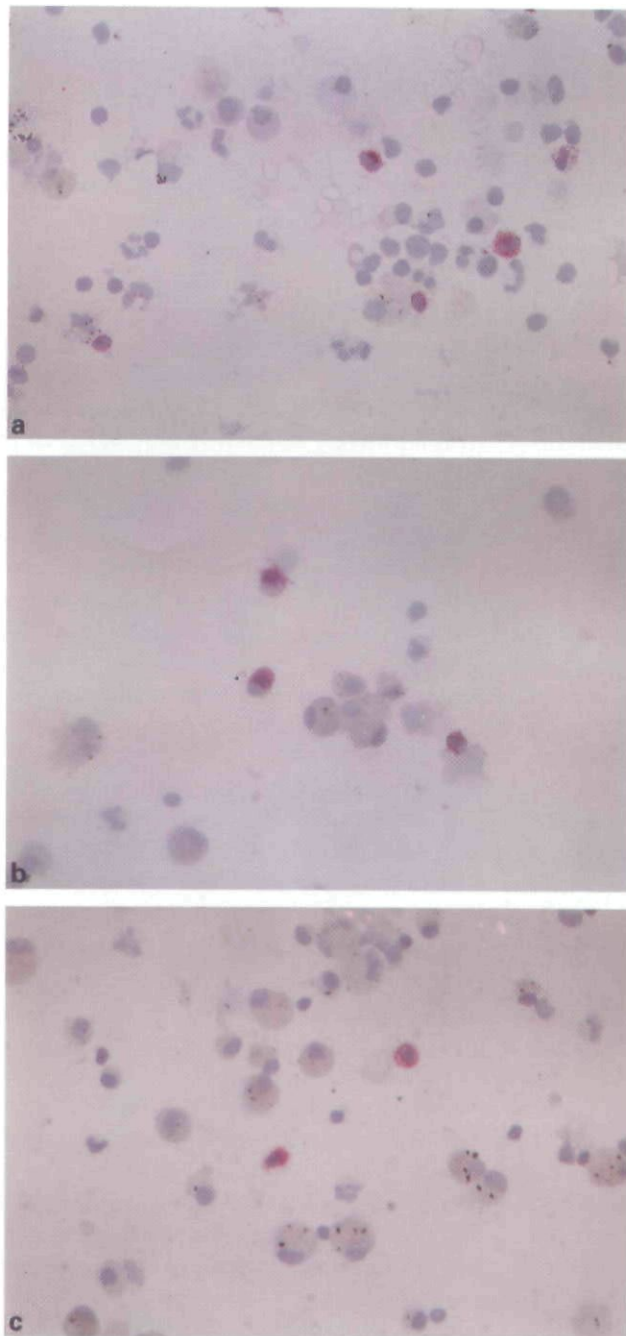


Fig. 2. Human lung mast cells stained with anti-tryptase antibody (AA-1). Human lung mast cells, which were reacted with buffer (a), 1/100 of anti-IgE (b) or 0.2 µg/ml of calcium ionophore A23187 (c), were stained with AA-1 antibody using APAAP method. The presence of tryptase is shown by red reaction product in the cytoplasm of mast cells (magnification: × 400).

directly into an airway segment of allergic asthmatics, and found that the 19 hr response to antigen challenge was characterized by a significant increase in basophils as well as eosinophils and lymphocytes [23]. Jarjour *et al.* reported the absence of correlation of tryptase with histamine in BAL fluid of allergic asthmatics, and suggested the participation of basophils in the pathogenesis of asthma [24]. Furthermore, it was demonstrated that spontaneous basophil release after allergen challenge was enhanced in subjects with asthma who developed a late asthmatic response after allergen inhalation [25]. We found that basophils were present in the airways of asthmatic patients who had died from asthmatic attacks. This finding suggests a role of basophils in the pathophysiology of asthma, although it is unclear whether the inflammation seen in the milder form of asthma is qualitatively or quantitatively different from that seen in fatal asthma.

Basophils were present beneath the basement membrane of the airway epithelium, in the submucosa and bronchial lumen of bronchioles. Unlike mast cells, basophils were not found in the submucosal glands, muscle bundles, and the alveolar septa. There were no basophils in the airways of the control group except in blood vessels. This finding suggests that basophils migrate to the airway epithelium and submucosa into the airway lumen from vessels.

Under normal conditions, basophils are only located in circulating blood. Therefore, the presence of basophils in the airways indicates that mechanisms exist which favour recruitment to these sites. The recruitment of circulating granulocytes from the circulation into tissue sites is dependent firstly on adhesive interactions between the leucocytes and the adjacent endothelial cells, secondly on subsequent transendothelial migration of leucocytes into the perivascular tissue, and thirdly on the local release of chemotactic and chemokinetic factors in tissues. Probably, the same is true for basophils. IL-3 has been shown to induce basophil adhesiveness for endothelial cells [26]. Recently, we have found that IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce migration of basophils in a dose-dependent fashion at picomolar concentrations, in addition to known effects of these cytokines on basophils, such as induction and priming of mediator release and enhancement of survival [27–32]. It has been reported that IgE-dependent stimuli, anti-IgE and antigen selectively promote basophil adherence to endothelial cells and CD11b expression [33]. Thus, allergen exposure may induce the localized recruitment of basophils by promoting their adherence to endothelial cells and transendothelial migration and subsequently by facilitating migration through cytokines produced by T lymphocytes and other inflammatory cells.

Finally, infiltrating basophils may release chemical mediators on stimulation with allergen.

In summary, we demonstrated the presence of basophils in lung tissues obtained from fatal asthma patients using immunohistochemical techniques. This finding supports the view that basophils play a role in the pathogenesis of bronchial asthma.

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