Rapid communication

Degranulation patterns of eosinophil granulocytes as determinants of eosinophil driven disease

J S Erjefält, L Greiff, M Andersson, E Ädelroth, P K Jeffery, C G A Persson

Abstract

Background—Degranulation of eosinophils in target tissues is considered a key pathogenic event in major chronic eosinophilic diseases. However, because of a lack of appropriate methods, little is known about degranulation of eosinophils in common eosinophilic diseases.

Methods—Using transmission electron microscopic (TEM) analysis, a novel approach has been devised and validated to quantify eosinophil degranulation in human tissues (assessed in individual cells as percentage granules with structural signs of protein release). Biopsy specimens from patients with inflammatory bowel disease, allergic rhinitis, asthma, and nasal polyposis were evaluated.

Results—All conditions displayed a similar degree of local tissue eosinophilia, with no differences being observed in eosinophil numbers in the airway mucosa of patients with airway diseases and the colonic mucosa of those with inflammatory bowel disease (IBD). In contrast, marked differences in the mean (SE) extent of eosinophil degranulation were observed between the patient groups; IBD 9.3 (1.4)% altered granules, artificial and natural allergen challenge induced allergic rhinitis 67.8 (6.8)% and 86.6 (3.0)%, respectively, asthma 18.1 (2)%, and nasal polyposis 46.6 (7.6)%.

Conclusions—This study provides the first quantitative data which show that different eosinophilic conditions, despite having similar numbers of tissue eosinophils, may exhibit markedly different degranulation patterns. The present assessment of piecemeal degranulation would thus make it possible to delineate the conditions under which eosinophils are likely to contribute to disease processes. This novel type of analysis may also guide and validate anti-eosinophilic treatment options.

(Thorax 2001;56:341-344)

Keywords: eosinophils; degranulation; allergy; airway pathology

Tissue eosinophilia is a central feature of major chronic diseases involving airway and gastrointestinal mucosa.¹ Local eosinophilia has also been used increasingly to diagnose major allergic and inflammatory diseases.² Eosinophil

granules contain cytotoxic proteins which, when released in the tissue by degranulation, cause injury and pathophysiological effects.³ An important consideration, beyond the mere presence of eosinophilia, is therefore whether or not degranulated tissue eosinophils can be identified and quantified in the target tissue. However, because no appropriate methods are available, little is known about degranulation in common eosinophilic diseases.

Molecular markers, including the monoclonal antibody EG2, have failed to distinguish degranulating eosinophils.⁴ Measurements of released granule proteins, although helpful, may not always reflect true release and cannot provide information about modes of degranulation.⁵ Fortunately, distinct ultrastructural features of eosinophils allow determination of piecemeal degranulation (PMD) and eosinophil cytolysis (ECL), the two major modes of eosinophil degranulation in human diseased tissues.⁵ However, no study has yet quantified and compared the occurrence of PMD and ECL in major eosinophilic diseases. By the use of a novel index of PMD (defined as percentage of granules displaying ultrastructural signs of granule protein release) and determination of ECL,⁶ we have examined eosinophil degranulation in asthma, allergic rhinitis, nasal polyposis, and inflammatory bowel diseases. Our data show that different eosinophilic conditions may exhibit markedly different eosinophil degranulation patterns.

Methods

PATIENTS AND TISSUE SAMPLING

All conditions examined are inflammatory mucosal conditions with local eosinophilia. Biopsy or surgical material were obtained from the diseased mucosal tissue, immediately immersed in fixative (PBS buffer containing 3% formaldehyde and 1% glutaraldehyde), and processed for light microscopy (EPO staining) and transmission electron microscopic analysis (see below).

To establish the usefulness of the present approach of quantifying eosinophil degranulation, it was considered advantageous to include patients representing a wide spectrum of eosinophilic conditions.

Asthma

All asthmatic patients (n=8) had a well documented history of asthma and atopy. Bronchial biopsy specimens were obtained out

Department of
Physiological Sciences,
Lund University
Hospital, 221 84 Lund,
Sweden
J S Erjefält

Department of
Otolaryngology, Lund
University Hospital
L Greiff
M Andersson

Department of Clinical
Pharmacology, Lund
University Hospital
C G A Persson

Department of
Respiratory Medicine
and Allergy, Umeå
University Hospital,
Sweden
E Ädelroth

Lung Pathology,
Department of Gene
Therapy, ICSTM,
National Heart & Lung
Institute, London, UK
P K Jeffery

Correspondence to:
Dr J Erjefält
jonas.erjefalt@mphy.lu.se

Received 20 December 2000
Returned to authors
15 January 2001
Revised version received
5 February 2001
Accepted for publication
5 February 2001

of the pollen season when disease was mild (as shown by symptoms, FEV₁, and PC₂₀ histamine values) and no steroids were taken.⁷

Allergic rhinitis

Nasal biopsy specimens were obtained from eight patients with symptomatic allergic rhinitis 24 hours after exposure to artificial allergen (daily provocations with allergen spray for 1 week).⁸ In a further five patients with symptomatic rhinitis nasal biopsy specimens were collected during natural allergen exposure (Swedish birch pollen season, 2000).

Nasal polyposis

Nasal polyps from three allergic and nine non-allergic subjects were obtained by routine surgery.

Inflammatory bowel diseases (IBD)

Biopsy specimens from the affected regions of the colon were obtained from patients with symptomatic inflammatory bowel diseases: ulcerative colitis (n=5), non-specific colitis (n=7), and Crohn's disease (n=8).

EOSINOPHIL PEROXIDASE (EPO) STAINING

Using light microscopy, the distribution of eosinophils was assessed in cryosections by histochemical staining of cyanide resistant eosinophil peroxidase.⁹ Briefly, cryosections (10 µm) were exposed to 100 µl incubation solution (PBS buffer, pH 7.6, containing 3,3-diaminobenzidine tetrahydrochloride (75 mg/100 ml, Sigma), H₂O₂ (100 µl/100 ml), and NaCN (50 mg/100 ml)) for 3 minutes at room temperature. After rinsing in tap water the samples were counterstained with Harris hematoxylin and mounted in DPX.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

After fixation, the biopsy specimens were rinsed in buffer, post fixed in 1% osmium tetroxide for 1 hour, dehydrated in graded acetone solutions, and embedded in Polarbed 812. One µm thick plastic sections were cut on an ultratome (Ultracut E, Leica, Germany), stained with toluidine blue, and examined under a light microscope (Axioscop, Zeiss, Germany). Areas with an intact epithelial lining were selected for further electron microscopical analysis. Ultrathin sections (90 nm) were cut and placed on a 200 mesh, thin bar, copper grid and stained with uranyl acetate and lead citrate.⁸ The specimens were examined by a Philips CM10 transmission electron microscope (Philips, Netherlands).

QUANTIFICATION

Tissue eosinophilia

Eosinophils were counted at a depth of 0–250 µm from the epithelial basement membrane and expressed as total numbers per 0.1 mm² tissue. The tissue area was calculated by computer assisted image analysis.

Ultrastructural analysis of piecemeal degranulation (PMD)

Each individual eosinophil was evaluated at ×5000 magnification. Each specific granule was examined and classified as either intact (no signs of degranulation—that is, intact core and matrix) or activated (various structural changes due to degranulation—for example, ragged loss of core material, coarsening of the granular matrix, or more or less empty granules).^{5–10} In order to express the degree of degranulation for each individual eosinophil, the percentage “activated granules” of the total granule number was calculated.⁹ TEM analysis also included identification of eosinophil cytolysis. Cytolytic death of eosinophils is characterised by chromatolysis, loss of plasma membrane integrity, partly dissolved cytoplasm, and release of membrane bound granules into the extracellular matrix.⁶

STATISTICAL ANALYSIS

Values of cell numbers and degranulation indices were logarithmically transformed before

Figure 1 (A) Numbers of tissue eosinophils and (B) extent of eosinophil degranulation in different eosinophilic conditions. Tissue eosinophils are expressed as numbers of cells/0.1 mm² subepithelial tissue. The eosinophil degranulation is expressed as mean percentage altered granules for individual eosinophils. On average, 103 eosinophils (~2100 granules) were assessed in each group. All data are presented as mean (SE). (C) Eosinophil degranulation patterns expressed as proportions of individual eosinophils displaying different levels of degranulation. Resting eosinophils: 0% altered granules; low piecemeal degranulation (PMD): 0–<20% altered granules; moderate PMD: 20–60% altered granules; extensive PMD: >60% altered granules; *artificial allergen exposure; #natural allergen exposure.

analysis and differences between the groups were examined using the Student's *t* test. *p* values of <0.05 were considered to be statistically significant. All statistical tests were performed with Microsoft Excel Version 5.0c; Astute Version 1.5.

Results

TISSUE EOSINOPHILIA

The cellular inflammation in all patient groups was characterised by a significant tissue eosinophilia (fig 1A). Eosinophils in biopsy specimens from patients with asthma, allergic rhinitis, and nasal polyposis were mainly located in the lamina propria tissue, just beneath the airway epithelial basement membrane. Eosinophil infiltration was also frequently observed in the airway epithelium (fig 2A). In the IBD material the highest density of eosinophils was typically present in the lamina propria tissue, close to the lumen and crypt epithelium (fig 2B). No, or exceedingly few, eosinophils were observed within the epithelial lining in material from patients with IBD.

GENERAL HISTOPATHOLOGICAL FINDINGS: TISSUE DAMAGE AND INFLAMMATION

A leucocyte rich inflammation was present in the subepithelial tissue from patients with allergic rhinitis, asthma, and nasal polyposis. The epithelial lining frequently displayed signs of damage repair processes such as the presence of undifferentiated repair cells.¹¹ Airway epithelial metaplasia with a stratified

cuboidal squamous epithelium was often observed in the nasal mucosa of patients with nasal polyps and rhinitis. A typical thickening of the basement membrane was observed in all airway conditions (fig 2A). In patients with IBD the subepithelial tissue was characterised by a leucocyte rich inflammation with extensive occurrence of cell apoptosis, necrosis, and scattered cell debris. The epithelial lining was typically intact with no or little signs of damage or leucocyte infiltration (fig 2B).

PIECEMEAL DEGRANULATION (PMD)

Despite a similar extent of tissue eosinophilia, profound differences in actual degranulation were observed in material from patients in the different clinical groups (figs 1B, 2C, 2D). For example, all nasal conditions had significantly higher degranulation than any of the IBD disorders (*p*<0.01). The extent of PMD in asthmatic patients was low to moderate (fig 1B). In the IBD groups the colon eosinophils displayed no, or only minor, signs of PMD (figs 1B, 2C). Subgrouping of eosinophils into degranulation levels revealed that resting eosinophils (containing only intact granules) were absent in the material from patients with symptomatic rhinitis where most of the eosinophils were involved in extensive degranulation (fig 1C). In contrast, resting eosinophils were frequently observed in material from patients with IBD or asthma (fig 1C). Correlation analysis found no relation between numbers of tissue eosinophils and the extent of degranulation in the same tissue.

Figure 2 Bright field (A, B) and transmission electron (C, D) micrographs showing EPO+ eosinophils in the colonic mucosa of a patient with Crohn's disease (B, C) or the nasal mucosa of a patient with active allergic rhinitis (A, D). Transmission electron microscopic analysis reveals that eosinophils in the colonic mucosa harbour granules which show minor signs of degranulation (C). By contrast, during active allergic rhinitis the typical nasal mucosal eosinophil has extensively altered granules. Scale bars: A=60 μ m, B=100 μ m, C and D=1.4 μ m.

EOSINOPHIL CYTOLYSIS (ECL), EXOCYTOSIS, AND APOPTOSIS

ECL was found to occur in all patient groups, being highest in those with allergic rhinitis where the proportions of cytolytic eosinophils in natural and artificial allergen induced disease were 27% and 33%, respectively. The extent of ECL in material from patients with nasal polyps and IBD was 10% and 14%, respectively. ECL was also observed in asthmatic subjects but, because of difficulties in discriminating between true ECL and mechanically produced cell rupture, no quantification was made.

Discussion

Using a novel approach to assess eosinophil degranulation in human tissues in vivo, the present study provides quantitative data which suggest that the extent of eosinophil degranulation may differ greatly between eosinophilic conditions. Hence, determinations of PMD and ECL appear as critical parameters in exploring the pathogenic role of eosinophils in inflammatory airway diseases.

TEM is the only technique currently available that can clearly identify and distinguish between different modes of degranulation.⁵ The large size and well defined structure and content of eosinophil specific granules make it possible to identify and quantify structural changes associated with granule protein release. Indeed, several previous in vitro studies, examining stimulated purified blood eosinophils, have shown that ultrastructural changes in eosinophil granules correspond to an actual extracellular release of granule proteins.¹²⁻¹³ However, lack of evident degranulation does not exclude the possibility that eosinophils are involved in important activities. For example, apart from defence against microorganisms (by degranulation), eosinophils have the capacity to participate in local immunoregulation or tissue repair by the release of cytokines and growth factors¹⁴ and their capacity to function as antigen presenting cells.

This study further strengthens the notion that PMD and ECL are the major cellular processes by which eosinophils release their cytotoxic granule proteins in human diseased tissues.⁵ Importantly, our data show that, despite the presence of local inflammation and a similar extent of tissue eosinophilia, the examined patient groups had marked differences in actual eosinophil degranulation. In allergic nasal mucosa and nasal polyps virtually all the mucosal eosinophils exhibited signs of pronounced PMD. Consequently, nasal eosinophilic diseases are perhaps more likely than the IBD disorders examined to be caused by the activity of local tissue eosinophils. The pronounced PMD and frequent ECL seen in biopsy material from patients with allergic rhinitis may reflect the symptom inducing allergen exposures that these patients experienced before tissue sampling, unlike the patients with mild asthma who had no prior exposure to allergens and displayed less degranulation. Recently reported ultrastructural observations¹⁵ suggest that eosinophils may also be subjected to extensive PMD and ECL in allergic skin diseases such as atopic dermatitis.

The present diverse degranulation patterns seen in the patient groups examined indicate that different types of mucosal inflammation are associated with different degrees of degranulation activities. Further studies are now warranted to distinguish between disease conditions with only marginal degranulation and those where eosinophils are likely to cause tissue disturbances through extensive degranulation. Such studies may also be essential to the evaluation of the effects of anti-inflammatory drugs on eosinophil degranulation. For example, identification of patient groups displaying extensive degranulation may be critical for optimal testing of the clinical efficacy of anti-eosinophilic approaches such as anti-IL-5 therapy.

In conclusion, by using and validating a novel approach for quantification of human eosinophil degranulation in vivo, this study has shown that eosinophil degranulation patterns differ markedly in different conditions. The assessment of PMD would thus make it possible to identify conditions under which eosinophils probably contribute to the disease process. This novel type of analysis may also guide and validate the increasing number of anti-eosinophilic treatment options.

This work was funded by The Swedish Medical Research Council, The Asthma and Allergy Association, The Heart & Lung Foundation, and The Vårdal Foundation, Sweden. The authors wish to thank Kristina Sjölund and Bodil Widerberg, Department of Gastroenterology, and Cecilia Ahlström-Emanuelsson, Department of Otolaryngology at Lund University Hospital for providing IBD tissues and nasal tissues.

- 1 Weller PF. Human eosinophils. *J Allergy Clin Immunol* 1997;100:283-7.
- 2 Persson CGA. Centennial notions of asthma as an eosinophilic, desquamative, exudative, and steroid-sensitive disease. *Lancet* 1997;350:1021-4.
- 3 Gleich GJ, Motojima S, Frigas E, et al. The eosinophilic leukocyte and the pathology of fatal bronchial asthma: evidence for pathologic heterogeneity. *J Allergy Clin Immunol* 1987;80:412-5.
- 4 Jahnsen FL, Brandtzaeg P, Halstensen TS. Monoclonal antibody EG2 does not provide reliable immunohistochemical discrimination between resting and activated eosinophils. *J Immunol Methods* 1994;175:23-36.
- 5 Erjefält JS, Persson CGA. New aspects on degranulation and fates of airway mucosal eosinophils. *Am J Respir Crit Care Med* 2000;161:2074-85.
- 6 Persson CG, Erjefält JS. Eosinophil lysis and free granules: an in vivo paradigm for cell activation and drug development. *Trends Pharmacol Sci* 1997;18:117-23.
- 7 Jeffery PK, Godfrey RW, Adelroth E, et al. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am Rev Respir Dis* 1992;145:890-9.
- 8 Erjefält JS, Greiff L, Andersson M, et al. Allergen-induced eosinophil cytotoxicity is a primary mechanism for granule protein release in human upper airways. *Am J Respir Crit Care Med* 1999;160:304-12.
- 9 Erjefält JS, Andersson M, Greiff L, et al. Cytotoxicity and piecemeal degranulation as distinct modes of activation of airway mucosal eosinophils. *J Allergy Clin Immunol* 1998;102:286-94.
- 10 Weller PF, Dvorak AM. Human eosinophils: development, maturation, and functional morphology. In: Busse WW, Holgate ST, eds. *Asthma and rhinitis*. Boston: Blackwell, 1994:255-74.
- 11 Erjefält JS, Persson CGA. Epithelial repair: breathtakingly quick and multipotentially pathogenic. *Thorax* 1997;52:1010-2.
- 12 Kita H, Weiler DA, Abu Ghazaleh R, et al. Release of granule proteins from eosinophils cultured with IL-5. *J Immunol* 1992;149:629-35.
- 13 Kroegel C, Dewar A, Yukawa T, et al. Ultrastructural characterization of platelet-activating factor stimulated human eosinophils from patients with asthma. *Clin Sci* 1993;84:391-9.
- 14 Gienbycz MA, Lindsay MA. Pharmacology of the eosinophil. *Pharmacol Rev* 1999;51:213-340.
- 15 Cheng JF, Ott NL, Peterson EA, et al. Dermal eosinophils in atopic dermatitis undergo cytolytic degeneration. *J Allergy Clin Immunol* 1997;99:683-92.