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Role of neutrophil elastase in hypersecretion in asthma

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Role of neutrophil elastase in hypersecretion in asthma. J.A. Nadel, K. Takeyama, C. Agustí. ©ERS Journals Ltd 1999

ABSTRACT: Goblet cell (GC) hyperplasia and mucous plugging are common in patients with acute asthma. These patients also show neutrophil recruitment into the airways. Neutrophils contain elastase, a potent secretagogue in airways. Therefore, it was reasoned that neutrophil recruitment, by releasing elastase, could result in GC hypersecretion. When neutrophil chemoattractants were instilled in the airways of guinea-pigs, time-dependent neutrophil recruitment and GC degranulation occurred. An inhibitor of leukocyte infiltration (NP15669) prevented both responses, implicating neutrophils. An inhibitor of neutrophil elastase (ICI 200,355) abolished GC degranulation, implicating elastase. Further studies implicate movement of elastase from cytoplasmic granules to the neutrophil surface, and they suggest a role for adhesion molecules on neutrophils and on GCs in neutrophil-dependent GC degranulation. Similarly, instillation of ovalbumin (OVA) into airways of OVA-sensitized guinea-pigs caused early recruitment of neutrophils and GC degranulation. GC degranulation was prevented by pretreatment with NP15669 or ICI 200,355. These results implicate neutrophil release of elastase in allergen-induced hypersecretion. The results suggest a mechanism for the mucous plugging that occurs in acute asthma; prevention of neutrophil recruitment, prevention of neutrophil-GC adhesion, or inhibition of elastase activity could provide effective therapy for this serious pathophysiological abnormality.

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Hypersecretion is generally considered to be a major cause of symptoms in chronic bronchitis [1, 2], bronchiectasis [3], and cystic fibrosis [4]. Hypersecretion is a more neglected component of asthma, but airway hypersecretion also plays a significant role in its pathogenesis, especially in acute asthma. *Post mortem* studies reported mucous obstruction in small airways [5], and subsequent studies have reported that goblet cell (GC) hyperplasia and mucous plugging are important findings in patients who die of acute asthma [6, 7].

In healthy subjects [8] and in pathogen-free animals [9], airways generally contain few GCs, but airways of asthmatics and of individuals with other hypersecretory diseases airways show GC hyperplasia [1–4, 6, 7]. Thus, GC hypersecretion is presumed to be an important factor in hypersecretory diseases, especially in peripheral airways where prolific GCs could easily cause plugging.

Goblet cell hypersecretion

GC hypersecretion involves two separate processes: 1) GC metaplasia; and 2) GC degranulation. This article focuses on neutrophil-dependent GC degranulation. The

mechanisms of hypersecretion are poorly understood, and there is no specific therapy. In chronic asthma, eosinophilic infiltrates predominate in airways, but acute, severe asthma is often associated with neutrophilic infiltration [10, 11]. In fatal attacks of short duration, neutrophils were reported to be predominant [12]. In early status asthmaticus, neutrophil infiltration is common [13].

Neutrophil elastase is a potent secretagogue in airway GCs [14, 15] and in airway submucosal glands [16] in various species, including humans [17] and provides a possible mechanism for neutrophil-dependent hypersecretion. Delivery of antigen into airways in allergic humans and in sensitized animals results in leukocyte recruitment. In the early phase, recruitment is mainly neutrophilic [9, 18–21]. It was reasoned that neutrophil recruitment, by releasing elastase, could cause GC degranulation. To examine this hypothesis, two series of experiments were performed in guinea-pigs *in vivo*, evaluating GC degranulation in the airways. Firstly, chemoattractants (interleukin (IL)-8 and *N*-formyl-leucyl-methionyl phenylalanine (fMLP)) were instilled into the trachea; in the second series of studies, ovalbumin (OVA) was instilled in the airways of sensitized animals. Neutrophil chemoattractants and antigen caused

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GC degranulation and neutrophil recruitment. The results indicate that chemoattractants cause neutrophil-dependent GC degranulation by adhesive interactions between neutrophils and GCs, resulting in elastase "release". Furthermore, the results implicate neutrophil elastase in allergen-induced GC degranulation. The results are reported in detail elsewhere [15, 22].

Methods

Methods of neutrophil-dependent GC degranulation are described in detail elsewhere [15] and will only be described briefly.

In vivo studies

Male Dunkin–Hartley outbred guinea-pigs (400–600 g; Simonsen Laboratories, Gilroy, CA, USA) were anaesthetized with pentobarbital sodium (35 mg·kg⁻¹, *i.p.*; Anthony Products, Arcadia, CA, USA). Drugs were delivered into the trachea *via* a 22 gauge angiocath catheter (Becton Dickinson, Sandy, UT, USA). At preselected times, the animals were perfused with 1% paraformaldehyde, the trachea was removed and placed in 4% paraformaldehyde (24 h), and then the trachea was cut and embedded in JB-4 plus monomer solution (Polyscience, Warrington, PA, USA). Sections (4 µm) were placed on a slide, stained with 3, 3'-diaminobenzidine (Sigma, St. Louis, MO, USA) to visualize migrated neutrophils. Neutrophils seen as myeloperoxidase-positive blue cytoplasmic cells were counted in 20 consecutive high-power fields of the epithelial layer. Slides were stained with Alcian blue/periodic acid–Schiff (PAS) and counterstained with haematoxylin and observed at ×400 magnification. IL-8 and fMLP were chosen to stimulate neutrophil migration, and the trachea was removed (1–4 h) after injection.

Quantification of goblet cell degranulation

The volume density of Alcian blue/PAS-stained mucosubstances on the mucosal surface epithelium was determined using a semiautomatic imaging system.

In vitro studies

The trachea was removed and cut into segments, bathed in Dulbecco's modified Eagle's medium (DMEM)-Hams F-12 medium containing HEPES buffer (25 mM) and incubated at 37°C in a 5% CO₂ water-jacketed incubator (Forma Scientific, Marietta, OH, USA). After the *in vitro* studies, the tracheal segments were prepared similarly to the *in vivo* studies.

Isolation of neutrophils

Because of the difficulty of obtaining sufficient guinea-pig neutrophils, human neutrophils were used in most experiments, purifying them from normal peripheral blood, as described elsewhere [15].

Sensitization and intratracheal challenge with OVA

Guinea-pigs were sensitized with OVA, 2.5 mg, *i.p.*; (grade V; Sigma), complexed with 50 mg alum in 0.5 mL NaCl (0.9%) on days 0 and 10. Intratracheal challenge with OVA was performed between days 20 and 22. The animals received an injection of pyrilamine maleate (10 mg·kg⁻¹, *i.p.*) 30 min before OVA challenge to prevent fatal anaphylactic shock. A 2% OVA solution in 150 µL saline was instilled.

Results

Effect of neutrophil chemoattractants on GC degranulation in vivo

Instillation of neutrophil chemoattractants fMLP and IL-8 (each at 10⁻⁷ M, 100 µL) intratracheally in guinea-pigs (n=5 animals per group) caused neutrophil recruitment (fig. 1a) and GC degranulation (fig. 1b) in a time-dependent fashion. Neutrophil recruitment and GC degranulation

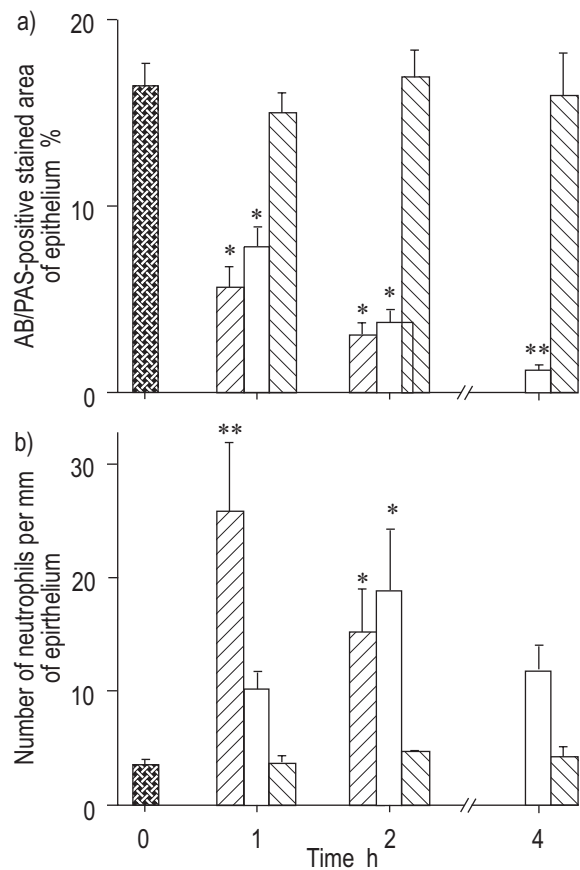


Fig. 1. – *In vivo* time course of effect of intratracheal injection of *N*-formyl-leucyl-methionyl-phenylalanine (fMLP; 100 µL, 10⁻⁷ M) and of interleukin (IL)-8 (100 µL, 10⁻⁷ M) on a) goblet-cell degranulation (expressed as a percentage of the area of epithelium stained with Alcian blue (AB) periodic acid–Schiff (PAS) and on b) neutrophil recruitment in guinea-pig trachea. ▨: control (animals euthanized immediately, without stimulation); ▤: intratracheal injection of fMLP (100 µL, 10⁻⁷ M); □: intratracheal injection of IL-8 (100 µL, 10⁻⁷ M); ▩: intratracheal injection of sterile phosphate-buffered saline (PBS; 100 µL). n=5 animals for each group. Responses are expressed as mean±SEM. *: p<0.05; **: p<0.01, significantly different from control values.

were dose-dependent. Pretreatment with NP-15669 ($10 \text{ mg}\cdot\text{kg}^{-1}$, *i.v.*), a molecule that prevents neutrophil migration [21], inhibited both neutrophil recruitment and GC degranulation completely ($p<0.01$). From these studies, it was concluded that IL-8-induced GC degranulation is neutrophil-dependent.

Pretreatment with ICI 200,355 ($700 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ *i.v.*; an inhibitor of neutrophil elastase) prevented IL-8-induced GC degranulation, implicating neutrophil elastase in neutrophil-dependent GC degranulation. Purified neutrophil elastase is a potent secretagogue in airway submucosal glands [16] and in GCs [14, 16] of various species [17], but when neutrophils are incubated with a neutrophil chemoattractant in a test tube, elastase is not normally released into the supernatant [23], indicating that elastase is not easily exocytosed from neutrophils stimulated by chemoattractants. Therefore, to discover how neutrophil elastase becomes available to degranulate goblet cells, *in vitro* studies were performed.

In vitro studies

Incubation of guinea-pig tracheal segments ($n=4$ animals per group) for 1 h with IL-8 (10^{-7} M), fMLP (10^{-8} M), or with neutrophils ($10^6 \text{ cells}\cdot\text{mL}^{-1}$) alone was without effect. However, incubation of tracheal segments with chemoattractant plus neutrophils together resulted in profound GC degranulation (fig. 2).

To determine whether GC degranulation was due to exocytosis of elastase and subsequent diffusion to the GC, or whether elastase remaining on the surface of neutrophils was responsible for degranulation, neutrophils were incubated with chemoattractants for 1 h. Then the neutrophils were harvested and washed with phosphate-buffered saline

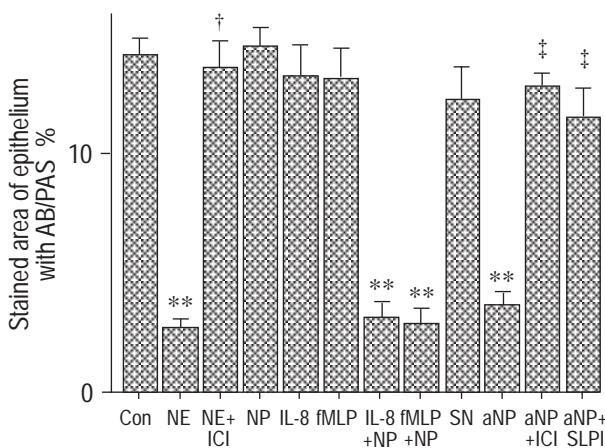


Fig. 2. – *In vitro* effect of neutrophils (NP) and of neutrophil chemoattractants on goblet cell degranulation (expressed as a percentage of the area of epithelium stained with Alcian blue (AB)/periodic acid-Schiff (PAS) in guinea-pig trachea. Various conditions examined include purified neutrophil elastase (NE; 10^{-7} M , used as positive control (Con)), NE ($10^6 \text{ cells}\cdot\text{mL}^{-1}$) alone, interleukin-8 (IL-8; 10^{-7} M) alone, *N*-formyl-leucyl-methionyl-phenylalanine (fMLP; 10^{-8} M) alone, NP plus chemo-attractant, NP "activated" (aNP) by IL-8 or their supernatant (SN). The effects of the NE inhibitors, ICI 200,355 (ICI; 10^{-5} M) and secretory leukocyte protease inhibitor (SLPI; 10^{-5} M) were also studied. Responses are expressed as mean \pm SEM; $n=4$ for each group. **: $p<0.01$, significantly different from control values. †: $p<0.01$, significantly different from response to NE. ‡: $p<0.01$, significantly different from the response to aNP.

(PBS) to avoid any further contamination with IL-8. The "activated" neutrophils caused GC degranulation, but the supernatant was without effect (fig. 2). Preincubation of the activated neutrophils with the elastase inhibitors ICI 200,355 (10^{-5} M) or secretory leukocyte proteinase inhibitor (SLPI; 10^{-5} M) prevented GC degranulation, indicating that elastase plays a role in the degranulation. These results suggest that elastase bound to neutrophils, and not elastase released into the free medium, causes GC degranulation. Therefore, the effect of neutrophil chemoattractants were studied on fibronectin-coated chamber slides to examine any possible translocation of elastase from the cytoplasm to the neutrophil surface.

Immunocytochemical localization of neutrophil elastase

Chemoattractants caused the surface expression of neutrophil elastase in a time-dependent manner. Adherent neutrophils incubated alone did not change shape and did not stain for elastase over a period of 45 min. Neutrophil incubation with chemoattractants resulted in changes in neutrophil shape; surface staining of elastase was present at 5 min and was at a maximum at 15 min. Surface staining was polarized, being stronger on one side of the neutrophils (fig. 3). These studies show that chemoattractants

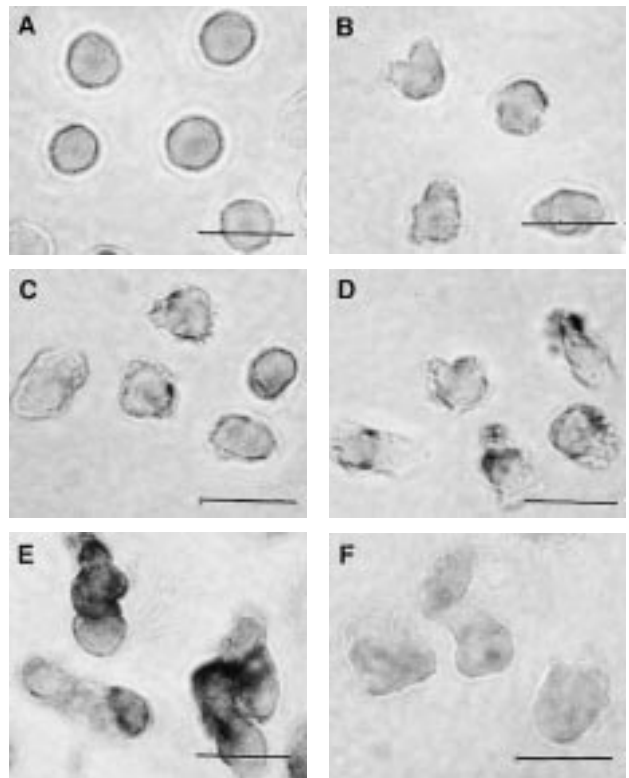


Fig. 3. – Time-course of the effect of *N*-formyl-leucyl-methionyl-phenylalanine (fMLP) on the surface appearance of neutrophil elastase in isolated neutrophils on fibronectin-coated chamber slides. Isolated neutrophils were incubated under various conditions on fibronectin-coated chamber slides. A: without fMLP; B: 2 min with fMLP (10^{-8} M); C: 5 min with fMLP (10^{-8} M); D: 10 min with fMLP (10^{-8} M); E and F: 15 min with fMLP (10^{-8} M). Cells were stained with a monoclonal mouse antihuman neutrophil elastase antibody (A–E) or with the antibody preabsorbed with a 100-fold excess of the purified neutrophil elastase (F). (Internal scale bars=160 μm .)

cause elastase to be expressed on the surface of neutrophils, where it is capable of interacting with tissue substrates (e.g. GCs).

To determine whether or not membrane-bound elastase is enzymatically active, the elastase activity of neutrophils was measured with an elastase-specific fluorogenic substrate (methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethylcoumarin; 200 μ M in 0.1 M HEPES buffer containing 0.15 M NaCl) for 25 min at room temperature. When neutrophils were incubated for 30 min with a chemoattractant, cleavage of the substrate by catalytically active membrane-bound elastase increased strikingly (fig. 4). These studies are compatible with the results of a recent study describing the binding of elastase to the neutrophil plasma membrane [24]. The studies by OWEN *et al.* [25] showed the importance of surface-bound elastase and of the pericellular environment in determining the enzymatic activity of elastase in tissues.

Role of adhesion molecules in neutrophil-dependent GC degranulation

Because elastase remains on the surface of stimulated neutrophils and is not secreted into the free medium, it was hypothesized that a close contact interaction between the surface of GCs and neutrophils is required for GC degranulation. Pretreatment of neutrophils with an anti-CD11b or anti-CD18 antibody inhibited chemoattractant-mediated GC degranulation. Similarly, preincubation of tracheal segments with an anti-intercellular adhesion molecule (ICAM)-1 antibody also prevented GC degranulation. These findings implicate adhesion molecules on neutrophils and GCs in neutrophil-dependent GC degranulation.

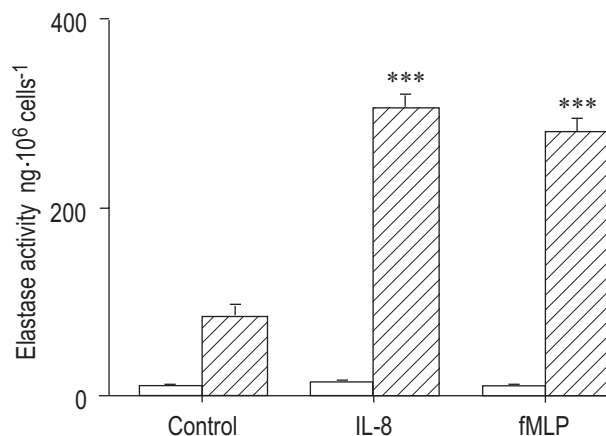


Fig. 4. – Effect of chemoattractants on catalytic activity of neutrophil elastase by methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin (AFC). Neutrophils (10^6 cells) were incubated with interleukin-8 (IL-8; 10^{-8} M) or with *N*-formyl-leucyl-methionyl-phenylalanine (fMLP; 10^{-8} M) for 30 min in a test tube. Cells were fixed with phosphate-buffered saline containing 3% paraformaldehyde and 0.5% glutaraldehyde (pH 7.4). Elastase activity was measured in the supernatant (▨) or on the surface of neutrophils (□) using AFC, a fluorogenic substrate that is specific for elastase. Data are expressed as elastase activity, ng · 10^6 cells⁻¹. Responses are expressed as mean \pm SEM; n=4 for each group. ***: $p < 0.001$, significantly different from control values.

Studies of human airways in vitro

Bronchial segments removed from four patients at the time of lung transplantation were selected because they contained GCs. The results were similar for guinea-pigs: incubation of bronchial segments with neutrophils plus IL-8 led to marked GC degranulation, an effect that was inhibited by an anti-CD18 antibody (fig. 5).

Effect of OVA challenge on GC degranulation in sensitized guinea-pigs

These studies are reported in detail elsewhere [22]. Intratracheal instillation of OVA (2%; 150 μ L) caused a marked GC degranulation that was significant within 1 h, persisted at 2 h and was associated with neutrophil recruitment into the tracheal epithelium. Pretreatment with NPC 15669 (10 mg · kg⁻¹, *i.v.*), a drug that prevents leukocyte migration, inhibited both neutrophil recruitment and GC degranulation. Pretreatment with an elastase inhibitor, ICI 200,355 (700 μ g · kg⁻¹, *i.v.*), also inhibited GC degranulation, but neutrophil recruitment was unaffected. Pretreatment with an anti-ICAM antibody (150 μ g · kg⁻¹ *i.v.*) significantly inhibited neutrophil recruitment and GC degranulation. These results suggest that antigen-induced GC degranulation is neutrophil-dependent, and that it involves a process involving adhesive interactions between the GC and neutrophil elastase acting at the neutrophil surface.

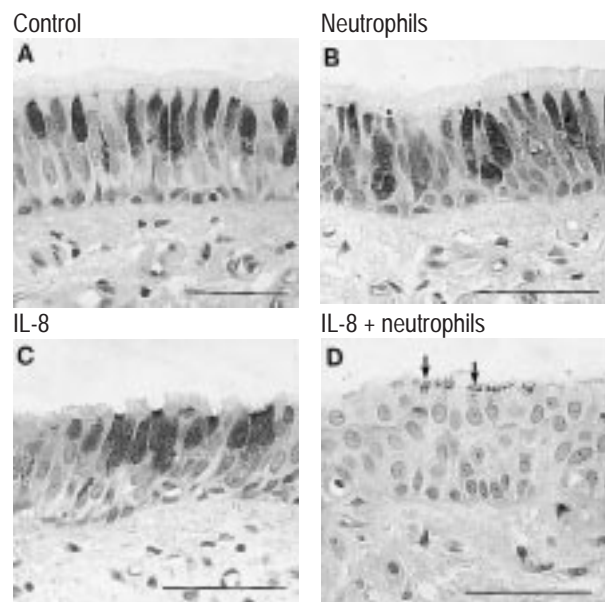


Fig. 5. – Photomicrographs of human bronchial epithelium removed from a patient with idiopathic pulmonary fibrosis at the time of lung transplantation and stained with Alcian blue/periodic acid-Schiff (PAS). In the unstimulated condition, Alcian Blue-staining cells are conspicuous in the bronchial epithelium (A). Bronchial segments that are incubated with neutrophils alone (B) or with interleukin (IL)-8 alone (C) do not show degranulation. However, segments incubated with IL-8 plus neutrophils show profound goblet cell degranulation (D; arrows). (Internal scale bars=50 μ m.)

Discussion

Purified neutrophil elastase is a potent secretagogue in airways [14–17], but when neutrophils are incubated with mediators that "activate" neutrophils *in vitro*, elastase is not normally released, so it is unclear whether or not intact neutrophils cause hypersecretion *in vivo*. Some effects of inflammatory cells have been shown to be due to intimate interactions between the inflammatory cell and tissue targets, and it was hypothesized that elastase could be released by a close contact interaction between neutrophils and GCs. Therefore, the effect of neutrophil chemoattractants on GC degranulation was studied in guinea-pigs *in vivo*. Instillation of neutrophil chemoattractants into the airways was shown to result in rapid neutrophil recruitment into the airway epithelium and GC degranulation. Inhibition of leukocyte motility (which inhibited neutrophil migration into the airways) prevented chemoattractant-induced GC degranulation, implicating neutrophils and their products in the response. Neutrophils contain three proteases capable of causing secretion: elastase, cathepsin G [16] and proteinase-3 [26, 27]. In the present studies, pretreatment with ICI 200,355, a selective inhibitor of elastase and proteinase-3 but not cathepsin G [28], prevented chemoattractant-induced GC degranulation. SLPI, an inhibitor of elastase and cathepsin G but not proteinase-3 [29], also prevented the GC degranulation. These findings implicate elastase in neutrophil-dependent GC degranulation.

To examine the mechanism of the elastase-induced effects, *in vitro* studies were performed. Neutrophils isolated from blood and incubated with tracheal tissue did not cause GC degranulation, but when the neutrophils were pre-incubated with chemoattractants and then incubated with airway epithelium, rapid and profound degranulation occurred, indicating that neutrophil activation was required for neutrophil-mediated GC degranulation.

Next, neutrophils were incubated with chemoattractants and the cells separated from the supernatant, and it was found that the neutrophils retained the ability to cause GC degranulation. It was concluded that elastase must have migrated from azurophilic granules in the cytoplasm, where it resides in resting neutrophils, to the neutrophil surface, where it is enzymatically active and available to tissue substrates (*e.g.* GCs).

When neutrophils are isolated and stained with an antibody, the antibody is too large a molecule to enter the cell, so only surface staining occurs. In the present study, resting neutrophils showed little surface staining, but when neutrophils were incubated with a chemoattractant, time-dependent surface staining occurred, which was marked by 15 min. These findings indicate that elastase activity in resting cells resides in the cytoplasm; during stimulation, surface expression of elastase occurs. These studies of surface elastase confirm the novel studies by OWEN *et al.* [24], who showed that in neutrophils incubated with chemoattractants, elastase moves to the surface and induces cleavage of elastase-specific substrates. The present studies implicate surface expression of elastase in the interaction with GCs.

Because elastase activity remained bound to the "activated" neutrophils, it was reasoned that a close interaction between neutrophils and GCs must occur to allow the elastase activity to signal GC degranulation. Adhesion

molecules can be expressed on neutrophils (*e.g.* MAC-1) and on airway epithelial cells (*e.g.* ICAM-1). These molecules have been characterized most extensively for their adhesive properties, but they are also reported to be involved in signal transduction in cells, especially in inflammatory responses [30]. In the present studies, blocking antibodies on epithelial cells (anti-ICAM-1) or on neutrophils (anti-MAC-1) inhibited neutrophil chemoattractant-induced GC degranulation. The exact mechanism by which the adhesive interactions permit surface-bound elastase on neutrophils to gain access to GCs is unknown. Perhaps MAC-1/ICAM-1 interaction stimulates the production of a second messenger in the neutrophils. The second messenger could change the conformation of surface-bound elastase, making it more accessible to GCs.

Another unknown is the exact molecular mechanism of elastase-induced degranulation of secretory cells. Proteinases, including neutrophil elastase [16], mast cell chymase [31], and cathepsin G [16] are the most potent secretagogues identified to date. Receptor-coupled secretion by agonists such as histamine occurs *via* cyclic adenosine monophosphate (cAMP), protein kinase C, and intracellular Ca^{2+} concentration-dependent pathways, but degranulation induced by elastase does not involve identified second messengers [32]. Elastase may activate degranulation directly, bypassing the signal transduction mechanisms necessary for receptor-mediated secretion.

What role could neutrophil-mediated hypersecretion play in disease? In chronic bronchitis [1, 2], bronchiectasis [3], and cystic fibrosis [4], where symptomatic hypersecretion is associated with neutrophil recruitment in the airways, the relevance is obvious. The relationship to asthma is more subtle! Chronic asthma is associated with eosinophilic infiltration of airways, especially in the submucosa. Because the half-life of eosinophils is measured in weeks (the half-life of neutrophils is days), it is assumed that inflammatory effects in asthma must be due, in large part, to eosinophils.

However, when neutrophils are recruited in the airways, they migrate from postcapillary venules located just below the epithelium. For example, in the present studies, airway recruitment of neutrophils by chemoattractants is seen in <1 h and is at a maximum in 2 h. Similarly, airway instillation of antigen stimulates neutrophil recruitment over a similar time period. During recruitment, neutrophils come in close contact with GCs, allowing a close contact interaction to occur. Thus, neutrophils travelling through the airway epithelium could play a major role in GC degranulation without prolonged residence in airway tissue.

An important question is what pathophysiological stimuli are most likely to promote neutrophil infiltration in airways in asthma? One can speculate on two possibilities: firstly, respiratory viruses induce the release of neutrophil chemoattractants from airway epithelial cells [33] and neutrophil infiltration, so viral infections could cause GC degranulation; and secondly, inhalation of antigens induces leukocytic infiltration characterized by early (1–4 h) neutrophil recruitment and later (12–48 h) eosinophil recruitment. Thus, inhalation of antigen could result in striking and rapid GC degranulation. Experimental models of asthma in rodents show that local delivery of antigen causes mucous plugging and leukocyte infiltration [34]; the present studies provide a mechanistic explanation for this effect.

The role of GC degranulation in clinical asthma remains unknown, and the solution may not be simple for several reasons. GC degranulation is likely to have the most significant effects in small airways, where degranulation of large GCs could easily completely obstruct individual airways. Obstruction of peripheral airways is not sensitively detected by the usual tests of pulmonary function [35], so alternative methods must be used. Airway plugging leads to areas of poor (or absent) ventilation, and this could lead to hypoxaemia (low ventilation compared to perfusion). Thus, a decrease in oxygen saturation without a drop in forced expiratory volume could suggest that peripheral mucus plugging is occurring. Furthermore, it would be useful if methods could be developed to visualize obstructed small airways.

Another important issue is the asthmatic population in which GC degranulation is most likely to be important. Again, this is unknown, but several facts are helpful. The post mortem findings were compared in asthmatic patients who died of severe acute asthma, asthmatics who died without evidence of severe acute asthma, and control subjects who died of nonrespiratory disorders. Airways of severe acute asthmatics showed 30-fold increases in GC area in peripheral airways with marked accumulation of mucus in airway lumens. However, in nonacute asthmatics, the GC areas and amounts of mucus in the lumens was small and not different from one another [7]. Similarly, SAETTA *et al.* [6] reported that asthmatics who died suddenly showed airway luminal occlusion with mucous plugs. These findings suggest that the morphological changes in acute, severe asthma differ from other asthmatics and that the risk factors in this population may be different.

One hypothesis is that susceptibility to mucous plugging of airways is determined by the existence and degree of goblet cell metaplasia that exists in the patient's peripheral airways. Thus, some patients may have massive overgrowth of peripheral airways by goblet cells, as seen in the airways of patients with mucous plugging who died of acute, severe asthma [7]. It is reasonable to assume that asthmatic individuals with marked goblet cell hyperplasia may be most at risk from stimuli (*e.g.* viral infections, inhalation of allergens) which may trigger goblet cell degranulation. An understanding of the mechanisms responsible for goblet cell growth and their prevention provides an important therapeutic strategy.

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