

Acute Allergic Responses Induce a Prompt Luminal Entry of Airway Tissue Eosinophils

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Traditionally, traffic and activation of eosinophils in asthmatic airways are thought to take place during the late-phase allergic reaction. The present study tests the hypothesis that when eosinophils are present in the tissue before allergen exposure, as in chronically inflamed asthmatic airways, acute anaphylactic reactions initiate an eosinophil response. Using a guinea-pig allergic model, where eosinophilia is present at baseline conditions, the traffic of resident eosinophils was examined *in vivo* immediately after allergen challenge. By 2 min after challenge, eosinophils had moved up to apical epithelial positions. Within 10 min, a marked migration of eosinophils into the airway lumen was demonstrated. Along with the allergen-induced egression of eosinophils, acute luminal entry of plasma proteins and eotaxin occurred. Eosinophil egression was effectively inhibited by the antiexudative drug formoterol, whereas the proexudative drug bradykinin could in naive animals evoke a prompt luminal entry of eosinophils. In conclusion, the present study demonstrates that acute allergic reactions initiate a prompt transepithelial migration of resident eosinophils. Our data further suggest that this response in part is initiated by the plasma exudation response, which may alter the transepithelial gradient of eosinophil chemoattractants including eotaxin. We propose that prompt eosinophil response is a significant component of the acute phase of allergic reactions when occurring in airways where these cells are already present in the mucosa.

Traditionally, the immediate anaphylactic airway response has been viewed as a mast cell-mediated event involving bronchoconstriction and edema formation (1). By contrast, the local recruitment and activation of leukocytes and subsequent development of an eosinophil-rich "cellular inflammation" has been considered as the main feature of the late phase allergic reaction (2, 3). However, although little studied, the acute allergic reaction may potentially also trigger significant leukocyte events, especially when it overlies chronic allergic conditions, where the airway mucosa constitutively harbors elevated numbers of leukocytes (4–6). For example, it has been little appreciated that the plasma extravasation, which occurs immediately after an

allergen provocation (7, 8), may have an effect on those leukocytes residing in the tissue at the time of challenge.

After leaving the circulation from high permeability venules, extravasated plasma, with its content of leukocyte-active molecules, is distributed throughout the mucosal tissue as well as into the airway lumen (7, 9). On its passage through the lamina propria tissue, across the epithelial basement membrane, and up between the airway epithelial cells, the bulk plasma exudate, through its rich content of cytokine-binding proteins (10), apparently picks up local tissue mediators and brings them to the mucosal surface (7). We hypothesized that such a change of the mucosal milieu would influence those inflammatory cells that are already present in the mucosal tissues at the time of challenge. In agreement, preliminary studies examining guinea-pig large airways (which, independent of sensitization, are constitutively rich in eosinophils [11, 12]) suggest that eosinophils residing at the level of the epithelial basement membrane move to an apical epithelial position shortly after allergen challenge (13). As suggested by measurements already at 1 h after allergen exposure many of these granulocytes may actually move into the airway lumen (13).

This study uses an *in vivo* model of acute allergic responses that specifically involves the tracheobronchial airways of guinea pigs, which are constitutionally characterized by mucosal eosinophilia as well as by a profuse superficial microcirculation (7). The focus of this study is on eosinophil trafficking induced by allergen challenge-induced immediate responses. In an attempt to explain luminal entry of eosinophils, we have further focused on acute plasma exudation responses, including the possibility that mucosal chemoattractant molecules will promptly appear on the mucosal surface along with a plasma exudate. Analysis of eotaxin was thought to be of interest because this chemokine has been shown to appear in airway lavage fluids after allergen challenge in humans (14, 15). Eotaxin has also been suggested to be responsible for the homing of eosinophils to the guinea-pig tracheal mucosa (16, 17).

Materials and Methods

Sensitization and Selective Allergen Challenge of the Tracheobronchial Airways

Male guinea pigs (Dunkin-Hartley, 200 g; Møllegaard, Ejby, Denmark) were sensitized to ovalbumin by one intraperitoneal injection of 0.5 ml saline containing 100 mg Al (OH)₃ (F2200; Anachemia, Montreal, Canada) and 2 µg ovalbumin (OVA) (grade III; Sigma, St. Louis, MO) (18). Three weeks after sensitization, animals were challenged by local allergen exposure of the tracheo-

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Abbreviations: bronchoalveolar lavage, BAL; eosinophil peroxidase, EPO; ovalbumin, OVA; phosphate-buffered saline, PBS; saline sodium citrate, SSC; tissue nonspecific alkaline phosphatase, TNAP.

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bronchial airways as previously described (13). Briefly, animals were anaesthetized by intramuscular administration (1 ml/kg) of a 2:3 ratio of Xylazine (Rompun, 20 mg/kg; Bayer, Leverkusen, Germany) and Ketamine (Ketalar, 50 mg/kg; Park Davies, Detroit, MI) and placed in an upright supine position. A thin plastic catheter was gently inserted via the mouth into the tracheal lumen 5 mm below the larynx. Next, using a microinjector (Carnegie Medical, Stockholm, Sweden) and a pump rate of 20 μ l/min, the allergen (10 pmol OVA in saline) was administered to the upper airways during 2 min (control animals received saline only). Importantly, this technique evenly and selectively distributes the allergen to the mucosal surface of the large airways only (i.e., the trachea and main bronchi) (19). The experiment was terminated at 2, 10, 30, and 60 min after initiation of the allergen challenge, when lavage and tissue samples were collected for further analysis.

In separate experiments the antiexudative capacity of β_2 -agonists (20, 21) was used to explore the correlation between plasma extravasation and eosinophil migration. At 5 min before allergen challenge, 100 μ g/kg of the β_2 -agonist formoterol (Astra-Zeneca, R&D, Lund, Sweden) was administered by intravenous administration. After the allergen provocation the animals were kept for 30 min before the experiment was terminated.

Airway Challenge with Bradykinin

In separate animals plasma extravasation, in the absence of an allergic inflammation, was assessed for its ability to induce an acute luminal influx of eosinophils. In nonchallenged animals, the proexudative drug bradykinin (Bradykinin acetate B-3259; Sigma) was administered by local tracheobronchial superfusion (10 nmol during 2 min) as previously described (19). At 30 min after bradykinin administration, the experiment was terminated, and luminal and tissue samples were collected.

Readministration of Tracheal Superfusates into the Upper Airways of Naive Animals

In separate animals the proexudative and chemoattractant properties of tracheal superfusates (obtained from previously OVA-challenged animals) were tested. Briefly, 250 μ l tracheobronchial lavage samples were obtained (as described below) 10 min after OVA challenge. The lavage cells were removed by centrifugation, and 100 μ l of the cell-free supernatant was readministered to the airway mucosa of naive animals by tracheal superfusion during 5 min.

Tracheobronchial Lavage

A previously validated technique was used to collect upper airway surface liquid without disturbing the baseline mucosal barrier functions (19). Briefly, immediately after killing, the airways (including larynx and lungs) were excised *in toto*. The lung lobes were gently tied off at the hilar region and a small incision was made in the left mainstream bronchus. A catheter (PE50) was introduced, secured and the airways were mounted vertically. Next, the upper airways were rinsed by pushing 0.25 ml saline three times through the trachea and the adjoining main bronchi.

Assessment of Plasma Extravasation and Exudation

^{125} I-labeled human serum albumin (Energiteknikk, Kjeller, Norway) was injected intravenously through a marginal ear vein 2 min before the OVA exposure. The extent of plasma extravasation

was determined by the ^{125} I-albumin content in airway lavage supernatants, as previously described in detail (19). Briefly, by determination of radioactivity in lavage samples and blood plasma (reference sample) in a Compugamma counter (Pharmacia-Wallac, Uppsala, Sweden) the amount of extravasated luminal plasma (expressed in μ l) was calculated using MULTICALC software package (Pharmacia-Wallac) (18).

Quantification of Luminal Eosinophils

Lavage samples were obtained, as described above, and 10 μ l was used to determine the total cell content using a haemocytometer. Twenty thousand cells were administered to glass slides using a cytospin centrifuge (Cytospin 3; Shandon, Astmoos, UK) and eosinophil numbers were determined after staining with May-Grünwald Giemsa. The extent of luminal eosinophils was expressed as total numbers per 0.2 ml lavage fluid.

Assessment of Tissue Expression of Eotaxin mRNA by *In Situ* Hybridization

Two 30-mer oligodeoxynucleotide probes complementary to nucleotides 115–144 (probe 1) and 286–315 (probe 2) in guinea pig eotaxin cDNA were designed (22). Because two different bases have been reported in position 299 in guinea pig eotaxin cDNA, these bases were mixed for the synthesis of the relevant probe (probe 2). The regions of eotaxin cDNA complementary to the probes show no homology with any other known cDNA as established by an EMBL/GenBank database search in Jan 2002. The probes were end-tailed with ^{35} S-dATP, using a terminal transferase (both supplied by NEN duPont, Stockholm, Sweden). After labeling, the probes were purified using Chroma spin-10 columns (Clontech, Intermedica, Stockholm, Sweden).

Frozen sections (10 μ m) were thaw-mounted onto chrome alum-coated slides and stored overnight at -20°C . They were then rapidly warmed to room temperature and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) for 30 min. The sections were rinsed (5 min $\times$ 2) in PBS and then acetylated by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Before hybridization; the sections were dehydrated through graded concentrations of ethanol, cleared in chloroform, and finally rinsed in 100% ethanol. The hybridization buffer contained 50% formamide, 4 $\times$ saline sodium citrate (SSC; 1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 $\times$ Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 10% dextran sulfate, 0.24 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, 1% sarcosyl, and 17 mM Na_2HPO_4 . The concentration of the probes was ~ 1.0 pmol/ml. Before hybridization, 0.1 M dithiothreitol was added to the hybridization buffer. To each slide, which typically contained two sections, 50 μ l of hybridization buffer was applied. The sections were covered with a parafilm slip to prevent evaporation. Hybridization was performed overnight in sealed moist chambers at 43°C . After hybridization, the coverslips were removed by immersion in 1 $\times$ SSC at room temperature. The sections were washed in 0.5 $\times$ SSC (4 $\times$ 15 min at 55°C and 1 $\times$ 30 min at room temperature). Finally, the sections were dehydrated by 70 and 96% ethanol, to which 0.3 M ammonium acetate had been added, followed by 100% ethanol, after which they were air-dried. For autoradiography the slides were dipped in Ilford's K5 film emulsion diluted 1:1 with distilled water, air-dried, and kept refrigerated at 4°C in light-sealed boxes. Exposure time was 2 wk. The autoradiographs were developed in Kodak D-19 and mounted in Kaiser's medium (glycerol-gelatin; Merck, Darmstadt, Germany). For control purposes, sections were incubated in RNase A (45 mg/ml; Sigma) for 30 min at 37°C before

hybridization. As additional controls, a 100-fold molar excess of unlabeled probe was added to the hybridization buffer.

Additional cryosections were subjected both to histochemical visualization of cyanide-resistant eosinophil peroxidase activity (13), detecting eosinophils, and *in situ* hybridization. Briefly, before the *in situ* hybridization protocol, cryosections were stained for eosinophil peroxidase (EPO; *see below*), rinsed, and then further processed according to the *in situ* hybridization protocol. In preliminary tests, it was established that neither the visualization of cyanide-resistant eosinophil peroxidase activity nor the probe labeling were altered due to the simultaneous labeling.

Luminal Content of Eotaxin

The content of eotaxin in tracheobronchial lavage samples was assessed by an enzyme-linked immunosorbent assay technique. Immunoreactive guinea pig eotaxin was measured using a mouse anti-guinea pig eotaxin monoclonal antibody (Clone 72D, a kind gift from Dr. T. Wells, Serono, Geneva, Switzerland) as the coat antibody and rabbit anti-guinea pig eotaxin IgG (B3, generated in house) as the detector and anti-rabbit IgG-HRP (Amersham Life Sciences, Buckinghamshire, UK). The reaction was developed by incubation with K-blue substrate (Neogen, Lexington, KY) and stopped by the addition of 100 μ l of 0.18M H₂SO₄, and the samples were read at OD450. The detection limit for the guinea pig eotaxin enzyme-linked immunosorbent assay was 20 pM.

Assessment of Eosinophil Distribution in Tracheal Sections

Two 4-mm tracheal rings, separated by 5 mm, were immersed in fixative (4% paraformaldehyde in 0.1 M PBS [pH 7.2]) overnight at 4°C. After rinsing in Tyrode buffer (PBS buffer supplemented with 10% sucrose), the rings were frozen on mounting stubs using Tissue Tec mounting medium (Sekura Finetek, Zoeterwoude, Netherlands). Eosinophils in tracheal mucosa were detected in 8 μ m transversally cut cross-sections by histochemical staining of cyanide-resistant eosinophil peroxidase (12). Tissue sections were incubated at room temperature for 8 min in PBS buffer supplemented with 3,3-diaminobenzidine tetrahydrochloride (75 mg/100 ml), 30% H₂O₂ (0.3 ml/100 ml), and sodium cyanide (120 mg/100 ml). Next, sections were counterstained with hematoxylin and mounted in Kaiser's medium (Merck). From each animal the total number of airway mucosal eosinophils (including intraepithelial eosinophils and the eosinophils present in the subepithelial tissue down to the perichondrial layer of the cartilage) were quantified at $\times 400$ magnification in two separate tracheal regions. Apical intraepithelial eosinophils (defined as eosinophils present within 0–10 μ m from the apical surface of the epithelium) were quantified using an internal scale and expressed as numbers per 1 cm epithelial lining. The occurrence of eosinophils in the apical aspect of the airway epithelium has previously been validated as a sensitive parameter of early eosinophil migration in response to acute epithelial damage (12).

Assessment of Eosinophil Distribution in Tracheal Whole-Mount Preparation

At the termination of the experiment, the trachea was cut along its dorsal side, gently stretched out on Sylgard-coated petri dishes, and immediately placed in fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.2) overnight at 4°C. For proper orientation, the apical surface of the airway epithelium was visualized by histochemical staining of tissue nonspecific alkaline phosphatase (TNAP), as previously described (13). Whole mount preparations were incu-

bated at room temperature for 5 min in a TRIS-HCL buffer (pH 9.0) containing 0.1% naphthol AS-BI phosphate (Sigma) and 0.1% Fast Blue (TR-salt; Sigma). In separate control experiments, it was demonstrated in cross-sectioned whole-mount preparations that the TNAP staining was restricted to the apical membrane of the epithelium. After rinsing in TRIS buffer, the preparations were subjected to eosinophil-peroxidase staining as described above. Next, the whole-mount preparations were mounted in Kaiser's medium and examined in a brightfield microscope. Using the present staining, eosinophils are identified as distinct dark brown spheres. The three-dimensional distribution of eosinophils was determined using X-Y scale (lateral distribution) and focal scale (depth) of the microscope. The surface epithelium (blue-dotted surface) and basement membrane (identified by the appearance of extracellular fibers) were used as depth reference points.

In Vivo Administration of Anti-Eotaxin Antibodies

To study the potential role of eotaxin in the present model, a neutralizing polyclonal antibody to guinea pig eotaxin was administered topically to the airway mucosa at the time of allergen exposure. Animals were sensitized to OVA and challenged as described above. At the time of challenge, anti-eotaxin antibodies (40 μ l anti-eotaxin rabbit IgG) were administered together with OVA by tracheal superfusion at a pump rate of 20 μ l per min (the neutralizing capacity of the present antibody has previously been validated in guinea pig skin [17]). Furthermore, 1/100 dilution of the same antibody completely blocks eosinophil shape change induced by 3 nM eotaxin *in vitro* (data not shown). Control animals received normal rabbit IgG. The experiment was terminated at 10 min after challenge, when samples were collected for analysis of eosinophil migration. Based on preliminary experiments, the appearance of eosinophils in the apical portion of the epithelium was selected as parameter of migration.

Statistics

All quantifications were performed in a blinded manner. Statistical differences between the various groups were determined by Mann Whitney's U test. The between-group variability was established using Kruskal-Wallis ANOVA on rank test. Determination of correlations between plasma extravasation and luminal content of eotaxin was performed by Spearman's rank correlation test. All tests were performed using certified statistical software (statistic add-in package for Microsoft Excel, Analyze-it: v1.62, 2,001). *P* values < 0.05 were considered statistically significant.

Results

Baseline Distribution of Eotaxin mRNA and Eosinophils in Guinea Pig Upper Airways

In the trachea of naive control animals, an intense expression of eotaxin mRNA was observed in the basal and mid-portion of the airway epithelium (Figures 1A and 1C). A similar epithelial expression was observed also in the large bronchi, but was absent in more peripheral airways. In separate experiments it was demonstrated that the expression of eotaxin mRNA was significantly increased at 4 h after allergen challenge (data not shown).

EPO staining of cryosections revealed a marked tissue eosinophilia in naive animals ($1,013 \pm 190$ eosinophils/tracheal sections). The eosinophils displayed a distribution pattern similar to that of the expression of eotaxin, i.e., an

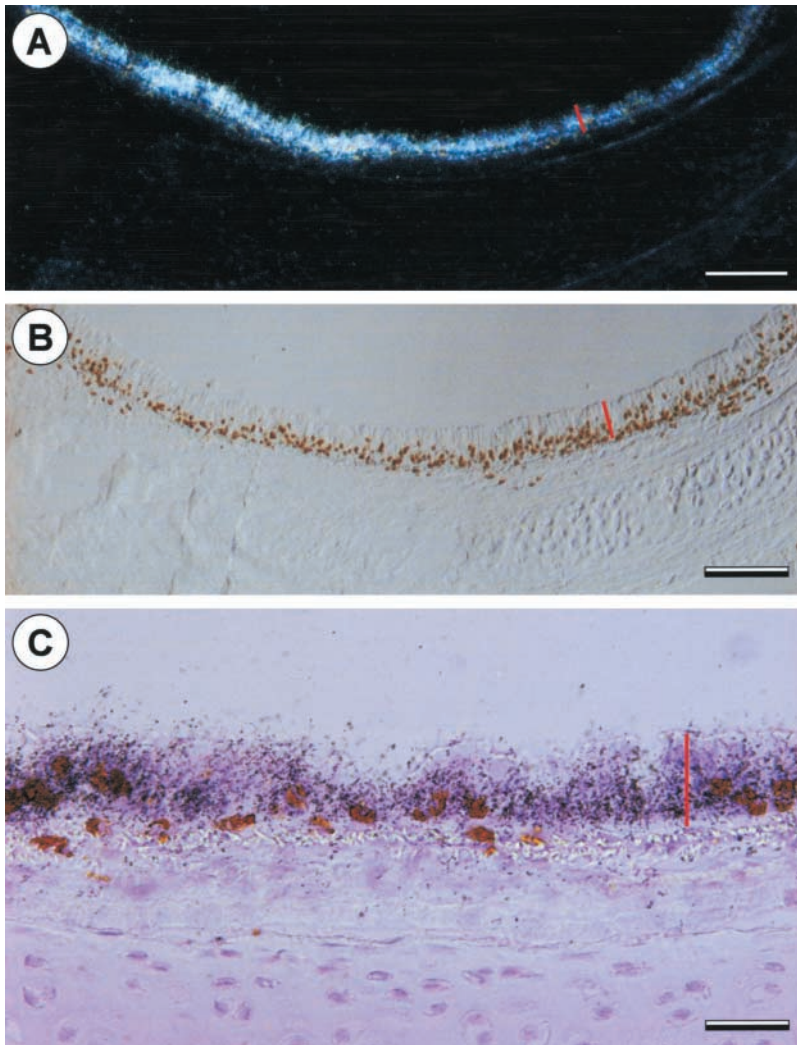


Figure 1. Darkfield (A) and brightfield (B and C) images of transversally cut tracheal sections from naive control animals. The expression of eotaxin mRNA was visualized by autoradiographic *in situ* hybridization (A). Note the intense accumulation of positive staining (i.e., *bright silver grains*) in the airway epithelium (red line). Enzyme histochemical staining of EPO revealed a marked tissue eosinophilia that co-localized with the eotaxin mRNA distribution (B). Higher magnification of sections, double-stained by EPO histochemistry and *in situ* hybridization, illustrates the close spatial relation between eotaxin mRNA and eosinophils under baseline conditions (C). Scale bars: A and B, 150 μ m; C, 50 μ m.

extensive occurrence that was restricted to the large airways (trachea and the large bronchi). The majority of the eosinophils were distributed within the basal portion of the airway epithelium or in the subepithelial tissue just under the basement membrane (Figures 1B and 1C). Exceedingly few eosinophils were present in the apical aspect of the airway epithelium, where expression of eotaxin mRNA was weak or absent.

Combined EPO staining and *in situ* hybridization demonstrated a close association between eotaxin mRNA expression and eosinophils. In some short stretches of the airway epithelial lining where eosinophils were absent, the eotaxin mRNA expression was low. Where epithelial eosinophils abounded, particularly high eotaxin mRNA expression occurred. Sensitized but not allergen-challenged control animals did not differ from naive animals in their displayed eotaxin expression or eosinophil distribution.

Allergen-Induced Acute Luminal Entry of Extravasated Plasma and Eotaxin

A significant plasma extravasation and exudation, assessed as luminal entry of previously injected plasma tracer, could

be detected already at 2 min after allergen exposure (Figure 2A). The luminal content of plasma was further increased at 10 min after challenge and remained elevated at the 30 and 60 min time points (Figure 2A) after challenge.

The levels of luminal eotaxin were significantly increased acutely after allergen challenge (Figure 2B), and remained elevated throughout the experiment. A significant correlation was observed between the emergence of plasma into the airway lumen and the luminal content of eotaxin ($P < 0.0001$, $r = 0.81$).

Allergen-Induced Transepithelial Passage of Eosinophils

In naive animals or saline-treated controls, only few scattered cells were present in the apical portion of the airway epithelium (Figure 3A). At 2 min after the initiation of allergen exposure, the numbers of eosinophils present in the apical portion of the epithelium were significantly increased, compared with time-matched PBS-challenged controls (Figure 4A). At this time point, no increase in luminal eosinophils could be detected. At 10 min after challenge, a further increase in apical, intraepithelial eosinophils was observed (Figures 3B and 4A). At this time point, the numbers of

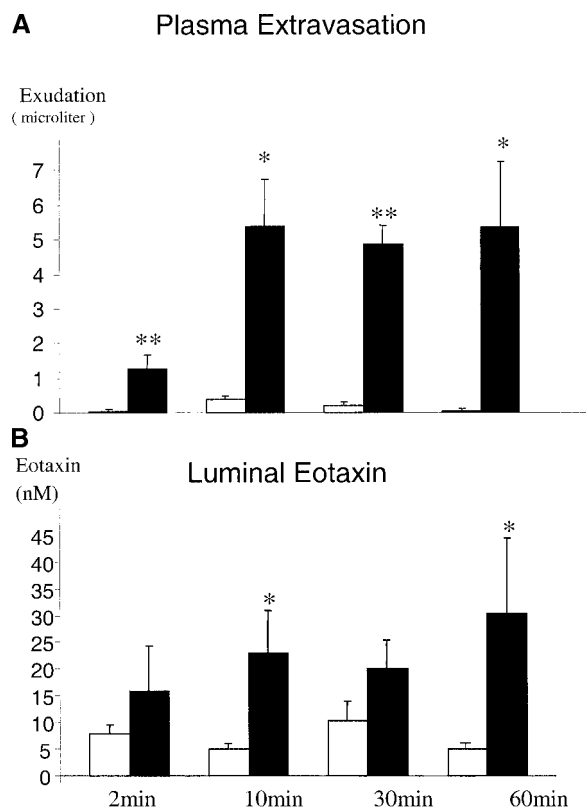


Figure 2. Levels of plasma extravasation (A) and eotaxin (B) in tracheobronchial lavage samples during the acute allergic reaction of OVA-exposed guinea pigs. The amount of extravasated plasma was calculated by determination of previously injected plasma tracer, ^{125}I -labeled albumin, in blood and lavage samples, as described in the methodology section. All data are presented as mean $\pm$ SEM. Significant differences between vehicle- (PBS) and OVA-challenged animals are indicated as * ($P < 0.05$) and ** ($P < 0.01$). Open columns, PBS-challenged animals; filled columns, OVA-challenged animals.

eosinophils in the airway lumen were also significantly increased (Figure 4B). The apical epithelial eosinophils declined at the 30 and 60 min time points, when a further increase in luminal eosinophils was observed (Figure 4).

The transepithelial passage of eosinophils was confirmed by analysis of EPO-stained tracheal whole-mount preparations. In naive animals or the PBS-treated controls no, or exceedingly few, eosinophils were present on the mucosal surface (Figure 5A). At 2 min after OVA challenge, scattered eosinophils were observed in the region of the epithelial apical surface (Figure 5B). At the later time points after OVA challenge, abundant eosinophils had accumulated in the epithelial surface region (Figure 5C).

Both Allergen-Induced Acute Plasma Extravasation and Luminal Entry of Eosinophils Is Effectively Inhibited by the β_2 -Agonist Formoterol

Formoterol, given intravenously before challenge, effectively inhibited the allergen-induced flow of extravasated plasma into the airway lumen (Figure 6A). As examined 30 min after challenge, the formoterol-treated animals had

significantly attenuated numbers of eosinophils in the airway lumen as compared with allergen-exposed nontreated animals (Figure 6B).

Local Administration of Tracheal Superfusates from Allergen-Exposed Animals Induces Luminal Entry of Eosinophils in the Absence of any Prior Plasma Extravasation

Our observation of increased levels of luminal eotaxin stimulated us to test the chemoattractant and pro-extravasation properties of superfusate alone, recovered from sensitized animals 10 min after allergen exposure. Topical administration of such superfusates to naive animals resulted in a marked transepithelial migration of eosinophils without inducing any plasma extravasation response (Figure 7). In separate control experiments, it was demonstrated that administration of superfusates from naive animals did not affect the luminal levels of eosinophils, nor were any effects on plasma extravasation observed (data not shown).

Bradykinin Induces a Prompt Luminal Influx of Eosinophils

Bradykinin, a well-known inducer of plasma extravasation, was given topically to the airways. Bradykinin administration induced a plasma extravasation response within 10 min (Figure 8A). Furthermore, bradykinin administration also resulted in increased luminal influx of eosinophils (Figure 8B).

Effect of Anti-Eotaxin Antibodies

OVA-challenged animals that received control rabbit IgG serum had significantly increased levels of apical intraepithelial eosinophils (117 ± 14 apical eosinophils per cm epithelial lining) compared with saline-challenged controls (12 ± 4 apical eosinophils per cm epithelium, $P = 0.002$). The numbers of apical transepithelial eosinophils in OVA-challenged animals that received neutralizing anti-eotaxin antibodies at the time of challenge displayed a clear trend toward a reduction (62 ± 18 apical eosinophils per cm epithelium, $P = 0.07$ compared with OVA-exposed control animals).

Discussion

By use of the well-controlled baseline situation provided by guinea pig large airways and a methodology that specifically assesses the airway region of interest, the present study demonstrates, for the first time, that an acute anaphylactic response evokes an immediate migration of resident mucosal eosinophils into the airway lumen. Thus, within 2 min after topical allergen challenge, eosinophils migrate to apical epithelial positions. Then, within 10 min after challenge, there is a marked migration of eosinophils into the airway lumen. Our data further suggest that acute-phase plasma extravasation, through its capacity to alter the local milieu and the transepithelial gradients of eosinophil chemoattractants including eotaxin, is likely to play a role in the present acute luminal entry of eosinophils. The speediness of the present eosinophil response suggests that luminal entry is a highly efficient airway mucosal response mechanism by which large numbers of tissue granulocytes can be acutely recruited to the mucosal surface. The occurrence of acute

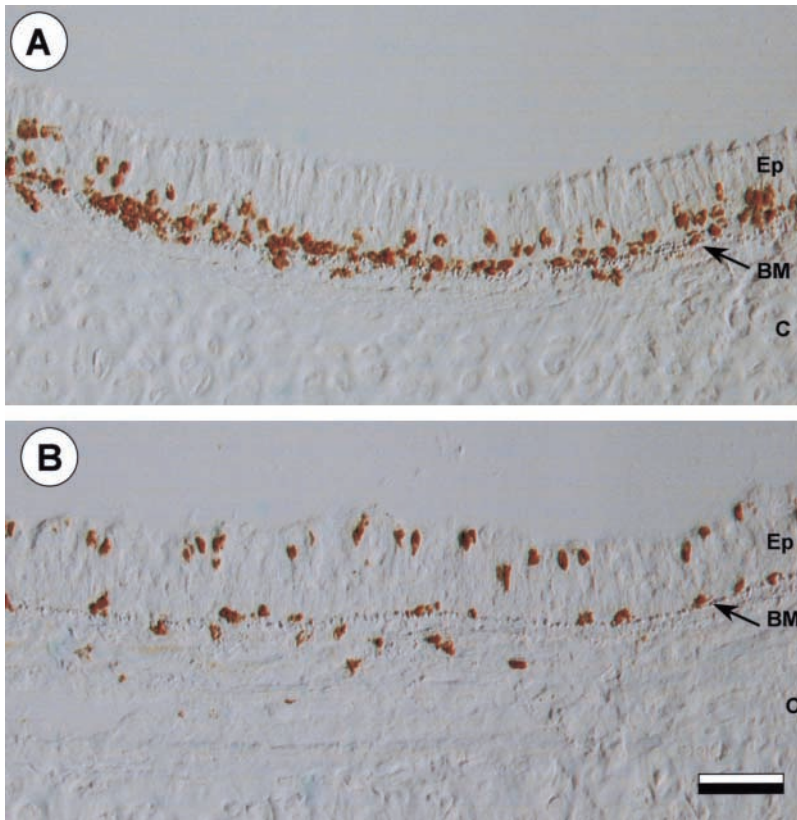


Figure 3. Brightfield micrographs demonstrating the representative distribution of EPO-stained eosinophils (*dark spots*) during control conditions (A) and at 10 min after topical allergen exposure (B). The structure of the non-stained areas of the section is visualized by Normasky optics. EP, epithelium; BM, basement membrane; C, cartilage. Scale bar: 50 μ m.

transepithelial traffic of mucosal inflammatory cells thus emerges as a first-line defense mechanism that also can be initiated during an acute anaphylactic response.

The traditional view of an allergic reaction implies that it is only during the late-phase allergic response that mucosal traffic of eosinophils occurs (1, 2). This assumption is largely based on studies in animal models where, in most cases, eosinophils are absent in the tissue until several hours after challenge (23, 24). However, in common clinical situations, allergen exposure and associated acute phase responses occur in airways that already at the time of exposure have elevated numbers of eosinophils in the airway tissue (4–6). The constitutive and stable tracheobronchial eosinophilia that is present in guinea pig large airway tissues was, therefore, considered relevant as well as advantageous for our experimental purposes, and so were the minimal baseline numbers of eosinophils in the lumen. Thus, this species offers a rare situation in which any immediate traffic of those eosinophils present in the tissue at the time of challenge can be examined in detail *in vivo*. Importantly, the present assessment of eosinophil movements was performed in a model that lacks the disturbance of surgical intervention and uses low traumatic techniques for mucosal exposure specifically of the area of interest (18, 19). The present lavage method is also selective to the large airways, thus differing from the common bronchoalveolar lavage (BAL) techniques that largely reflect the contribution of small intrapulmonary airways where, at least in guinea-pigs, the mucosal eosinophilia may not be pronounced until the late-

phase eosinophilia develops. Indeed, studies involving the common BAL techniques in allergic guinea pigs may not detect any significant increase in luminal eosinophilia during the first hours after allergen challenge, whereas studies involving specific large airway lavage methodology do so (13).

As demonstrated in the many animal models that lack airway tissue eosinophils at the time of allergen exposure, it takes 5–10 h after allergen exposure until eosinophils enter the airway lumen (23, 25). The present data on transepithelial migration show that in a mucosa that harbors eosinophils close to the epithelial surface, the speed by which eosinophils can be mobilized onto the airway surface increases greatly. Such immediate luminal entry of granulocytes would represent a paramount mechanism in the mucosal defense system. In accord, in the urinary and gastrointestinal tract an efficient luminal entry of granulocytes seems essential for successful neutralization of infectious organisms (26–28). An exceedingly prompt luminal entry, as clearly demonstrated in this study, further supports the possibility that a major anti-infectious feature of mucosally lined organs is to let granulocytes neutralize and destroy invading organisms before their entry into the tissue, i.e., already on the mucosal surface. This mechanism would significantly reduce the risk of inflammation and damage to the host tissue. Hence, the prolonged mucosal eosinophilia occurring in chronic allergic conditions, or in healthy guinea pig airways as well as in the human gastrointestinal tract (29, 30), may represent a defense strategy providing the mucosa with the capacity to immediately mobilize leukocytes onto the mucosal sur-

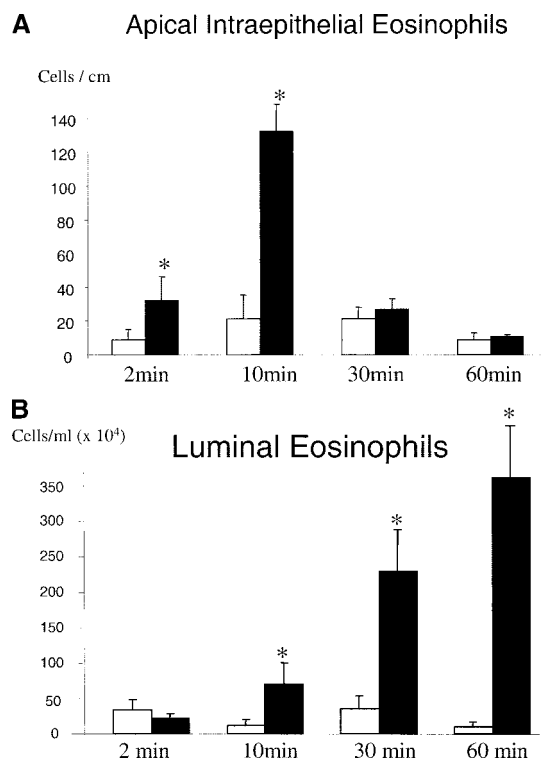


Figure 4. Numbers of apical intraepithelial eosinophils (*A*) and airway luminal eosinophils (*B*) during the first hour after allergen challenge. Number of eosinophils in the apical portion of the tracheal epithelium (defined as 0–10 μ m from the apical epithelial surface) was assessed by quantification of transversally cut 10- μ m EPO-stained sections (*A*). The numbers (mean $\pm$ SEM) represent occurrence of apical intraepithelial eosinophils per cm epithelial lining. Luminal eosinophils were assessed as eosinophils in tracheo-bronchial lavage samples at different time points after challenge (*B*). Significant differences between vehicle- (PBS; open columns) and OVA-challenged animals (filled columns) are indicated as $^*(P < 0.05)$.

face. An acute activation of resident eosinophils may, apart from direct defense mechanisms against invading organisms, also serve immunomodulatory roles. For example, eosinophils have the capacity to release a variety of growth factors and cytokines, as well as function as antigen-presenting cells (31, 32). Further studies are warranted to investigate the effect of acute allergen-induced activation of eosinophils on the subsequent development of late phase or chronic inflammatory conditions.

As indicated from the numbers of apical intraepithelial eosinophils, the transepithelial flow of cells peaks already around 10 min. Yet the luminal content of eosinophils continues to increase at the later time points. This finding may be explained by a relatively slow clearance of luminal eosinophils by the mucociliary transport system, leading to a gradual accumulation of eosinophils on the mucosal surface during the first hour after the initial massive transepithelial migration.

As indicated by the present kinetic data, any mechanisms involved in the present luminal influx of leukocytes would be operating within one or two minutes after challenge.

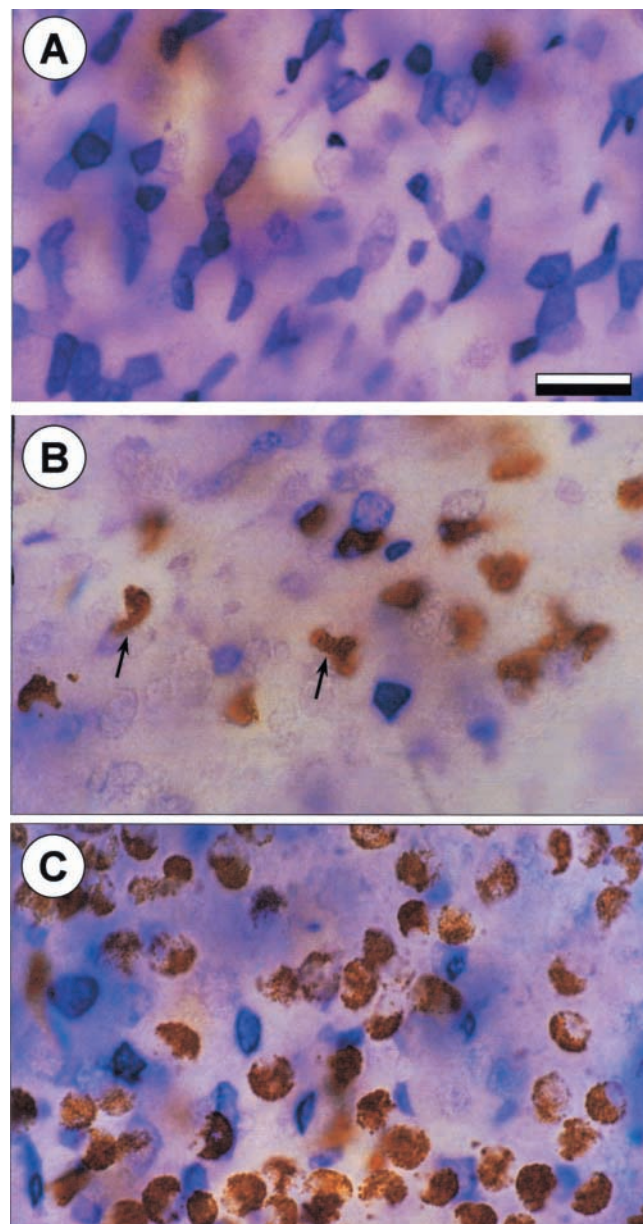


Figure 5. Tracheal whole-mount preparations where the apical membrane of epithelial cells (viewed from above) has been visualized by enzyme histochemical staining of TNAP (blue staining). Under baseline conditions eosinophils, stained brown by EPO histochemistry, are rarely found in the epithelial surface region (*A*). At 2 min after OVA exposure an increased number of eosinophils (arrows) can be seen in the surface region (*B*). Abundant eosinophils have accumulated on the epithelial surface 30 min after allergen challenge (*C*). Scale bar: 25 μ m.

We hypothesized that plasma extravasation, a major acute allergic response in the airways, could be involved. Supporting this hypothesis, the allergen-induced vascular permeability response in the present model starts within 30 s after challenge by formation of endothelial gaps at postcapillary venules in the mucosal microcirculation (13, 18). Initially, bulk extravasated plasma is distributed in the lamina pro-

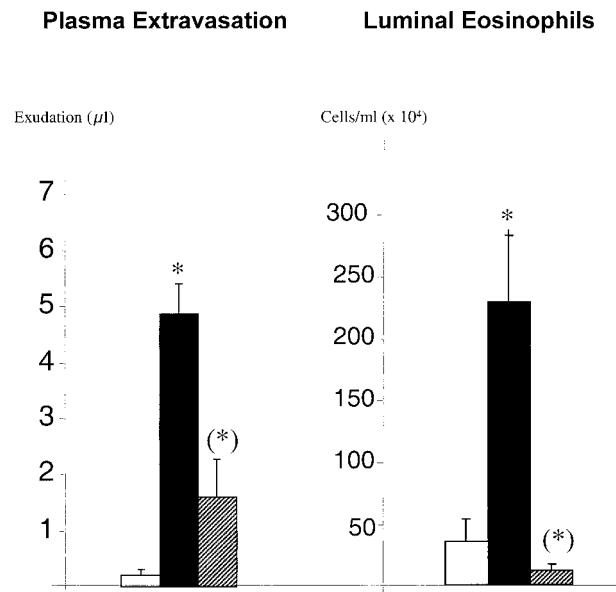


Figure 6. Effect of the β_2 -agonist formoterol on extravasation of plasma (A) and transepithelial migration of eosinophils (B) during the acute allergic reaction. Formoterol was administered by an intravenous injection 5 min before allergen challenge. All data are presented as mean $\pm$ SEM. Significant differences between vehicle- (PBS; open columns) and OVA-challenged animals (filled columns) are indicated as * ($P < 0.05$) and ** ($P < 0.01$). Asterisk within parentheses indicates significant difference between pretreatment with saline and formoterol before OVA challenge (striped bars), $P < 0.05$.

pria tissue. Then it moves through the epithelial basement membrane and swiftly exudes into the airway lumen through ubiquitous paracellular epithelial pathways (7, 33). Experimental data indicate that the size-independent mechanisms of luminal entry of a bulk plasma exudate reflects a hydrostatic pressure operated valve-like feature of the junctions between airway epithelial cells that unidirectionally will let through plasma macromolecules without disturbing the

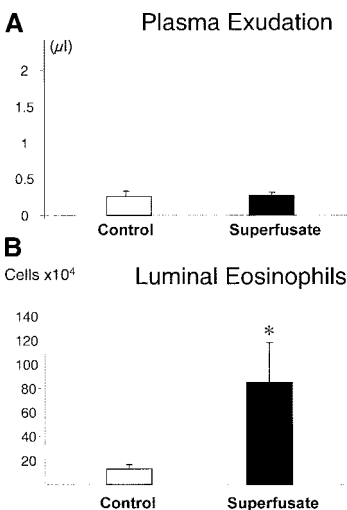


Figure 7. Plasma exudation (A) and tracheal luminal content of eosinophils (B) 30 min after topical airway exposure of naive animals by tracheal superfusate (the administered superfusate was collected 30 min after previous OVA exposure of sensitized animals). Significant difference between vehicle and superfusate-challenged animals is indicated as * ($P < 0.05$). All data are presented as mean $\pm$ SEM.

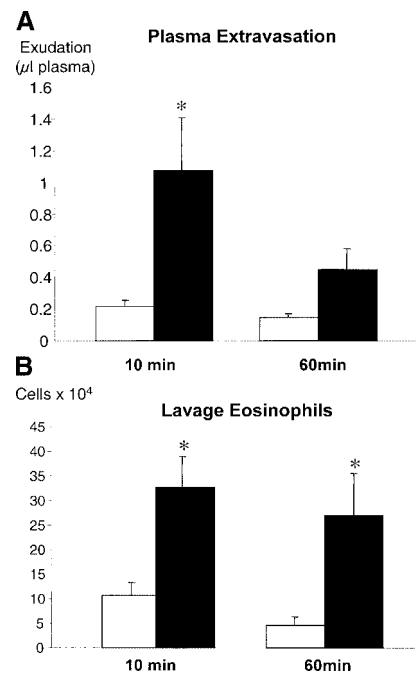


Figure 8. Plasma exudation (A) and tracheal luminal content of eosinophils (B) at 10 and 60 min after topical airway exposure of bradykinin (filled columns). Significant differences between vehicle- (PBS; open columns) and OVA-challenged animals are indicated as * ($P < 0.05$). All data are presented as mean $\pm$ SEM.

integrity of the epithelial lining as an absorption barrier (7). Hence, just before the initiation of the eosinophil migration, important routes into the airway lumen have been exposed to an exudate containing plasma proteins capable of acting as leukocyte activators or carriers for chemoattractants (7). Thus, through plasma exudation chemoattractant proteins may accumulate on the mucosal surface to alter the transepithelial gradients of leukocyte-active molecules. This latter aspect is supported by the present strong correlation between the appearance of exuded plasma and eotaxin in the mucosal surface fluid. Furthermore, the present application of a tracheal superfusate was sufficient to bring about luminal entry of eosinophils (in the absence of any prior plasma extravasation), suggesting that the acute allergic response did result in a biologically significant increase in surface fluid chemoattractants. Collectively, these observations suggest that chemoattractants including eotaxin, moved into the lumen by extravasated plasma, contributed to the present migration of eosinophils to the mucosal surface. In other words, luminal chemoattraction is increased, whereas there is less chemoattraction holding back the eosinophils within the mucosa.

Interestingly, the plasma exudation-associated luminal entry of eosinophils was not dependent on allergen being the exudative stimulus. Thus we demonstrate here that a single pro-inflammatory mediator such as bradykinin produced prompt luminal entry of both plasma and eosinophils. Also, confirming our original demonstration of formoterol-induced inhibition of allergen-induced plasma exudation (20) we could now, in addition, demonstrate that this vascular

antipermeability drug action was associated with abolition of the acute allergen-evoked luminal entry of eosinophils. A possibility of such an effect on cell traffic may need consideration also in human clinical studies, because formoterol has been shown to attenuate plasma exudation also in human bronchi (34). Interestingly, in agreement with the present increase in luminal eotaxin recently studied in humans suggests that experimental induction of a plasma extravasation can be used to transport tissue mediators into the lumen (35). This finding and the present results suggest the possibility that similar extravasation-induced luminal entry of mediators operates in humans and guinea pigs. Further work is now needed to explore potential species differences in relation to, e.g., eosinophil homing and transepithelial migration mechanisms.

The traffic of eosinophils during the late phase of allergic inflammation has been extensively studied in guinea pig airways (13, 25). In this context, a role of the CC-chemokine eotaxin has been established, and the kinetics of eotaxin production and its strong relationship to the allergen-induced late phase eosinophilia has been examined (17, 36). In contrast to the earlier studies in this field, the present study examines the distribution of eotaxin and its correlation with eosinophil traffic during the early stages of the acute allergic reaction. Eotaxin is constitutively expressed in guinea pig airways, and the airway epithelium has been suggested as the major site of its production (16, 37). In support of a role of eotaxin in the tissue homing of eosinophils, the present demonstration of eotaxin mRNA expression in the basal and mid-portion of the epithelium correlated well with the actual distribution of eosinophils. Our data showing that even within local tissue regions there is a spatial correlation between the expression of eotaxin and local distribution of eosinophils further underscores the view that eotaxin may regulate the homing of tracheobronchial eosinophils in naive animals (17). This notion is in line with the emerging concept that in addition to tissue recruitment, chemokines also have important roles in relocation of leukocytes within the local tissue compartments (38).

It is of note that when applied topically onto human or guinea pig airways, recombinant eotaxin can, by itself, evoke an increase in BALF numbers of eosinophils (39, 40). The suggested role of eotaxin in both tissue homing and migration upon challenge complicate the use of neutralizing agents to dissect the role of eotaxin in the present model. For example, neutralization of eotaxin may disturb the normal baseline distribution of eosinophils but also attenuate a challenge-induced transepithelial migration. Nevertheless, in the present study we observed that administration of anti-eotaxin antibodies produced a trend toward a reduction of apical intraepithelial eosinophils after allergen challenge. This finding, together with the present rapid alteration of the transepithelial gradient of eotaxin, suggests a contributory role of eotaxin in the acute luminal entry of eosinophils. Considering the complexity of the mucosal molecular milieu *in vivo* immediately after challenge, it is likely that other chemokines or epithelial and plasma-derived molecules also participate.

In conclusion, this study examining guinea pig eosinophil-rich tracheobronchial airways *in vivo* has demonstrated

that the acute plasma exudation response to challenge with allergen or bradykinin is associated with an almost equally quick luminal entry of resident eosinophils. The speedy plasma exudation response by its content of pluripotent proteins appears instrumental to this cell traffic, and so may cell-derived chemokines that are transported by exuding plasma. For example, mucosal eotaxin appears to be moved promptly (within 120 s) to the mucosal surface along with extravasated bulk plasma that first has flooded the mucosal interstices. We propose that luminal entry of eosinophils is a significant component of the most acute phase of allergic reactions occurring in airways where these cells are already present in the mucosa. On a more general note, the present demonstration of acute luminal entry of potent leukocytes across the airway mucosa indicate a potential for prompt and efficient cellular host defense mechanisms getting into operation before infectious organisms or other topical insults have made their way into the tissue.

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