

Airway inflammation in a murine model of chronic asthma: evidence for a local humoral immune response

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Summary

Background Asthma is an acute-on-chronic inflammatory disease of the airways characterized by recruitment of eosinophils into the epithelial layer, chronic inflammation in the lamina propria, as well as variable accumulation of mast cells in the airway wall. The role of local production of allergen-specific immunoglobulins in triggering mast cell-mediated asthmatic inflammation is unknown.

Methods We used a chronic inhalational exposure model of asthma in ovalbumin-sensitized BALB/c mice to examine the phenotype of immunoglobulin-secreting cells and mast cells in the airway wall. In parallel, we assayed ovalbumin-specific IgG and total IgE in the plasma of these animals.

Results In sensitized mice exposed to aerosolized ovalbumin for 6 weeks, aggregates of chronic inflammatory cells consisted of a majority of plasmacytoid cells, including numerous IgG-synthesizing cells, which were significantly increased in sensitized animals compared to controls. IgA-synthesizing cells were also present, but were not increased in the sensitized exposed mice. Immunoglobulins in the cytoplasm of the plasma cells were demonstrated to be antigen-specific. No IgM- or IgE-synthesizing cells were observed, although levels of total IgE in the plasma were significantly increased. There was no recruitment of mast cells of either the mucosal or the connective tissue phenotype into the lamina propria or the epithelium.

Conclusion In this experimental model of chronic asthma, the pattern of inflammation in the airway wall is consistent with development of a local IgG-mediated humoral immune response. However, there is no evidence of local production of IgE or recruitment of mast cells.

Clinical and Experimental Allergy, Vol. 30, pp. 1486–1492. Submitted 22 October 1999; revised 20 January 2000; accepted 2 March 2000.

Introduction

Asthma is characterized by acute and chronic inflammation of the airway mucosa, with recruitment of eosinophils which are characteristically present within the epithelial layer [1], as well as accumulation of chronic inflammatory cells such as lymphocytes, plasma cells and macrophages in the lamina propria [2,3]. Increased numbers of mast cells may be present in both the airway epithelium and lamina propria [3,4]. The relationship between airway inflammation and the clinical manifestations of asthma remains

incompletely understood. Experimental studies suggest that in atopic/allergic asthma, the early response to inhalation of allergen is a result of an IgE-dependent type I hypersensitivity reaction driven by mast cell-derived mediators, whereas the late response develops as a result of activation of antigen-specific T cells [5].

The secretion of cytokines by activated T-lymphocytes, mast cells and injured airway epithelial cells, which may contribute to the pathogenesis of airway hyperreactivity and other features of asthma [6–8], has been the focus of considerable recent interest. However, the role of cells participating in generation of the early asthmatic response has otherwise received relatively little attention. Human and animal experimental studies indicate that the humoral

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immune response to inhaled antigens may be compartmentalized to the lung [9,10]. It is therefore possible that local production of allergen-specific immunoglobulins could play a role in triggering mast cell-mediated asthmatic inflammation, but no data are available about the phenotype and specificity of immunoglobulin-synthesizing cells that accumulate in the airway wall. Furthermore, subsets of mast cells defined on the basis of protease expression demonstrate heterogeneity in terms of their production of pro-inflammatory cytokines [11] but little information has been reported about whether there is selective accumulation of these subpopulations in the airway wall. Experimental studies of asthma have thus far not addressed these issues, largely because no suitable model of the chronic inflammatory component of the asthmatic response has been available.

We have recently described a model of chronic asthma in mice [12] which involves systemic sensitization of BALB/c mice with ovalbumin and subsequent long-term inhalational challenge with low mass concentrations of antigen. In contrast to other reports in which long-term challenge is associated with down-regulation of the inflammatory and immunological response [13,14], this approach consistently elicited lesions typical of chronic human asthma. These include recruitment of numerous intraepithelial eosinophils; chronic inflammation in the lamina propria with accumulation of lymphocytes, plasmacytoid lymphocytes and macrophages; together with epithelial hypertrophy, mucous cell hyperplasia and subepithelial fibrosis. The inflammatory changes are maximal after 6 weeks of inhalation exposure and are associated with sustained high titres of antigen-specific IgE [12]. Importantly, and in sharp contrast to virtually all previously reported murine experimental models of asthma, there is no evidence of alveolitis associated with the airway lesions. The mice also exhibit marked hyperreactivity to cholinergic stimuli, delivered either systemically [12] or by aerosol to conscious unrestrained animals [15], which can be attributed to disease of the airways rather than to parenchymal lesions.

In this study, we quantified and characterized immunoglobulin-synthesizing cells in the lamina propria of the airways in this novel experimental model. In parallel, we examined the distribution and phenotype of mast cells in the airway wall. Our findings are consistent with induction of a local humoral immune response in chronic asthma.

Materials and methods

Animals

Specific pathogen-free female BALB/c mice aged 8–10 weeks were obtained from CULAS, Sydney, Australia. Animals were maintained in a laminar flow holding unit

(Gelman Sciences, Sydney, Australia) and housed in autoclaved cages on autoclaved bedding in an air-conditioned room on a 12-h light/dark cycle. Autoclaved food and acidified water were provided *ad libitum* throughout. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (ref. no. 98/29),

Sensitization and inhalational exposure

This was performed as previously described [12]. In brief, mice were sensitized by two intraperitoneal injections of 10 µg of alum-precipitated chicken egg ovalbumin (Grade V, ≥98% pure, Sigma, St. Louis, MO, USA; unless otherwise specified, all chemicals were obtained from this source) administered 21 days and 7 days before inhalation exposure. An experimental group of six mice was exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 6 weeks in a whole-body inhalation exposure system (Unifab Corporation, Kalamazoo, MI, USA). This time interval was selected because we had previously demonstrated that both inflammation and airway wall remodelling are maximal after 6 weeks [12]. During the exposure, the mice were held in wire flow-through cage racks and filtered air was drawn through the 0.5 m³ inhalation chamber at a flow rate of 250 L/min. A solution of 2.5% ovalbumin in normal saline was aerosolized by delivery of compressed air to a sidestream jet nebuliser (Trimed, Sydney, Australia) and injected into the airstream entering the chamber. The concentration and size distribution of particles within the breathing zone of the mice were continuously and cumulatively monitored using a light scatter device (PCAM-TX, PPM, Knoxville, TN, USA). Particle concentration was maintained in the range 10–20 mg/m³ by controlling the airflow into the nebuliser. Two groups of animals were studied as controls: nonsensitized animals exposed to aerosolized ovalbumin as above, as well as nonsensitized, unexposed normal animals.

Blood and tissue collection

At the beginning and the end of the exposure period, mice were bled by retro-orbital puncture using heparinized capillary tubes. Plasma was diluted 1:20 in phosphate-buffered saline and antibody levels assayed as described below. Tissues for histopathological examination were collected 24 h after the last inhalational exposure and were fixed overnight, either in 10% neutral buffered formalin or at –20 °C in acetone containing protease inhibitors [16]. Formalin-fixed tracheas were embedded in paraffin and sections cut at 5 µm. Acetone-fixed tracheas were embedded in glycol methacrylate resin (JB-4, Polysciences, Warrington, PA, USA) in the presence of methyl benzoate [16] and

sections cut at 2 μm . Tracheas were orientated longitudinally during embedding to facilitate subsequent morphometry.

Immunohistochemistry

Immunostaining was performed using a capillary action staining system as previously described [17]. For identification of cells secreting IgG, IgA or IgM, polyclonal goat antibodies to these mouse immunoglobulins (Kirkegaard & Perry, Gaithersburg, MD, USA) were used at appropriate dilutions determined on the basis of preliminary experiments. For sections of formalin-fixed tissues, antigen retrieval was performed by boiling in 0.01 M citrate buffer, pH 6.0, for 10 min. Sections were washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and blocked with 50% normal rabbit serum for 10 min at 37 °C. They were then incubated for 30 min at 37 °C with an appropriate concentration of the primary antibody. Sections were washed four times with PBS-Triton after this and each subsequent incubation. Bound antibody was detected by sequential 30 min incubations with biotinylated rabbit anti-goat immunoglobulins followed by a complex of streptavidin (Boehringer Mannheim, Sydney, Australia) and biotinylated horseradish peroxidase in PBS 1% bovine serum albumin (BSA). Diaminobenzidine was used as the substrate and sections were lightly counterstained with methyl green. For identification of cells secreting IgE, monoclonal rat antimouse IgE (R35-92 or R35-72, PharMingen, San Diego, CA, USA) was used as the primary antibody. Staining was performed as above except that blocking was with 50% goat serum and detection was with biotinylated rabbit antirat immunoglobulins.

Phenotypic subsets of mast cells were identified on the basis of immunohistochemical staining for mouse mast cell protease (mMCP) 2, which is characteristically expressed by mast cells of the so-called mucosal phenotype, and mMCP 5 which is associated with so-called connective tissue mast cells [18]. Affinity-purified rabbit polyclonal antibodies to specific, non cross-reactive peptide sequences of mMCP-2₅₆₋₇₁ [19] and mMCP-5₁₄₆₋₁₆₂ [20] were used for this purpose. For demonstration of mMCP-5, antigen retrieval with citrate buffer was performed as above, but for mMCP-2 the use of Tris buffer at pH 9.5 [21] was necessary for satisfactory staining. The procedure was essentially as described above except that blocking was with 50% goat serum and detection was with swine antirabbit immunoglobulins followed by rabbit peroxidase-antiperoxidase complex (both from Dako, Sydney, Australia).

Anti-ovalbumin immunoglobulins in the cytoplasm of plasmacytoid cells were identified by immunostaining of acetone-fixed tissues embedded in glycol methacrylate resin. Endogenous peroxidase activity was inhibited by incubation for 30 min with 0.3% H₂O₂ in PBS containing

0.1% sodium azide. Sections were washed with PBS containing 0.075% Brij-35 and blocked with 50% goat serum. They were then incubated overnight with 250 $\mu\text{g/mL}$ of biotinylated ovalbumin (kindly prepared by Dr Craig Harrison, School of Pathology, University of New South Wales, Sydney, Australia) and washed four times with PBS-Brij. Bound protein was detected by incubation with streptavidin-biotinylated horseradish peroxidase complex for 2 h. After incubation with diaminobenzidine substrate, sections were lightly counterstained with haematoxylin.

Morphometry

Quantification of the profile density of immunoreactive cells was performed using a Leica Q500MC image analysis system (Leica, Cambridge, UK) calibrated with a reference measurement slide. Using a $\times 40$ objective, nuclear profiles in the lamina propria (defined as the region bounded by the epithelial basement membrane and the luminal border of the tracheal cartilage plates) were counted in consecutive microscopic fields and the length of the epithelial basement membrane was measured in each field. The process was repeated until the full length of the tracheal section had been examined and the data were used to calculate the mean number of cells per 100 μm of epithelial basement membrane in the lamina propria for individual animals.

Enzyme immunoassay

IgG antibodies to ovalbumin in plasma samples from individual animals were quantified using an enzyme immunoassay. Microplate wells coated with 100 $\mu\text{g/mL}$ of ovalbumin were incubated for 1 h with 50 μL of each of the test samples or with serial dilutions of a pool of plasma from sensitized animals in PBS containing 0.05% Tween-20. Detection of bound antiovalbumin IgG was with biotinylated affinity-isolated rabbit antimouse IgG (Dako, Sydney, Australia), followed by incubation with streptavidin-biotinylated horseradish peroxidase complex in PBS 1% BSA. Colour development was by incubation with ABTS substrate in citrate-phosphate buffer (pH 4.0) and the reaction product was read using a Titertek Multiskan photometer (ICN Biomedicals, Sydney) with a 405-nm filter. Absorbance data for serially diluted standard samples were fitted to a sigmoidal dose-response curve and results for test samples were expressed as units relative to the standard, which was arbitrarily assigned a value of 100 units/mL.

Total IgE levels were measured using a sandwich immunoassay in which monoclonal rat antimouse IgE (R35-72, PharMingen, San Diego, CA, USA) was coated on to microplate wells at 2 $\mu\text{g/mL}$ in carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. Captured IgE in plasma samples was detected using biotinylated monoclonal rat

antimouse IgE antibody (R35-92, PharMingen) at 2 µg/mL. To enhance the sensitivity of the assay, enzyme complex amplification [22] was used as the reporter system. The reporter reagent was prepared by assembling streptavidin-biotinylated horseradish peroxidase complexes in PBS 1% BSA, then cross-linking these with biotinylated goat anti-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) and additional streptavidin. Monoclonal mouse IgE (SPE-007) was used to generate a standard curve. Results were expressed as ng/mL. The assay was reliably able to detect 3 ng/mL of IgE.

Statistical analysis

Results are presented as arithmetic mean $\pm$ standard error for each experimental group. To examine differences between

groups, an initial one-way analysis of variance was followed by Newman-Keuls multiple comparison test. Curve fitting and data analysis were performed using the software package GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Immunoglobulin-secreting cells in the airway wall

In normal mice, occasional cells exhibiting cytoplasmic immunoreactivity for IgA were identified in the lamina propria of the trachea (Fig. 1a). Cells staining for IgG were rarely observed whereas no cells staining for IgM or IgE were detected.

Non-sensitized mice exposed to aerosolized ovalbumin for 6 weeks did not develop significant airway inflammation.

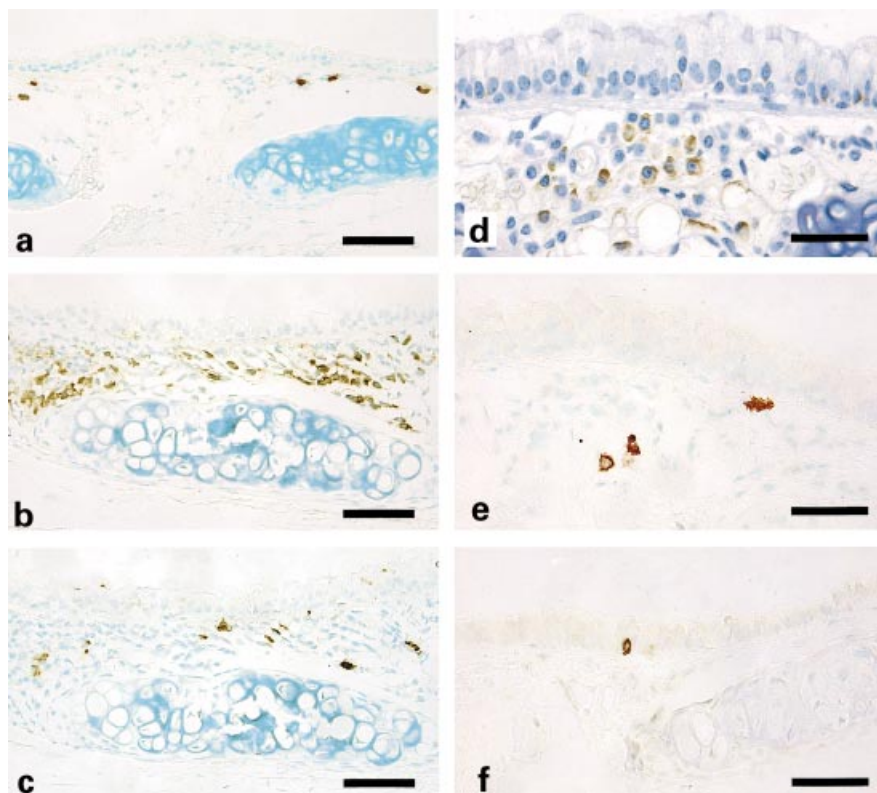


Fig. 1. Immunoglobulin-producing cells and mast cells in experimental asthma. (a) Occasional IgA-reactive cells in the lamina propria of the trachea in a normal animal. Bar = 50 µm. (b) Numerous IgG-reactive cells among the aggregates of inflammatory cells in the lamina propria of the trachea from a sensitized mouse exposed to aerosolized ovalbumin for 6 weeks. Bar = 50 µm. (c) Infrequent IgA-reactive cells in a consecutive section. Bar = 50 µm. (d) Binding of biotinylated ovalbumin by immunoglobulins in the cytoplasm of plasma cells in the lamina propria of the trachea from a sensitized exposed animal. Bar = 35 µm. (e) Mast cells reactive for mMCP-5 in the lamina propria of the trachea from a sensitized exposed animal. Bar = 35 µm. (f) Mast cell reactive for mMCP-2 in the epithelium of the trachea from a nonsensitized exposed animal. Bar = 35 µm. Immunoperoxidase staining. A-C, E, F counterstained with methyl green, D counterstained with haematoxylin.

Table 1. Numerical density of immunoreactive cells in lamina propria of the trachea

	Normal	Non-sensitized exposed	Sensitized exposed
IgA	2.33 ± 0.68	0.37 ± 0.19	3.25 ± 1.15
IgG	0.25 ± 0.10	0.16 ± 0.08	5.71 ± 1.06***
mMCP5	0.87 ± 0.39	0.71 ± 0.31	0.58 ± 0.21

***Significantly different from both normal and nonsensitized mice, $P < 0.001$.

Cytoplasmic staining for immunoglobulins was observed in only a few cells in the lamina propria (not shown).

In contrast, sensitized mice chronically exposed to aerosolized ovalbumin developed widespread multifocal accumulation of chronic inflammatory cells in the lamina propria of the trachea, as previously described [12]. The focal aggregates included a substantial proportion of cells that exhibited cytoplasmic staining for IgG (Fig. 1b) and relatively few cells that stained for IgA (Fig. 1b). Again, no cells staining for IgM or IgE were found.

Morphometric quantification of the density of immunoreactive cells established that there was a statistically significant increase in the number of IgG-positive cells in the airways of sensitized exposed mice, compared to both normal and nonsensitized mice (Table 1). No such increase was demonstrable for IgA-positive cells. Although the numerical density of the latter cells was unexpectedly decreased in nonsensitized mice, this difference was not statistically significant compared to normal controls (Table 1).

In sections of acetone-fixed, glycol methacrylate-embedded tissue, the presence of functionally intact anti-ovalbumin immunoglobulins was demonstrated by cytoplasmic binding of biotinylated ovalbumin (Fig. 1d). The population of immunoreactive cells frequently exhibited the characteristic 'clock-face' nuclear morphology of plasma cells.

Mast cells in the airway wall

Cells immunoreactive for mMCP-5 (so-called connective tissue mast cells) were present in the lamina propria of the trachea in all three groups of experimental animals (Fig. 1e). No mMCP-5-positive cells were present in the epithelial layer. There was no significant difference in the numerical density of these cells in either nonsensitized or sensitized mice chronically exposed to aerosolized ovalbumin (Table 1).

Cells immunoreactive for mMCP-2 (so-called mucosal mast cells) were rarely present in sections of the trachea and

Table 2. Immunoglobulin levels in plasma

	Normal	Non-sensitized exposed	Sensitized exposed
IgG (units/mL)	Not detectable	0.15 ± 0.03	56.4 ± 5.0
Total IgE (ng/mL)	≤ 60	230 ± 90	2470 ± 1050

were only found within the epithelial layer (Fig. 1f). These cells were too sparse to permit reliable morphometric quantification.

Immunoglobulins in plasma

Plasma from normal animals did not contain any ovalbumin-specific IgG. Following chronic inhalation exposure of nonsensitized mice, very low levels of specific IgG were reproducibly detectable in the plasma. Much higher concentrations were present in the plasma of sensitized mice (Table 2).

In the 1 : 20 diluted samples that were tested, total IgE in the plasma of normal mice was below the limit of detection of the enzyme immunoassay. Plasma concentrations of IgE were modestly elevated in the nonsensitized exposed animals and strikingly increased in the sensitized mice (Table 2).

Discussion

In this study we have demonstrated that airway inflammation in an experimental model of chronic asthma is associated with significant accumulation of immunoglobulin-synthesizing cells. This study provides, for the first time, information about the relative proportion of plasma cells synthesizing different classes of immunoglobulins in the airway wall in chronic asthmatic inflammation. We found that whereas IgG-synthesizing cells were undetectable in normal airways, the overwhelming majority of immunoglobulin-producing cells in mice chronically challenged with aerolized antigen exhibited cytoplasmic immunoreactivity for IgG. Cells synthesizing IgM were not detectable in the inflammatory infiltrate. Although the time point selected for study was associated with maximal accumulation of chronic inflammatory cells in the airway wall, there was no increase in the number of cells synthesizing IgA and we were unable to demonstrate the presence of cells producing IgE.

Using immunohistochemical staining of sections of resin-embedded tissue, the cytoplasmic immunoglobulins in the plasma cells in the airway wall were demonstrated to be antigen-specific. Production of antigen-specific immunoglobulins has previously been shown in an experimental

model of acute allergic bronchopulmonary inflammation [23] but so far as we are aware, this is the first demonstration of allergen-specific immunoglobulin synthesis by plasma cells within the airway wall in a model of chronic asthma.

In nonfatal asthma, the presence of plasma cells in the airway wall has long been recognized [24]. Increased numbers of B cells have also been found in the sputum of asthmatics [25]. However, immunoglobulin-synthesizing cells in the airways of asthmatics have received relatively little attention. This may in part be a consequence of the difficulty of reliably identifying plasma cells on the basis of morphology. Resin embedding of tissues may be necessary to achieve this [10]. In those studies which employed resin embedding of tissues obtained by bronchial biopsy, a large proportion of the cells in the lamina propria was noted to be plasma cells [3,24]. This finding is consistent with evidence from bronchoalveolar lavage studies of local production of IgA, IgG3 and IgG4 in asthmatics [26,27].

The role of locally synthesized IgG in the pathogenesis of asthma is unknown. In mice, IgG (especially IgG1) may play a role in type I hypersensitivity responses [28,29]. Murine IgG1 is considered to be the counterpart to human IgG4 [27] which, as noted above, may be locally synthesized in asthmatics. However, we did not define the IgG subclass produced by the plasmacytoid cells in the airway wall. Despite systemic induction of an IgE response, demonstrated in these experiments by elevation of total plasma IgE and in a previous study by the presence of high titres of ovalbumin-specific IgE [12], we could not demonstrate the presence of IgE-synthesizing cells in the airway wall. This suggests that local immunoglobulin production does not contribute significantly to the induction of an IgE-mediated response.

While examining local production of immunoglobulins, it was pertinent to investigate the phenotypes and distribution of mast cells, which function as effector cells in type I hypersensitivity responses. Numerous metachromatically staining cells, including mast cells and basophils, are present in bronchoalveolar lavage fluid and induced sputum of asthmatics [30,31], but there is controversy about whether mast cells accumulate in the airway wall. Some studies have demonstrated increased numbers of mast cells, both in the epithelium and the lamina propria [3,4]. Others have asserted that there is no such increase [32,33], although there may be changes in phenotype as assessed by expression of proteases associated with different subsets of mast cells [11]. In mice, mast cell proteases (mMCP) 1 and 2 are characteristically expressed by mast cells associated with mucosal epithelial cells whereas mMCP 5 is associated with mast cells in connective tissues [18]. We used antibodies to these proteases to quantify mast cells in the airway wall in animals chronically exposed to inhaled antigen, but found

no evidence of accumulation of mast cells or of selective recruitment of a subset of mast cells.

In conclusion, this study demonstrates that antigen-specific IgG-synthesizing cells selectively accumulate in the airway wall in an experimental model of chronic asthma. Further investigation of the pathogenetic role of local synthesis of IgG and other immunoglobulins in asthma is warranted.

Acknowledgements

We thank Ms. Angelina Enno for preparation of sections of paraffin-embedded tissues. Jason Temelkovski was supported by an Australian Postgraduate Award.

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