

Reversibility of airway inflammation and remodelling following cessation of antigenic challenge in a model of chronic asthma

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Summary

Background Asthma is associated with recruitment of eosinophils, accumulation of chronic inflammatory cells in the airway walls, subepithelial fibrosis and other structural changes of airway wall remodelling. The role of ongoing exposure to allergens in their pathogenesis remains unclear.

Objective To examine whether changes of inflammation and remodelling were reversible following cessation of antigenic challenge in a mouse model of chronic asthma.

Methods BALB/c mice sensitized to ovalbumin (OVA) were chronically challenged by inhalation of a low mass concentration of antigen for 8 weeks, leading to development of acute-on-chronic airway inflammation, subepithelial fibrosis and other changes of airway wall remodelling. Epithelial injury was assessed by immunohistochemistry, while inflammation and remodelling were quantified by appropriate histomorphometric techniques. Regression of lesions was assessed in animals examined at 1, 2 and 4 weeks after exposure to OVA ceased.

Results We did not find evidence of airway epithelial injury in this model of low-level chronic inhalational exposure to antigen. Persistence of the recruitment of eosinophils and chronic inflammatory cells in the airway walls was dependent on continuing antigenic challenge, as was persistence of mucous cell hyperplasia/metaplasia. Subepithelial fibrosis and epithelial hypertrophy exhibited delayed reversibility following cessation of exposure to antigen, possibly related to matrix-associated accumulation of transforming growth factor- β_1 .

Conclusion In chronic asthma, low-level antigenic challenge may be required to maintain the inflammatory response in the airway wall, but airway remodelling may persist in its absence.

Keywords chronic inflammation, eosinophil recruitment, epithelial injury, subepithelial fibrosis, transforming growth factor- β

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Long-standing asthma is accompanied by the development of structural changes collectively referred to as airway wall remodelling [1], which include subepithelial fibrosis, goblet cell hyperplasia/metaplasia, smooth muscle hypertrophy/hyperplasia and increased vascularity. These changes are associated with the development of a component of irreversible airflow obstruction [2, 3], which has led to the suggestion that remodelling of the airway wall may be a potentially important therapeutic target for anti-asthma drugs [4].

Subepithelial fibrosis is a distinctive lesion of asthma that may be the single most useful marker of airway wall remodelling, as it is related to the severity of the disease [5] and correlates with other evidence of remodelling [6]. The pathogenesis of subepithelial fibrosis is incompletely understood, but appears to be related to epithelial injury, leading to the release of growth factors that promote the accumulation of myofibroblasts and synthesis of collagen and related

extracellular matrix proteins by these cells [7, 8]. The role of the inflammatory response in the development of subepithelial fibrosis – and of airway wall remodelling in general – remains contentious.

The extent to which the changes of airway wall remodelling are reversible is unclear, as is their relationship with ongoing exposure to antigen. Much of the available evidence regarding regression of remodelling in response to treatment is contradictory. However, subepithelial fibrosis associated with diisocyanate-induced occupational asthma has been reported to be reversible after cessation of exposure [9]. In allergic asthmatics, reversal of subepithelial fibrosis has been demonstrated following prolonged treatment with high doses of corticosteroids [10, 11].

We have developed a chronic antigenic challenge model of asthma in BALB/c mice, which replicates several key inflammatory and structural changes of this disease, including subepithelial fibrosis [12]. Following systemic sensitization to ovalbumin (OVA), animals are subjected to inhalational challenge with controlled low levels of aerosolized antigen for 6–8 weeks. In contrast to models involving uncontrolled

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exposure to high levels of aerosolized OVA, this exposure protocol is not associated with the development of perivascular and peribronchiolar inflammation in the lung parenchyma but instead elicits lesions confined to the conducting airways. The model thus emulates mild-to-moderate chronic asthma and has proved useful for investigation of cytokines regulating inflammation, remodelling and airway hyper-reactivity (AHR) (reviewed in [13]) as well as the relationship between airway epithelial expression of growth factors and the development of remodelling [14]. In the present study, we assessed evidence of epithelial injury associated with development of subepithelial fibrosis and epithelial hypertrophy; the regression of inflammatory changes in the airways after cessation of antigenic challenge; and its relationship with the regression of changes of airway wall remodelling. Our data suggest that in this experimental model of chronic asthma, persistence of airway inflammation and mucous cell hyperplasia/metaplasia are temporally related to ongoing antigen challenge, whereas subepithelial fibrosis and epithelial hypertrophy exhibit delayed reversibility following cessation of exposure to antigen.

Materials and methods

Animals

Specific pathogen-free female BALB/c mice, routinely screened serologically for relevant respiratory pathogens, were obtained from the Biological Resources Centre, University of New South Wales. Animals were aged approximately 8 weeks at the commencement of experimental studies. They were maintained in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) and housed in autoclaved cages on autoclaved bedding in an airconditioned room on a 12 h light/dark cycle. Irradiated food and acidified water were provided *ad libitum* throughout. Each of the eight experimental groups comprised six animals. All procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (ref. no. 01/34.1).

Sensitization and exposure

The protocols we used for sensitization and inhalational challenge have previously been described in detail [12, 15]. Briefly, specific pathogen-free female BALB/c mice were sensitized by two intraperitoneal injections of 50 µg alum-precipitated chicken egg OVA (Grade V, ≥98% pure, Sigma, Sydney, Australia; unless otherwise specified, all chemicals were obtained from this source) 21 days and 7 days before inhalational challenge commenced. Animals were exposed to aerosolized OVA for 30 min/day on 3 days/week for 8 weeks in wire flow-through cage racks in a 0.5 m³ whole-body inhalation exposure chamber (Unifab Corporation, Kalamazoo, MI, USA). Filtered air was drawn through the chamber at a flow rate of 250 L/min and an aerosol of OVA, generated from a 2.5% solution by delivery of compressed air to a sidestream jet nebulizer (Trimed, Sydney, Australia), was injected into the airstream entering the chamber. Particle concentration within the breathing zone of the mice was

continuously monitored and maintained at $\approx 3 \text{ mg/m}^3$ as assessed by a real-time monitor (DustTrak 8520, TSI, St Paul, MN, USA) (equivalent to $\approx 18 \text{ mg/m}^3$ as assessed by the PCAM-TX instrument used in our previous reports) by controlling the airflow into the nebulizer. Animals were examined at the end of the 8-week period of inhalational exposure and at 1, 2 and 4 weeks after cessation of exposures. Control groups were non-sensitized non-exposed (i.e. naïve) as well as non-sensitized chronically exposed animals examined at the end of the period of exposure.

Histopathology

Tissues for histopathological examination were collected 18 h after the last inhalational exposure and fixed in 10% buffered formalin overnight. The longitudinally oriented trachea and a horizontal slice from the mid-zone of the single-lobed left lung were embedded in paraffin. Ribbons of two to three sections cut at 4 µm were stained with haematoxylin and eosin for assessment of inflammatory changes in sections of the longitudinally oriented trachea; with Gordon and Sweet's reticulin stain for assessment of collagen deposition; or with Alcian blue-periodic acid Schiff for enumeration of mucin-secreting cells in the intrapulmonary conducting airways.

Immunohistochemistry

This was performed as previously described [14, 16]. Expression of CD44v10, CD44s and intercellular adhesion molecule type 1 (ICAM-1) in the epithelium of the intrapulmonary airways was demonstrated using a commercial ABC-Peroxidase technique (Vectorstain Elite Kit, ALEXIS Deutschland GmbH, Grünberg, Germany) according to the manufacturer's protocol. Monoclonal rat antibodies were directed against CD44v10 (clone K926; kindly provided by M. Zöller, Heidelberg, Germany), CD44s (clone KM201; Genzyme, Rüsselsheim, Germany) and ICAM-1 (clone KAT-1; Genzyme). Expression of transforming growth factor (TGF)- β_1 was demonstrated using an affinity-purified rabbit polyclonal antibody to a peptide mapping at the carboxy terminus of the precursor form of human and mouse TGF- β_1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-146). This antibody detects TGF- β_1 in the absence of its latency-associated peptide and is thus considered to react with the cleaved, potentially biologically active form of TGF- β_1 [17]. The smooth muscle layer surrounding the intrapulmonary airways was demonstrated by staining for α -smooth muscle actin (monoclonal mouse antibody, clone asm-1; Cymbus Biotechnology Ltd, Hants, UK), using a commercial mouse on mouse Kit (DakoCytomation, Hamburg, Germany) according to the manufacturer's instructions.

Morphometry

The validity and reliability of the morphometric techniques that we used for quantifying inflammation and remodelling have been established in previous reports [12, 15, 18]. To enumerate inflammatory cells in sections of the trachea, images were captured with a Spot Cooled Color Digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) that was calibrated with a reference measurement slide. Nuclear profiles in haematoxylin and eosin-stained sections

were counted in every second consecutive microscopic field in the lamina propria and the length of the epithelial basement membrane was measured by tracing using the Spot software. 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.

In parallel, the full length of the epithelial surface was examined using a $\times 100$ oil immersion objective to enumerate intraepithelial eosinophils, which were readily identified on the basis of their strongly staining cytoplasmic granules. The data were expressed as number of cells per millimetre of epithelial basement membrane for individual animals.

Reticulin-stained sections of the trachea were used for assessment of subepithelial collagenization. Multiple measurements of the thickness of the reticulin-stained zone underneath the tracheal epithelium were taken using the Spot software and the mean thickness for each animal was calculated. The mean thickness of the epithelial layer was also measured in these sections. The thickness of the smooth muscle layer surrounding the left main bronchus was similarly quantified in sections of lung tissue immunostained for α -smooth muscle actin.

Metaplasia and/or hyperplasia of mucus-secreting goblet cells in the intrapulmonary airways was assessed in sections of lung tissue stained with Alcian blue-PAS. The percentage of cells staining positive for mucins was enumerated in the largest visible airway and each animal was categorized according to a semi-logarithmic scale as grade 0 = <1% positive cells, grade 1 = 1–3%, grade 2 = 4–10%, grade 3 = 11–30% and grade 4 = $\geq 31\%$.

The intensity of immunoreactivity was semi-quantitatively scored as grade 0 = no staining, grade 1 = weak staining, grade 2 = moderate staining and grade 3 = strong staining. All grading was performed by a single observer blinded to the identity of the samples and slides were presented in random order for examination.

Statistical analysis

Cell counts and measurements of thickness are presented as arithmetic means and standard errors for each experimental group. An initial one-way ANOVA followed by Dunnett's test was used to examine differences compared with the naïve and non-sensitized control groups. Grading of the proportion of mucin-positive cells and the intensity of immunoreactivity are presented as median values and interquartile ranges. For these data, a non-parametric Kruskal–Wallis test followed by Dunn's test was used to assess differences compared with the naïve control group. The software package GraphPad Prism 4.01 (GraphPad Software, San Diego, CA, USA) was used for all data analysis and preparation of graphs.

Results

Airway inflammation

As previously described [12, 15], sensitized BALB/c mice exhibited accumulation of significant numbers of eosinophils within the epithelial layer following repeated exposure to aerosolized antigen ($P < 0.01$ compared with both control groups) (Fig. 1a). The mean number of intraepithelial

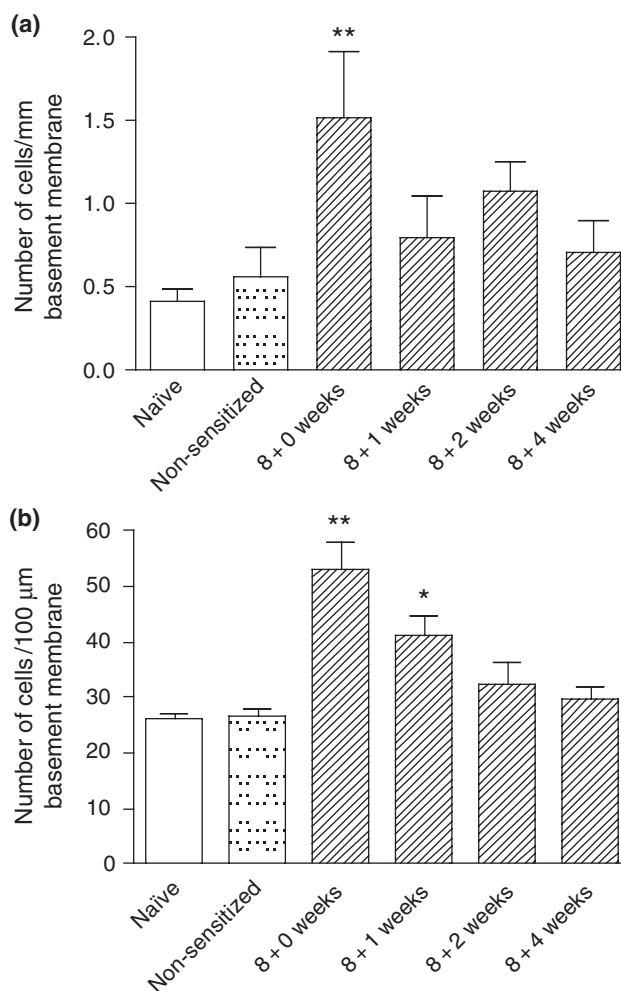


Fig. 1. (a) Intraepithelial eosinophils and (b) inflammatory cells in the lamina propria of the trachea of sensitized mice after 8 weeks of antigenic challenge and 0, 1, 2 or 4 weeks of recovery, compared with naïve and non-sensitized chronically challenged animals. Significant differences compared with naïve controls are shown as * $P < 0.05$, ** $P < 0.01$.

eosinophils declined rapidly after cessation of exposure to aerosolized antigen and was not significantly greater than in controls at 1, 2 or 4 weeks post-exposure.

In sensitized chronically challenged mice, there was a statistically significant increase in the mean profile density of cells in the airway wall as a consequence of accumulation of lymphocytes, plasma cells and other mononuclear inflammatory cells (Fig. 1b) ($P < 0.01$ compared with controls). The mean number of cells declined within the first week after cessation of antigenic challenge, although it was still significantly increased compared with controls ($P < 0.05$). There was a further decline in the second week post-exposure, by which time the number of cells was no longer significantly greater than in controls.

Changes of remodelling

As previously described, a prominent subepithelial reticulin-stained zone was apparent in tracheal sections from sensitized mice exposed to antigen. The thickness of this zone was significantly greater than in control animals ($P < 0.01$) (Fig. 2a). In marked contrast to the findings with respect to airway

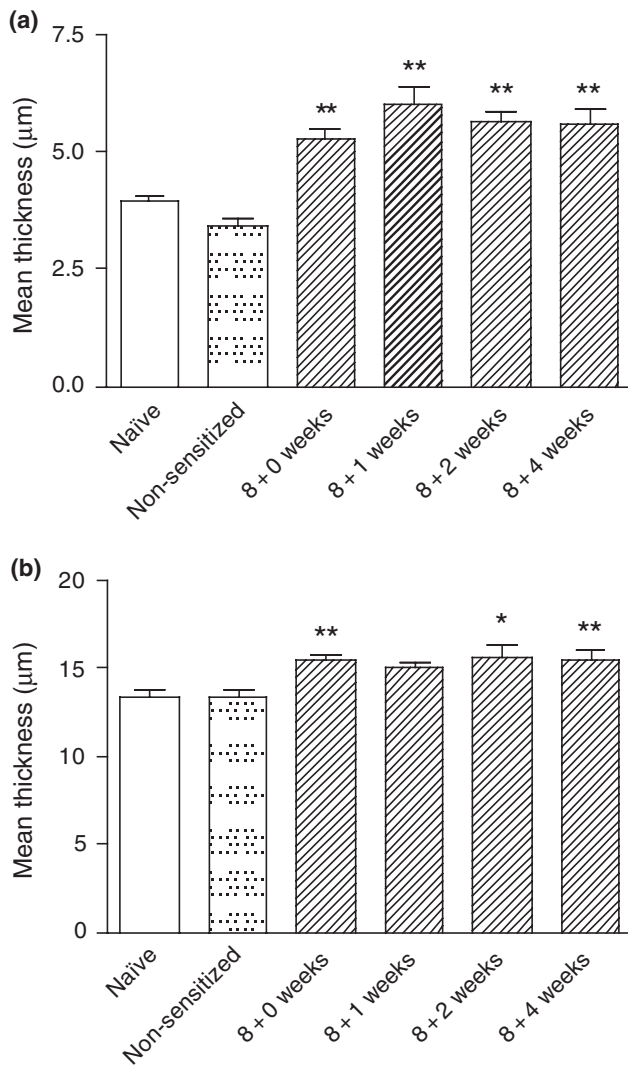


Fig. 2. (a) Subepithelial collagenization and (b) hypertrophy of the tracheal epithelium in sensitized mice after 8 weeks of antigenic challenge and 0, 1, 2 or 4 weeks of recovery, compared with naïve and non-sensitized chronically challenged animals. Significant differences compared with naïve controls are shown as * $P < 0.05$, ** $P < 0.01$.

inflammation, there was no evidence of any decrease in the thickness of the subepithelial fibrotic zone in the 4 weeks following cessation of antigenic challenge.

The mean thickness of the airway epithelium was significantly increased in sensitized BALB/c mice after 8 weeks of inhalational exposure to antigen ($P < 0.01$ compared with control animals) (Fig. 2b). Again, there was no evidence of a decrease in the thickness of epithelium in the 4 weeks following cessation of antigenic challenge.

Mucus-secreting goblet cells were rare or absent in the intrapulmonary airways of naïve mice and were only occasionally observed in non-sensitized chronically exposed mice. Consistent with our previous reported studies, there was a striking increase in the proportion of goblet cells in the intrapulmonary airways of sensitized chronically exposed mice (Fig. 3) with the median grade rising from 0.5 to 4 ($P < 0.01$). The median grade declined in the first week after cessation of antigenic challenge, although it remained

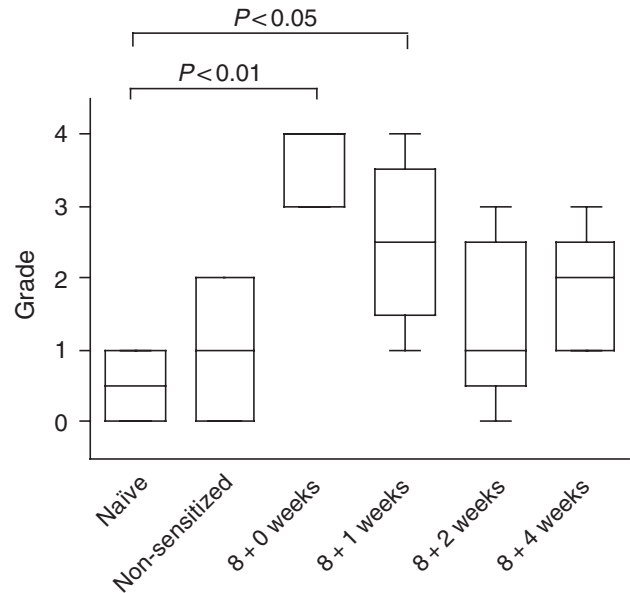


Fig. 3. Mucous cells in the intrapulmonary airways of sensitized mice after 8 weeks of antigenic challenge and 0, 1, 2 or 4 weeks of recovery, compared with naïve and non-sensitized chronically challenged animals. Significant differences compared with naïve controls are as shown.

significantly greater than in naïve controls ($P < 0.05$). By the second week post-exposure, the proportion of mucous cells was no longer significantly increased compared with controls.

In naïve control animals, the mean thickness of the α -smooth muscle actin-positive zone surrounding the intrapulmonary airways was $8.6 \pm 0.5 \mu\text{m}$. The thickness of the smooth muscle layer in sensitized chronically challenged mice was essentially unchanged at $8.9 \pm 0.7 \mu\text{m}$.

Immunohistochemical evidence of epithelial injury

Staining for CD44v10 in naïve control animals was generally restricted to the basolateral regions of epithelial cells of the intrapulmonary airways, with little if any expression on the free surface. Almost all cells exhibited basolateral staining of moderate intensity (median grade 2.0, interquartile range 1.5–3.0). There was no evidence of an increase in the intensity of staining in sensitized chronically exposed mice (median grade 2.0, interquartile range 1.5–3.0) nor was there any change in the distribution of immunoreactivity. Essentially identical results were obtained for immunostaining for CD44s. ICAM-1 was strongly expressed on the endothelium of both large vessels and alveolar capillaries, but airway epithelial cells in naïve animals were only weakly immunoreactive, primarily along the apical and lateral borders (median grade 1.0, interquartile range 1.0–2.0). Epithelial staining for ICAM-1 in sensitized chronically challenged animals was comparable with that in naïve mice (median grade 1.0, interquartile range 1.0–2.5).

Immunohistochemical staining for transforming growth factor- β_1

As we have recently reported [14], whereas naïve animals exhibited little if any staining for TGF- β_1 in the airway wall, there was widespread staining for this growth factor in the

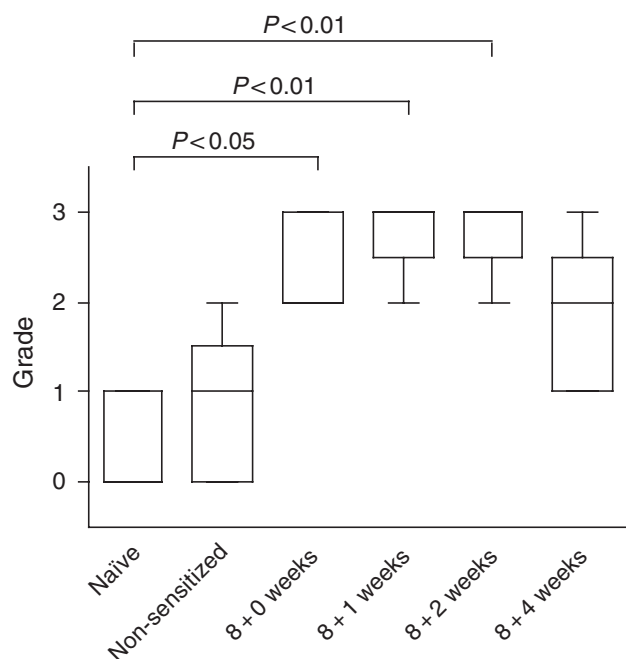


Fig. 4. Intensity of immunoreactivity for active transforming growth factor- β_1 in the subepithelial zone of the trachea in sensitized mice after 8 weeks of antigenic challenge and 0, 1, 2 or 4 weeks of recovery, compared with naive and non-sensitized chronically challenged animals. Significant differences compared with naive controls are as shown.

subepithelial zone of connective tissue in sensitized chronically exposed mice and the median grade of the intensity of staining was significantly greater than in controls ($P < 0.05$) (Fig. 4). Staining was even stronger at 1 and 2 weeks post-exposure but then declined somewhat at 4 weeks.

Discussion

The results of this study clearly demonstrate that in this model of chronic asthma, persistence of intraepithelial eosinophils and of chronic inflammatory cells within the lamina propria was dependent on continuing antigenic stimulation. This finding may be relevant to the long-established observation that even in patients with early and/or mild asthma, there is significant inflammation of the airways [19, 20]. As has recently been shown, exposure to allergens may induce inflammation without eliciting obvious clinical manifestations [21]. Our data are consistent with the inference that in allergic asthmatics, low-level antigenic challenge may be responsible for maintaining the inflammatory response in the airway wall.

This experimental model involves controlled exposure to low mass concentrations of aerosolized antigen (particle concentrations of $\approx 3 \text{ mg/m}^3$) and closely replicates many of the lesions of human asthma [13]. It has proved particularly useful for examining the pathogenesis of airway wall remodelling. Data from the present study suggest that ongoing exposure to allergens may be important in maintaining the hyperplasia of goblet cells with significantly increased mucin content that characterizes human asthma [22]. In murine experimental models of asthma, the goblet cell response is dependent on signalling by T-helper type 2

cytokines including IL-4, -9 and -13 [23–25]. In the context of the relatively rapid regression of the chronic inflammatory response in the airway wall observed in this study, it is therefore not surprising that the proportion of mucous cells in the epithelium diminishes in parallel.

In contrast, following cessation of antigenic challenge in this model, there was no evidence of spontaneous reversal of the changes of subepithelial fibrosis and epithelial hypertrophy within the period of follow-up. This is of interest in the light of our observations that in established disease, these changes of remodelling are readily reversed by treatment with a high dose of systemic corticosteroids or with the novel phosphodiesterase inhibitor roflumilast [26]. The data are consistent with evidence from recent studies that a high dose of inhaled glucocorticoids may similarly be necessary to reverse subepithelial fibrosis in asthmatics [10, 11]. Delayed reversibility of changes of remodelling has also been demonstrated in mouse models of chronic allergic inflammation involving ongoing challenge with high doses of antigen, by uncontrolled inhalational exposure to aerosols or by intranasal instillation [27–29]. While such models are confounded by marked parenchymal inflammation, which may undergo down-regulation after repeated challenge, the reported studies lend credence to the notion that although airway inflammation may be dependent on continuing exposure to antigen, airway remodelling may persist in its absence.

We speculate that the marked subepithelial immunoreactivity for TGF- β_1 , which was still evident for weeks following cessation of antigenic challenge, could provide at least a partial explanation for the delayed reversibility of airway remodelling. In this model, we have previously shown that subepithelial accumulation of immunoreactive TGF- β_1 precedes the accumulation of collagen, while its absence correlates with the lack of remodelling in IL-13-deficient mice and mice chronically treated with anti-CD4 [14]. The antibody used for immunostaining recognizes the cleaved, biologically active form of TGF- β_1 and it is therefore plausible that the matrix-associated TGF- β_1 drives the formation and/or maintenance of collagenous connective tissue in the subepithelial zone.

We have shown that the accumulated active TGF- β_1 is apparently derived from latent TGF- β_1 in the cytoplasm of airway epithelial cells and is rapidly released following antigenic challenge [14]. Thus, it was of considerable interest to assess whether there was any evidence of epithelial injury associated with the increased immunoreactivity for TGF- β_1 in this model. In asthmatics with disease of moderate severity, the hyaluronan receptor CD44 is well documented as a marker of epithelial injury, with increased intensity of staining especially on the free surface of exposed basal cells in areas of damage [30, 31]. Moreover, CD44 can provide a cell surface receptor for matrix metalloproteinase-9 and this complex, which is proteolytically active, can activate latent TGF- β [32]. However, we found no evidence that there was increased intensity of immunostaining for CD44 in the airway epithelium of sensitized chronically exposed animals, nor was there any evidence of staining of the free surface of the cells. ICAM-1 has also been demonstrated to be up-regulated in the airway epithelium of asthmatics [33] and is of interest because enhanced expression of this adhesion molecule could contribute to the intraepithelial accumulation of eosinophils [34].

However, we similarly found no evidence of increased intensity of immunoreactivity for ICAM-1 in the airway epithelium of chronically challenged mice. Nevertheless, epithelial release of TGF- β_1 following antigenic challenge indicates that these cells are undoubtedly activated in this model.

Several studies in asthmatics have suggested that airway wall thickening – and in particular subepithelial fibrosis – correlates with AHR [5, 35, 36]. However, in our model, AHR is no longer detectable 48 h after the last allergen challenge [37] so that persistence of the subepithelial and epithelial changes of remodelling clearly is not associated with persistence of AHR. Moreover, we have previously demonstrated a lack of correlation between AHR and remodelling, as well as between remodelling and airway wall inflammation, in gene-targeted animals deficient in IL-5 [18]. In contrast, in some high-level chronic antigen challenge models, AHR assessed as increased respiratory system resistance in response to methacholine has been demonstrated weeks after cessation of allergen exposure [28, 29]. While parenchymal lesions in such models may contribute substantially to respiratory system resistance [37], the difference could also be related to the induction of smooth muscle hypertrophy and/or hyperplasia that is observed in these high-level challenge models. Increased smooth muscle mass is a major feature of severe human asthma [38, 39]. However, this aspect of airway remodelling is not replicated in our model of low-level antigenic challenge [40] and the previously reported histological finding was confirmed in the present study by immunohistochemical assessment of α -smooth muscle actin-positive cells in the airway walls. Whether smooth muscle hypertrophy and/or hyperplasia is critical to the development of persistent AHR remains an intriguing question.

In summary, we have shown that in an experimental model of asthma involving chronic challenge with low mass concentrations of inhaled antigen, persistence of the recruitment of intraepithelial eosinophils and accumulation of chronic inflammatory cells in the airway walls appear to be dependent on continuing antigenic challenge. Persistence of mucous cell hyperplasia and/or metaplasia is similarly related to ongoing exposure to antigen. In contrast, subepithelial fibrosis and epithelial hypertrophy exhibit delayed reversibility following cessation of exposure to antigen. This may at least in part be explained in terms of matrix-associated accumulation of active TGF- β_1 derived from airway epithelial cells, which appear to be activated even in the absence of morphological or immunohistochemical evidence of significant epithelial injury in this model.

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