CD4⁺ T-Lymphocytes Regulate Airway Remodeling and Hyper-Reactivity in a Mouse Model of Chronic Asthma

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SUMMARY: Asthma is an acute-on-chronic inflammatory disease of the airways, characterized by airflow obstruction and hyper-reactivity of the airways to a variety of stimuli. Chronic asthma is associated with remodeling of the airway wall, which may contribute to hyper-reactivity and fixed airflow obstruction. We used an improved mouse model of chronic asthma to investigate the role of CD4⁺ T-lymphocytes in airway remodeling and hyper-reactivity. Animals functionally depleted of CD4⁺ T-lymphocytes by repeated administration of a monoclonal antibody exhibited markedly decreased airway responsiveness. In addition, these mice had greatly diminished subepithelial fibrosis, epithelial thickening, and mucous cell hyperplasia/metaplasia. Chronic inflammation in the airway wall was moderately reduced, with a marked decrease in the accumulation of immunoglobulin-synthesizing plasma cells. However, intraepithelial accumulation of eosinophils was not significantly inhibited and airway epithelial expression of eotaxin was undiminished. This work provides the first experimental evidence that CD4⁺ T-lymphocytes play a crucial role in the pathogenesis of the lesions of chronic asthma and lends support to the notion that functional inhibition of these cells may be an important therapeutic target. (*Lab Invest* 2002, 82:455–462).

Asthma is an acute-on-chronic inflammatory disease of the airways, clinically characterized by episodic airflow obstruction and abnormally enhanced reactivity to a variety of stimuli (Chung and Adcock, 2000). Chronic asthma is associated with a variety of changes of airway wall remodeling, including subepithelial and airway wall fibrosis, epithelial metaplasia and increased smooth muscle mass (Hegele and Hogg, 1996). Pathogenetic mechanisms remain incompletely defined, but it has been suggested that acute inflammation is primarily responsible for episodic bronchoconstriction, whereas chronic inflammation and airway wall remodeling contribute to airway hyper-reactivity (AHR) and to the fixed airflow obstruction that develops in chronic asthmatics (Bousquet et al, 2000).

Studies in human asthmatics indicate that CD4⁺ T-lymphocytes are likely to play a crucial role in the development of asthma and AHR. Progressive accumulation of activated CD4⁺ T cells in the lamina propria of the airways is associated with development of the clinical features of asthma in individuals who previously exhibited asymptomatic AHR (Laprise et al,

1999). Increased numbers of activated CD4⁺ T cells are recruited into the airways of asthmatics following allergen challenge (Bentley et al, 1993) and T cell-specific allergen peptides are able to trigger a late asthmatic response (Haselden et al, 1999). Furthermore, treatment of severe asthmatics with a humanized monoclonal antibody to CD4⁺ T-lymphocytes leads to marked improvement in peak expiratory flow (Kon et al, 1998).

Studies in animal models of acute allergic bronchopulmonary inflammation provide additional support for the pathogenetic role of CD4⁺ T-lymphocytes. Mice lacking CD4⁺ T cells because of deficient ability to express class II major histocompatibility complex antigens fail to develop eosinophil-dominated inflammation or AHR following exposure to antigen (Brusselle et al, 1994). Adoptive transfer of CD4⁺ T cells from sensitized mice is associated with recruitment of eosinophils and the development of AHR following antigen challenge (Hogan et al, 1998a; Kaminuma et al, 1997). Conversely, depletion of CD4⁺ T-lymphocytes by administration of antibody abrogates AHR (Gavett et al, 1994; Hogan et al, 1998b; Mould et al, 2000). Depletion of CD4⁺ T cells typically also inhibits eosinophil recruitment (Garlisi et al, 1995; Gavett et al, 1994; Nakajima et al, 1992). However, both animal and human studies indicate that there is no consistent relationship between the accumulation of eosinophils and the development of AHR (Crimi et al, 1998; Hessel et al, 1997; Leckie et al, 2000; Mould et al, 2000; Nagai et al, 1996; Wilder et al, 1999).

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An important, and as yet unresolved, question is whether CD4⁺ T-lymphocytes play a significant role in airway wall remodeling in asthma. This is of particular interest because AHR in asthmatics may correlate with the development of changes of remodeling, especially subepithelial fibrosis (Chetta et al, 1996; Hoshino et al, 1998). Increasing subepithelial fibrosis has also been associated with progression from asymptomatic AHR to asthma (Laprise et al, 1999). This issue cannot be satisfactorily addressed in studies using short-term inhalational exposure to antigen, because the characteristic changes of remodeling fail to develop in such experimental models.

We have described an improved model of chronic asthmatic inflammation of the airways in mice (Temelkovski et al, 1998). This involves systemic sensitization of high IgE-responder BALB/c mice to ovalbumin and subsequent chronic inhalational challenge with low levels of aerosolized antigen for 30 minutes/day, 3 days/week for 6 weeks. This approach consistently elicits airway lesions typical of chronic human asthma, including acute and chronic inflammation in the epithelial layer and lamina propria, together with epithelial hypertrophy, mucous cell hyperplasia/metaplasia, and subepithelial fibrosis. In contrast to previously reported mouse models of asthma, in which the inflammatory response is characterized by a high density of lymphocytes and eosinophils surrounding the airways and extending to the blood vessels (Cohn, 2001), there are fewer total inflammatory cells and no evidence of pulmonary parenchymal inflammation. However, numerous intraepithelial eosinophils are seen in the upper airways. In addition, the mice exhibit marked airway hyper-reactivity to an inhaled cholinergic agonist, which can be attributed to abnormalities of the airways rather than to parenchymal lesions. Thus, the model replicates most features of the chronic human disease (Kumar and Foster, 2001).

To test the hypothesis that CD4⁺ T-lymphocytes play a crucial role in acute and chronic inflammation of the airways, remodeling of the airway wall, and development of airway hyper-reactivity in chronic asthma, we used our improved experimental model to examine these responses in mice depleted of CD4⁺ T-lymphocytes by chronic administration of antibody. Our results establish a crucial role for CD4⁺ T cells in airway wall remodeling and the development of AHR.

Results

Long-term administration of anti-CD4 and control monoclonal antibodies had no apparent deleterious effects upon the experimental animals. At the end of the period of treatment, there was a slight decrease in the mean weight of the anti-CD4-treated group relative to the control group (18.36 ± 0.32 g versus 19.07 ± 0.24 g, $n = 16$, $p < 0.05$). There was no evidence of pulmonary infection or other significant abnormalities in the anti-CD4-treated group at autopsy.

Airway Inflammation

We have previously shown that sensitized BALB/c mice develop widespread multifocal accumulation of lymphocytes, plasmacytoid lymphocytes, and other chronic inflammatory cells in the lamina propria of the trachea after long-term inhalation exposure to aerosolized ovalbumin (Temelkovski et al, 1998). This finding was reproduced in mice treated with the control monoclonal antibody β GL113. In comparison, there was a statistically significant reduction in the mean profile density of cells in the lamina propria in mice treated with the anti-CD4 antibody GK1.5 ($p < 0.05$) (Fig. 1A).

Whereas eosinophils are rarely identifiable within the tracheal epithelium of normal BALB/c mice, sensitized BALB/c mice treated with the control monoclonal antibody β GL113 exhibited recruitment of signifi-

Figure 1.

Inflammation in the airways in sensitized, chronically-exposed mice treated with β GL113 control monoclonal antibody or GK1.5 monoclonal antibody to CD4. A, Inflammation in the airway wall, as assessed by quantifying the numerical density of cells in the lamina propria of the trachea. B, Numerical density of intraepithelial eosinophils.

cant numbers of eosinophils, comparable with that previously described in untreated animals. Accumulation of eosinophils was unaffected in mice treated with the anti-CD4 antibody GK1.5 (Fig. 1B).

Airway Wall Remodeling

As has previously been demonstrated in untreated animals, a prominent subepithelial reticulin-stained zone was apparent in sensitized mice exposed to antigen and treated with control monoclonal antibody. The mean thickness of this zone was significantly decreased in mice treated with anti-CD4 antibody ($p < 0.001$) (Fig. 2A) and was equivalent to that in naïve mice. In parallel, treatment with anti-CD4 prevented the development of thickening of the airway epithelium that was observed in mice treated with control monoclonal antibody ($p < 0.001$) (Fig. 2B).

Mucus-secreting goblet cells, which are virtually absent in the intrapulmonary airways of naïve mice, were strikingly increased in sensitized exposed mice treated with the control antibody. These animals had a median grade of 3.5, whereas mice treated with anti-CD4 exhibited significantly decreased mucous cell hyperplasia/metaplasia, with a median grade of 1.0 ($p < 0.05$) (Fig. 2C).

Airway Responsiveness

As reported previously for sensitized, chronically-exposed BALB/c mice, animals treated with the control monoclonal antibody exhibited a left-shifted dose-response curve and increased maximal reactivity, characteristic of airway hyper-reactivity (Fig. 3). In contrast, animals treated with anti-CD4 exhibited markedly decreased airway responsiveness, comparable with that of naïve mice. The difference in enhanced Pause (Penh) was statistically significant at all methacholine concentrations tested.

Immunohistochemistry

Consistent with our previously reported observations in sensitized, chronically-exposed BALB/c mice (Kumar et al, 2000), there were numerous cells immunoreactive for cytoplasmic Ig in the lamina propria of the airways in animals treated with the control monoclonal antibody (Fig. 4A). Immunostaining for IgG subclass revealed the presence of a corresponding number of IgG1-positive cells (Fig. 4B) and no evidence of IgG2a-positive cells (Fig. 4C). In contrast, Ig-positive cells were virtually absent in mice treated with anti-CD4 antibody. The profile density of these cells was reduced from 3.79 ± 0.95 to 0.19 ± 0.07 cells/100 μm basement membrane ($p < 0.01$).

There was no evidence of diminished expression of growth factors of the epidermal growth factor (EGF) family by airway epithelial cells in animals chronically treated with anti-CD4. For TGF- α , the median grade for intensity of immunoreactivity of the epithelium was 2.0 in mice treated with the control antibody and 2.5 in mice treated with anti-CD4, whereas for amphiregulin

Figure 2.

Changes of remodeling in sensitized, chronically-exposed mice treated with monoclonal antibodies. A, Subepithelial fibrosis, as assessed by measuring the thickness of the reticulin-stained zone. B, Hypertrophy of the tracheal epithelium. C, Mucous cell hyperplasia/metaplasia in the intrapulmonary airways.

Figure 3.

Airway reactivity of sensitized, chronically-exposed mice, assessed by change in enhanced Pause (Penh) in response to increasing concentrations of aerosolized β -methacholine. Significant differences between mice treated with β GL113 control monoclonal antibody or GK1.5 monoclonal antibody to CD4 are shown as ** = $p < 0.01$, *** = $p < 0.001$.

the corresponding figures were 2.0 and 3.0. These differences were not statistically significant.

We have previously shown that in chronically challenged mice, expression of eotaxin by airway epithelial cells is maximal 3 hours after inhalational exposure (Kumar et al, 2002b). Airway epithelium was strongly immunoreactive for eotaxin both in mice treated with control antibody and in those treated with anti-CD4. Indeed, expression of eotaxin seemed to be moderately elevated in the latter animals, with median grades of 2.0 and 3.0, respectively ($p < 0.05$).

Discussion

In this investigation, we have demonstrated the feasibility and effectiveness of long-term administration of an anti-CD4 antibody to study the role of these T cells in an experimental model of chronic asthma. In mice that received injections of anti-CD4 over a period of 6 weeks, there was no evidence of illness, and although there was a small decrease in mean weight compared with mice that received the control antibody, there was no evidence of pulmonary infection or other significant abnormalities at autopsy.

That the anti-CD4 antibody was effective in blocking CD4 T cell function was clearly demonstrated by the abrogation of the local humoral immune response in the airway wall in the treated animals. We have previously shown that long-term inhalational exposure of sensitized mice to antigen is associated with accumulation of numerous IgG-producing plasma cells in the lamina propria of the airways (Kumar et al, 2000). In the present study, this finding was replicated in mice treated with control antibody, while staining for IgG subclasses revealed that the plasma cells were essentially all IgG1-positive and IgG2a-negative, which is consistent with a Th2 pattern of response (Finkelman et al, 1990). In contrast, the response was abrogated in mice treated with anti-CD4, presumably as a consequence of the lack of helper T cell function for antibody production.

Figure 4.

A, Many cells strongly immunoreactive for cytoplasmic immunoglobulin were present in the lamina propria of the trachea of sensitized, chronically-exposed mice treated with β GL113 control monoclonal antibody. B, Immunostaining for cytoplasmic IgG1 demonstrated numerous immunoreactive cells (some indicated by arrows). C, In contrast, virtually no cells immunoreactive for cytoplasmic IgG2a could be identified in a consecutive section. Immunoperoxidase, counterstained with hematoxylin (A) or methyl green (B and C).

The most striking feature of this study was that in comparison with mice treated with control antibody, there was marked suppression of airway wall remodeling in mice treated with anti-CD4. This was evident both with respect to collagen deposition in the subepithelial region and epithelial hypertrophy. Subepithelial fibrosis is a distinctive lesion of human asthma that is well reproduced in our chronic inhalation exposure model (Temelkovski et al, 1998) but is not amenable to study in animal models of acute allergic bronchopulmonary inflammation. Our investigation is the first to demonstrate the crucial role of the T cell-mediated response in regulating airway wall remodeling in chronic asthma. Furthermore, these findings are consistent with our earlier work using experimental models of pulmonary fibrosis, which demonstrated a role for T cells in regulation of the

production of macrophage-derived growth factors for fibroblasts (Kumar, 1989; Li et al, 1992), including growth factors of the epidermal growth factor receptor (EGFR) ligand family (Kumar et al, 1993). Recent studies have emphasized the potential role of EGFR ligands in airway wall remodeling in human asthma (Davies et al, 1999; Puddicombe et al, 2000), particularly with respect to epithelial injury and repair. Therefore, we investigated whether there was any diminution of epithelial expression of TGF- α or amphiregulin in the airways of mice treated with anti-CD4, because these are the major EGFR ligands produced by airway epithelial cells (Tsao et al, 1996). However, we were unable to demonstrate any differences compared with animals treated with control antibody, which might indicate that other growth factors could be more important in the induction of epithelial hypertrophy. Nevertheless, our observations do not exclude a role for EGFR ligands produced by macrophages (Madtes et al, 1988), by eosinophils (Brach et al, 1994), or by T cells themselves (Blotnik et al, 1994) in the pathogenesis of subepithelial fibrosis.

Another significant finding to emerge from the present study was that CD4⁺ T cells played a crucial role in the development of AHR. This result was not unexpected, because the role of CD4⁺ T cells in AHR has previously been demonstrated in a variety of short-term, high-level exposure models of allergic bronchopulmonary inflammation (Gavett et al, 1994; Hogan et al, 1998a, 1998b; Mould et al, 2000). However, this work provides the first experimental evidence that CD4⁺ T cells regulate AHR in chronic asthma. This finding is entirely consistent with data from long-term follow-up studies in human asthmatics (Laprise et al, 1999).

An interesting question in this context is whether there is a pathogenetic relationship between the suppression of remodeling and the decrease in airway reactivity following treatment with anti-CD4 in this model of chronic asthma. Human studies reveal an association between severity of remodeling and AHR in asthma (Chetta et al, 1996; Hoshino et al, 1998), as well as a correlation between increasing subepithelial fibrosis and progression from asymptomatic AHR to asthma (Laprise et al, 1999). However, our previous studies using this experimental model suggest that remodeling and development of AHR can be uncoupled in IL-5-deficient animals, which develop significant airway wall remodeling but not AHR (Foster et al, 2000), as well as in IL-13-deficient animals, which do not exhibit remodeling but continue to be hyper-reactive to methacholine (Kumar et al, 2002a).

With respect to the inflammatory changes in the airway walls, we wish to emphasize that because these animals do not develop a significant alveolitis, relatively few inflammatory cells are present in bronchoalveolar lavage fluid and lavage is thus an inappropriate method of quantifying inflammation in this model. We, therefore, employed morphometric quantification of eosinophils within the airway epithelium and of chronic inflammatory cells within the lamina propria. In mice treated with anti-CD4, there was a diminution in the number of

chronic inflammatory cells in the airway wall, as demonstrated by morphometric quantification of nuclear profiles. This was possibly related to the absence of the local humoral immune response and the lack of accumulation of plasma cells (Kumar et al, 2000). Unexpectedly, however, there was no diminution in the intraepithelial recruitment of eosinophils in anti-CD4-treated mice. In experimental studies using short-term, high-level challenge models of allergic bronchopulmonary inflammation, recruitment of eosinophils was shown to be abrogated by administration of the GK 1.5 antibody against CD4⁺ T cells (Gavett et al, 1994), and the T cell dependence of eosinophil recruitment was shown to be related to induction of eotaxin. However, in our study using chronic low-dose challenge, expression of eotaxin by airway epithelium was undiminished in mice treated with anti-CD4 antibody. We have recently demonstrated up-regulation of the induction of eotaxin and other eosinophil chemoattractants in this model as compared with short-term, high-level exposure models (Kumar et al, 2002b), and we speculate that this may account for the lack of effect of administration of anti-CD4 on eosinophil recruitment.

In conclusion, our studies in this model of chronic asthma have facilitated investigation of the pathogenesis of changes in the airway wall, which simply could not be carried out with conventional short-term models of allergic bronchopulmonary inflammation. They have also highlighted some striking differences between the pattern of inflammation observed in our model and in short-term, high-level exposure models. The results emphasize the critically important role of CD4⁺ T-lymphocytes in asthma and lend support to the notion that functional inhibition of these cells may be an important therapeutic target (Kon and Kay, 1999).

Methods

Animals

Specific pathogen-free female BALB/c mice were obtained from the Biological Resources Centre, University of New South Wales, Sydney, Australia. 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 air-conditioned room on a 12 hour light/dark cycle. Irradiated 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, Antibody Administration, and Inhalational Exposure

This was performed as previously described (Temelkovski et al, 1998), except that, to ensure reproducible induction of an antiovalbumin IgE response, mice were sensitized by an intraperitoneal injection of 50 μ g of alum-precipitated chicken egg ovalbumin (Grade V,

≥98% pure; Sigma, St. Louis, Missouri; unless otherwise specified, all chemicals were obtained from this source) 21 days and 7 days (booster injection) before inhalational exposure. Experimental groups comprised of 7 to 9 immunized and boosted animals were administered weekly intraperitoneal injections of 0.75 mg of ammonium sulfate-purified anti-CD4 monoclonal antibody GK1.5 or isotype control monoclonal antibody β GL113 (Hogan et al, 1998b), beginning 3 days before inhalation exposures were commenced. Mice were exposed to aerosolized ovalbumin for 30 minutes/day on 3 days/week for 6 weeks in a whole-body inhalation exposure system (Unifab Corporation, Kalamazoo, Michigan). During the exposure, the animals 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/minutes. A solution of 2.5% ovalbumin in normal saline was aerosolized by delivery of compressed air to a sidestream jet nebulizer (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, Tennessee). Particle concentration was maintained in the range of 10 to 20 mg/m³ by controlling the airflow into the nebulizer.

Histopathology

Tissues for histopathologic examination were collected 18 hours after the last inhalational exposure and fixed in 10% buffered formalin overnight. Tissues for immunostaining were collected 3 hours after the last exposure. The longitudinally-oriented trachea and a horizontal slice from the mid-zone of the single-lobed left lung were embedded in paraffin. Ribbons of 2 to 3 sections, cut at 5 μ m, were stained with hematoxylin and eosin for assessment of inflammatory changes in sections of the longitudinally-oriented trachea, with Gordon & Sweet's reticulin stain for assessment of collagen deposition, or with Alcian blue-PAS for enumeration of mucin secreting cells in the intrapulmonary conducting airways.

Immunohistochemistry

To identify Ig-producing cells within the airway wall, immunostaining was performed using a capillary action staining system as previously described (Kumar et al, 1989). Sections of formalin-fixed, paraffin-embedded tissue were subjected to antigen retrieval by boiling in 0.01M citrate buffer (pH 6.0) for 10 minutes in a microwave. Sections were washed with PBS containing 0.1% Triton X-100 and nonspecific binding was blocked with 50% normal goat serum for 10 minutes at 37° C. They were then incubated for 30 minutes at 37° C with an appropriate concentration of a biotinylated rabbit antibody to mouse immunoglobulins (Dako, Sydney, Australia). Sections were washed four times with PBS-Triton after this and each subsequent incubation. Bound antibody was detected by

incubation for 30 minutes with a complex of streptavidin (Boehringer Mannheim, Sydney, Australia) and biotinylated horseradish peroxidase in PBS-1% BSA. Diaminobenzidine was used as the substrate and sections were lightly counterstained with hematoxylin for 30 seconds.

To assess relative production of IgG1 and IgG2a by the Ig-producing cells, immunostaining was performed using rabbit antibodies specific for each of these immunoglobulin subclasses (Zymed, San Francisco, California) (5 μ g/ml). Antigen retrieval and blocking of nonspecific binding were performed as above. Following incubation with the primary antibody, detection was by sequential 30-minute incubations with a 1:50 dilution of swine anti-rabbit immunoglobulins, followed by a 1:100 dilution of rabbit peroxidase-antiperoxidase complex (Dako).

Expression by airway epithelium of growth factors of the EGF family and of eotaxin were also assessed by immunostaining. Expression of EGF family ligands was demonstrated using rabbit polyclonal antibodies to transforming growth factor- α (TGF- α) (Santa Cruz Biotechnology, Santa Cruz, California, sc-9043) (10 μ g/ml) and amphiregulin (NeoMarkers, Fremont, California, Ab-1) (20 μ g/ml), and the procedure was as described above. Eotaxin expression was demonstrated using a goat polyclonal antibody to a 19-amino acid peptide corresponding to an epitope at the carboxy terminus of mouse eotaxin (Santa Cruz, sc-6182) (2.5 μ g/ml). The procedure was similar except that blocking was with rabbit serum and detection was with a 1:200 dilution of rabbit anti-goat immunoglobulins, followed by a 1:100 dilution of goat peroxidase-antiperoxidase complex (Dako).

Morphometry

The validity and reliability of the morphometric techniques we employed for quantifying inflammation and remodeling have been established in previous reports (Foster et al, 2000; Temelkovski et al, 1998). To enumerate inflammatory cells and Ig-secreting cells, images were captured with a Spot Cooled Color Digital camera (Diagnostic Instruments, Sterling Heights, Michigan), which was calibrated with a reference measurement slide. Nuclear profiles in hematoxylin and eosin-stained sections, or numbers of cells exhibiting cytoplasmic immunoreactivity for immunoglobulins, 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 μ m 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 millimeter of epithelial basement membrane for individual animals. The thickness of the epithelial layer

was also measured in these sections and the mean thickness for each animal was calculated.

Reticulin-stained sections were used for assessment of subepithelial collagenization. The thickness of the reticulin-stained zone underneath the tracheal epithelium was measured and the mean thickness for each animal was calculated.

Metaplasia and/or hyperplasia of mucus-secreting goblet cells 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 semilogarithmic scale as grade 0 = <1% positive cells; grade 1 = 1 to 3%; grade 2 = 4 to 10%; grade 3 = 11 to 30%; and grade 4 = ≥31%.

Intensity of immunoreactivity of airway epithelial cells was semiquantitatively 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.

Airway Reactivity

Responsiveness to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, New York). This system yields a dimensionless parameter known as Penh, reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurement was performed essentially as previously described (Hamelmann et al, 1997). Briefly, mice were placed in the chamber and baseline readings were taken and averaged for 3 minutes. Aerosolized methacholine (concentrations in solution ranging from 3.125–50 mg/ml) was then delivered through an inlet into the chamber for 3 minutes and readings averaged over a period of 3 minutes after each dose was administered.

Statistical Analysis

Results of morphometry and assessment of airway reactivity were, in general, expressed as arithmetic mean ± standard error for each experimental group, with comparisons between experimental groups by an unpaired *t* test. Because measurements of the proportion of mucin-positive cells in the intrapulmonary airways and the intensity of immunoreactivity were based upon grading, these results were expressed as median values and comparisons were obtained by a nonparametric Mann-Whitney test. The software package GraphPad Prism (GraphPad Software, San Diego, California) was used for all data analysis and preparation of graphs.

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