ORIGINAL ARTICLE

Histopathologic Changes in Two Mouse Models of Asthma

D Olmez,[1] A Babayigit,[1] G Erbil,[2] O Karaman,[1] A Bagriyanik,[2] O Yilmaz,[1] N Uzuner[1]

[1] Department of Pediatric Allergy, Dokuz Eylul University Hospital, Izmir, Turkey
[2] Department of Histology and Embriology, Dokuz Eylul University Hospital, Izmir, Turkey
[3] Department of Experimental Animal Laboratory, Dokuz Eylul University Hospital, Izmir, Turkey

Abstract

Background: No studies to date have compared mouse models of asthma by evaluating airway histopathology.
Objective: To compare 2 such models by studying chronic histopathologic changes of the airways using light and electron microscopy.
Methods: Twenty-one male BALB/c mice were divided into 3 groups: a nebulization group sensitized via an intraperitoneal injection of 10 µg ovalbumin on days 0 and 14 and exposed to 2.5% aerosolized ovalbumin 3 days a week over the subsequent 8 weeks; an intranasal group sensitized via 2 intraperitoneal injections of 100 µg ovalbumin on days 0 and 14 and administered an intranasal dose of 500 µg ovalbumin on days 14, 27, 28, 29, 47, 61, 73, 74, and 75; and a control group that received nothing. Airway histopathologies were evaluated.
Results: Basement membrane, epithelium, and subepithelial smooth muscle layer thicknesses and mast and goblet cell numbers were significantly higher in the nebulization group than in the control group. With the exception of mast cell numbers, these parameters were also significantly higher in the intranasal group than in the control group. On comparing the intranasal and the nebulization group, goblet cell numbers were significantly higher in the former and mast cells in the latter.
Conclusion: Both models replicated all the structural parameters of asthma except for mast cell numbers in the intranasal group (no significant difference with respect to the control group). Our findings do not provide sufficient evidence that one protocol is superior to the other. Larger studies are needed to compare different asthma protocols.

Key words: Mice. Asthma. Remodeling. Nebulized ovalbumin. Intranasal ovalbumin

Resumen

Antecedentes: Ningún estudio hasta la fecha ha comparado los modelos murinos de asma mediante la evaluación de la histología de la vía aérea.
Objectivo: Comparar 2 modelos tales mediante el estudio de los cambios crónicos histopatológicos de la vía aérea usando microscopía de óptica y electrónica.
Métodos: Veintiún ratones machos BALB/c fueron divididos en 3 grupos: un grupo "nebulización" sensibilizado mediante inyección intraperitoneal de 10 µg de ovalbúmina en los días 0 y 14 y expuesto a 2,5% de albúmina aerosolizada 3 días por semana a lo largo de 8 semanas consecutivas; un grupo "intranasal" sensibilizado mediante 2 inyecciones intraperitoneales de 100 µg de ovalbúmina en los días 0 y 14 y una dosis intranasal de 500 µg de ovalbúmina administrada en los días 14, 27, 28, 29, 47, 61, 73, 74, 75; y un grupo control que no recibió nada. Se evaluó la histología de la vía aérea.
Resultados: El grosor de la membrana basal, el epitelio, y la capa de músculo liso subepitelial, así como el número de mastocitos y las células caliciformes fueron significativamente mayores en el grupo "nebulización" que en el grupo control. Con la excepción del número de mastocitos, estos parámetros fueron también significativamente mayores en el grupo "intranasal" comparado con el grupo control. Comparando el grupo "intranasal" con el grupo "nebulización", el número de las células caliciformes fue significativamente superior en el primero y el de los mastocitos superior en el último.
Conclusión: Ambos modelos reproducen todos los parámetros estructurales del asma excepto por el número de mastocitos en el grupo "intranasal" (no diferencias significativas con respecto al grupo control). Nuestros hallazgos no nos proporcionan suficientes evidencias para afirmar que un protocolo es superior al otro. Son necesarios estudios más extensos para comparar los diferentes protocolos de asma.

Palabras clave: Modelo experimental con ratón. Asma. Remodelado. Ovoalbúmina nebulizada. Ovoalbúmina intranasal.

Introduction

Asthma is a chronic inflammatory disease characterized by reversible airflow limitation and airway hyperresponsiveness [1]. Sensitization to antigens is thought to be a prerequisite for initiating the inflammatory cascade in bronchial asthma, and repeated and continuous allergen exposure causes inflammation of the airway mucosa and submucosa, orchestrated by type 2 helper T (T_H2) cells [2]. Animal models of asthma have highlighted the importance of T_H2-driven allergic responses in the development of asthma [3]. Persistent inflammation in asthma may lead to structural changes known as airway remodeling [1], and components of remodeling in asthmatic airways of humans have been successfully reproduced in several animal models [4].

Immune responses to allergens can be influenced by many factors. Asthma-like phenotypes in murine models vary according to the asthma protocol used [2]. Although several methods have been used to induce asthma in mice, only a few studies have compared the efficiency of different methods [5]. To the best of our knowledge, no studies to date have compared asthma models in mice by evaluating chronic histopathologic changes associated with asthma. This was our aim, using 2 models of asthma to evaluate such changes in BALB/c mice.

Methods

Experimental Animals

Six-to-8-week-old female BALB/c mice, weighing 18 g to 20 g, were obtained from the Bornova Veterinary Control and Research Institute in Izmir, Turkey and kept in hygienic cages in a pathogen-free laboratory at our university. They were subject to a 12-hour light/dark cycle in air-conditioned rooms. The study complied with the recommendations of the Animal Care and Ethics Committee at Dokuz Eylul University Hospital. Twenty-one mice were divided into 3 groups, each containing 7 mice.

Sensitization and Inhalational Exposure

One group of mice (nebulization group) was sensitized via 2 intraperitoneal injections of 10 µg of ovalbumin (grade V, ≥98% pure; Sigma, St. Louis, Missouri, USA) with alum adjuvant on days 0 and 14 of the experiment. Starting on day 21, the mice, housed in whole-body exposure chambers, were exposed to 2.5% aerosolized ovalbumin for 30 minutes a day, 3 days a week, for 8 weeks. The temperature was kept at 20ºC to 25°C and the relative humidity at 40% to 60% [6,7]. A second group of mice (intranasal group) received an intraperitoneal injection of 100 µg of ovalbumin complexed with alum on days 0 and 14, followed by an intranasal dose of 500 µg of ovalbumin on days 14, 27, 28, 29, 47, 61, 73, 74, and 75 [8,9]. The third group (control group) received nothing. An outline of the study procedures and time intervals for the 2 study groups is given in Figure 1.

Histologic and Morphometric Analysis

Two investigators blinded to the groups interpreted structural and ultrastructural changes. The animals were

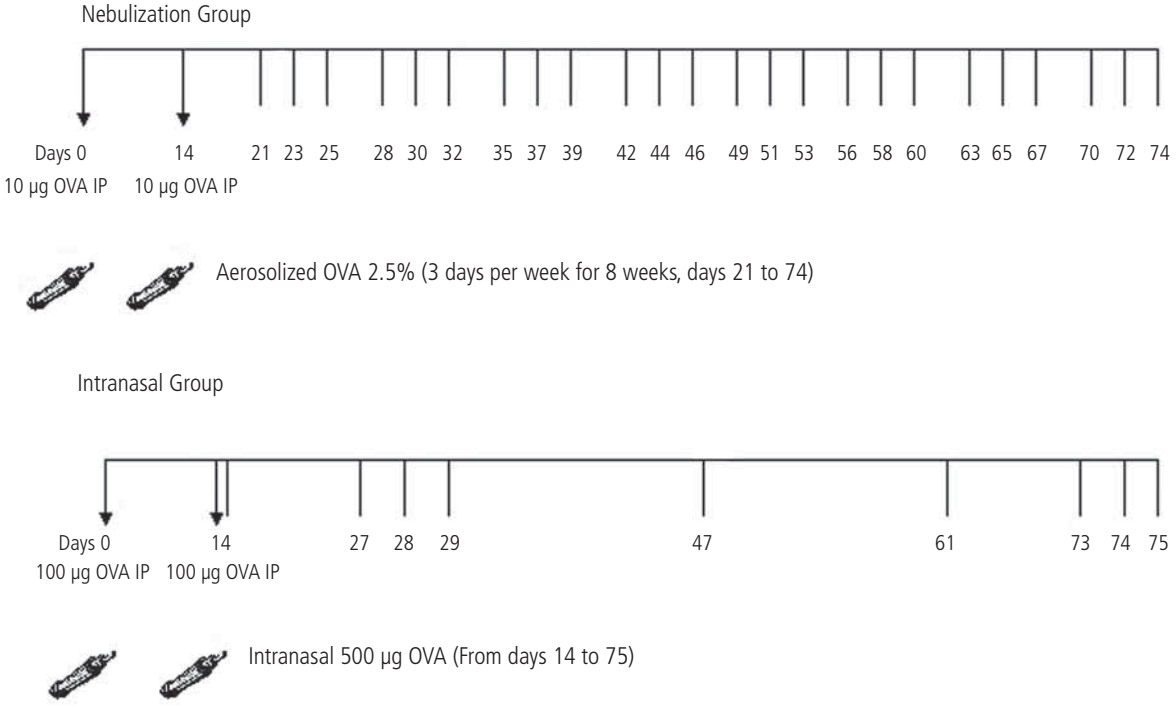

Figure 1. Schematic diagram showing procedures and timelines for the nebulization and intranasal groups. OVA indicates ovalbumin; IP intraperitoneal.

sacrificed with an overdose of ketamine 24 hours after the last ovalbumin exposure and histologic specimens were collected. Tissue specimens were taken from the mid zone of the left lung of the mice. Specimens measuring 1 mm^3 to 2 mm^3 were stored for electron microscopic evaluation and others were fixed in 10% formalin and embedded in paraffin using routine histologic procedures for subsequent light microscopic evaluation. Serial sections measuring 5 µm in thickness were then cut from the paraffin blocks and selected for staining. Ten sections were taken from every mouse. (Every 10th section starting from a randomly chosen section was selected). Three staining methods were used for light microscopic evaluation. The first ten sections were stained with hematoxylin and eosin (H&E), the next ten sections with toluidine blue, and the rest with periodic acid-Schiff (PAS). The slides stained with H&E were analyzed for tissue structure and morphometric features such as the thickness of the epithelium and the subepithelial smooth muscle layers of the medium and small airways. For these measurements, photomicrographs of 3 fields from each section containing airways were taken using a digital camera (JVC TK-890-E; JVC, Yokohama, Japan) fitted to an Olympus BH-2 RFCA microscope (Olympus Optical Co. Ltd, Tokyo, Japan) [10]. Morphometric analysis was carried out using version 3 of the UTHSCSA Image Tool for Windows (The University of Texas Health Science Center, San Antonio, Texas, USA) [11]. Epithelium and subepithelial smooth muscle layer thicknesses were measured using a calibrated micrometric analyzer at 8 different points on 2 to 3 different airways. Photomicrographs were taken randomly of 5 fields in each section stained with toluidine blue. A standard area measuring 16400 μm^2 was used for the enumeration of mast cells and the same method was used to count goblet cells in 10 PAS-stained sections from each mouse. In each section, 3 to 5 randomly selected airways were photographed, airway circumference measured, and the number of goblet cells in the standard area recorded. For standardization, goblet cell numbers in 100 µm were analyzed using the formula: total goblet cell numbers / total airway circumference × 100 [12].

Tissue samples obtained for electron microscopic evaluation using a Libra 120 microscope (Carl Zeiss, Oberkochen, Germany) were fixed with 2.5% glutaraldehyde and postfixative osmium tetraoxide. After routine electron microscopic procedures, the tissues were embedded in Epon blocks and semithin sections were used to mark the respiratory tracts. Ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs were taken using a TRS Sharp:eye dual speed CCD camera (Troendle, Moorenwies, Germany) fitted to the electron microscope. Basement membrane thicknesses of respiratory epithelium samples were measured at 20 points in each mouse using electron microscopy and the iTEM software package (version 5.0) (Olympus Soft Imaging Solutions GmbH, Münster, Germany) [13].

Table 1. Histologic Parameters Analyzed for 3 Groups[a]

Parameter	Nebulization Group[b] (n = 7)	Intranasal Group[c] (n = 7)	Control Group[d] (n = 7)
Basement membrane thickness, nm	439.99 (68.29) (346.02-601.61)[e]	403.22 (56.28) (299.47-522.53)[f]	308.16 (38.93) (245.19-389.47)
Subepithelial smooth muscle thickness, µm	13.54 (4.16) (7.71-20.16)[e]	1121.79 (4.26) (6.18-19.26)[f]	7.19 (1.38) (4.92-10.12)
Epithelium thickness, µm	33.56 (7.08) (21.33-43.98)[e]	30.43 (3.41) (24.6-35.27)[f]	19.39 (1.91) (16.79-22.76)
Mast cells, No./16400 μm^2	17.8 (9.22) (1-38)[e,h]	5.26 (3.28) (1-12)	7 (3.04) (2-11)
Goblet cells, No./100 µm	1.47 (0.64) (0.42-2.76)[e]	3.50 (1.73) (0.6-6.35)[f,g]	0.43 (0.33) (0-1)

[a] Data are presented as means (SD) and range (minimum-maximum).
[b] Sensitized with 1 injection of 10 µg ovalbumin on days 0 and 14 and exposed to 2.5% aerosolized ovalbumin 3 days a week for 8 weeks.
[c] Sensitized with 1 injection of 100 µg ovalbumin on days 0 and 14 and administered 500 µg intranasal ovalbumin on days 14, 27, 28, 29, 47, 61, 73, 74, and 75.
[d] Received nothing.
[e] $P < .001$ for nebulization group vs control group.
[f] $P < 001$ for intranasal group vs control group.
[g] $P < .01$ for nebulization group vs intranasal group.
[h] $P < .001$ for nebulization group vs intranasal group.

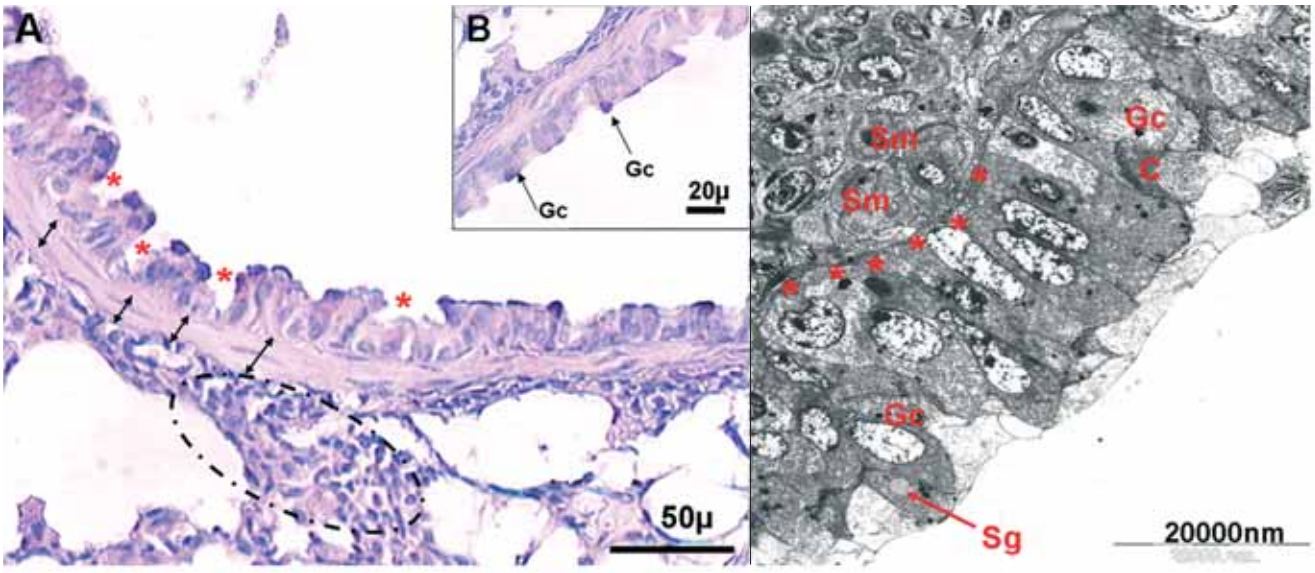

Figure 2. Light and electron microscopic findings for the nebulization group.
A, light microscopy revealed an abnormal epithelium surrounding the airways (*), markedly thickened subepithelial smooth muscle (arrow with 2 heads), and mononuclear infiltration in peribronchial parenchymal areas (ellipsoid area). B, periodic acid-Schiff-positive goblet cells. Electron microscopy revealed a thickened subepithelial smooth muscle (Sm) layer, regular basement membrane, goblet cells (Gc), secretory granules (Sg), and epithelial cells with cilia (C).

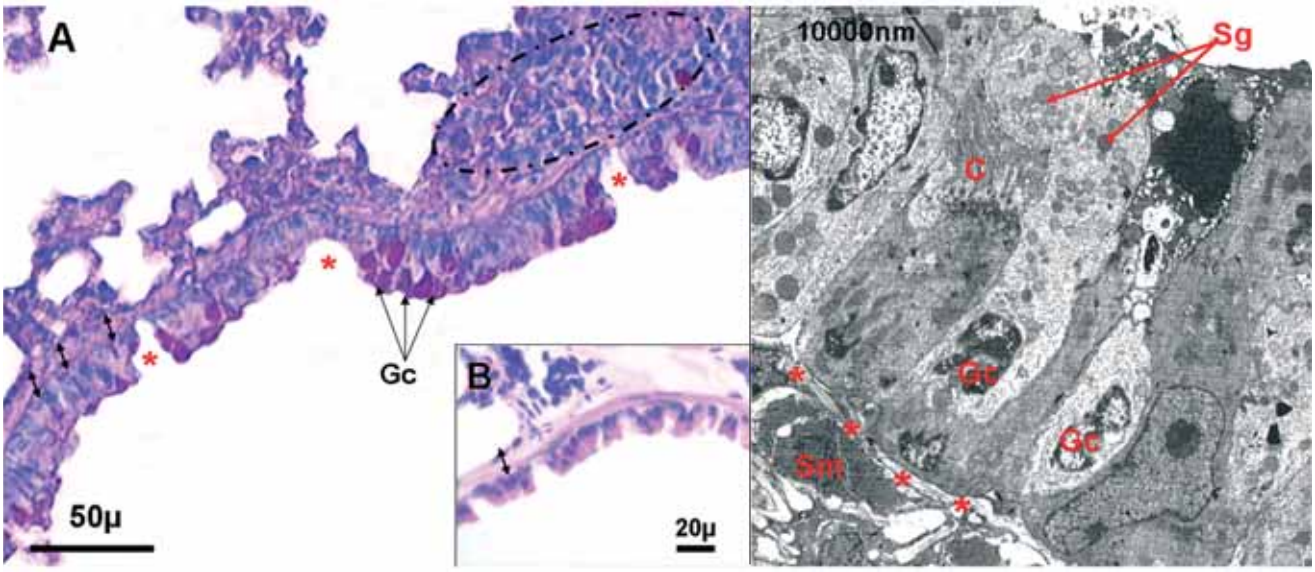

Figure 3. Light and electron microscopic findings for the intranasal group.
A, light microscopy revealed abnormal respiratory epithelium (*), thickened epithelium, and high numbers of goblet cells (Gc). Also seen were thickened subepithelial smooth muscle (arrow with 2 heads) and peribronchial mononuclear infiltration with periodic acid-Schiff staining (ellipsoid area). B, irregular, disrupted epithelium and thickened smooth muscle (arrow with 2 heads) with hemotoxylin and eosin staining. Electron microscopic findings revealed a regular basement membrane (*) and smooth muscle cells (Sm) in the tranverse section. Healthy respiratory epithelium with and without cilia (C) and goblet cells (Gc) filled with secretory granules (Sg).

Statistical Analysis

Data were presented as means (SD) and ranges (minimum-maximum) and between-group comparisons were made using the Kruskal-Wallis method. When differences were statistically significant, the Mann-Whitney U test was used. A P value of $<.05$ was considered statistically significant. Statistical analysis was performed using version 11 of the SPSS software package (SPSS Inc, Chicago, Illinois, USA).

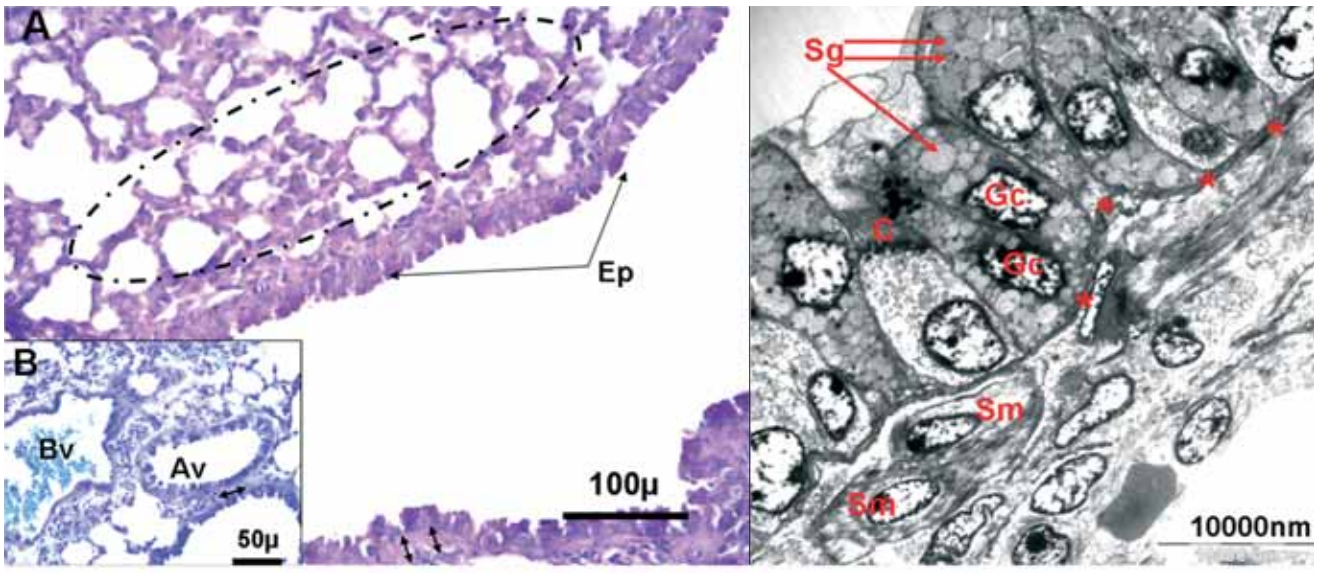

Figure 4. Light and electron microscopic findings for the control group.
A, light microscopic findings revealed a regular respiratory epithelium (Ep), a thin, regular subepithelial smooth muscle layer (arrow with 2 heads), and normal periodic acid-Schiff-stained parenchymal structures (ellipsoid area). B, parenchymal structures, blood vessels (Bv) and perivascular area and airways (Av) with toluidine blue staining were regular. Electron microscopy revealed goblet cells (Gc), secretory granules (Sg) and healthy epithelial cells with cilia (C). The basement membrane was thin and regular (*), and there were 3 to 4 layers of subepithelial smooth muscle cells (Sm).

Results

Basement membrane, epithelium, and subepithelial smooth muscle layer thicknesses and mast cell and goblet cell numbers were significantly higher in the nebulization group than in the control group ($P < .001$ in all cases).

With the exception of mast cell numbers (for which no significant differences were found), the above parameters were also found to be significantly higher in the intranasal group than in the control group ($P < .001$ in all cases, Table 1). These results show that the asthma model was successfully established by both protocols, with 1 exception (mast cell numbers in the intranasal group).

Abnormal respiratory epithelium, markedly thickened subepithelial smooth muscle, mononuclear infiltration in peribronchial parenchymal areas, and increased numbers of PAS-positive goblet cells were compatible with asthma in both the nebulization (Figure 2) and intranasal groups (Figure 3). Figure 4 shows normal lung structures with regular respiratory epithelium, basement membrane, parenchymal structures, and a thin subepithelial smooth muscle layer.

A comparison of the nebulization group and the intranasal group revealed a significantly higher number of mast cells in the former ($P = .001$) and a significantly higher number of goblet cells in the latter ($P < .001$) (Table 1).

Vascularization was similar in the nebulization and intranasal groups but vascular density was slightly increased in both the nebulization and intranasal groups when compared to the control group.

Discussion

Allergic asthma has been modeled successfully in mice [14]. An ideal mouse model for asthma should replicate the major features of the disease in humans: high levels of allergen-specific immunoglobulin (Ig) production, immediate hypersensitivity with early-phase and late-phase responses, airway inflammation characterized by an influx of T cells, eosinophils and, to a lesser extent, neutrophils, development of airway hyperresponsiveness, and airway remodeling [2,15,16].

Airway eosinophilia and changes in lung function have been documented in mouse models of asthma using a variety of protocols [14], and airway remodeling has also been successfully established in animal models [4]. These structural changes consist of goblet cell hyperplasia in the epithelium, mucous gland hyperplasia, reticular basement membrane thickening, increased vascularity of mucosa, and thickening of the smooth muscle layer [17]. No mouse models to date, however, have reproduced all of the features of human asthma [2,15,16]. In the present study, goblet cell numbers and basement membrane, epithelium, and subepithelial smooth muscle layer thicknesses were significantly higher in the 2 asthma groups as compared to the control group, demonstrating that the models were well established. Furthermore, mast cell numbers were significantly higher in the nebulization group than in the control group. Temelkovski et al [6] suggested that a chronic inhalation exposure mouse model replicated most of the features of chronic human asthma. However, in our

study, mast cell numbers in the intranasally challenged asthma group were not significantly different from those in the control group. On establishing the same asthma mouse model as that used in our intranasal group, Henderson et al [8] reported increased eosinophil and mononuclear cell inflammatory response, thickening of the airway smooth muscle layer, and collagen deposition beneath the airway epithelial cell layer when compared to control mice. However, they did not evaluate mast cells. Mast cells are the main effector cells of hypersensitivity reactions, especially early-phase allergic reactions and immediate asthmatic response [18]. Although the role of these cells in acute-phase allergic reactions has been shown in several studies, their role in chronic allergic inflammatory responses of the lung is not fully known [19]. Takeda et al [19] showed that neither mast cells nor IgE-mast cell activation was required for the development of eosinophilic inflammation and airway hyperresponsiveness in allergen-sensitized mice. William et al [20], in turn, reported that the contribution of mast cells to airway hyperresponsiveness and chronic inflammation associated with murine models of asthma depended on the experimental model used. On investigating the role of mast cells in the development of airway remodeling using 2 kinds of mast cell–deficient mice, Masuda et al [16] suggested that these cells had no role in the development of airway inflammation, epithelial remodeling, or airway hyperresponsiveness. The divergent results for mast cells in our study may be related to differences in the sensitization and challenge protocols used.

The genetic background of the animal, the route, dose, and frequency of allergen, and the type of adjuvant used all influence the T_H2-biased immune response in mouse models of asthma. We used BALB/c mice as these are known to be IgE-high responders to many allergens. Systemic sensitization and repeated allergen challenges are necessary to induce peripheral priming of the immune response and to establish airway inflammation, respectively [2]. In the majority of models available, mice are sensitized with an intraperitoneal injection of ovalbumin, often combined with a T_H2 skewing adjuvant (alum in our case). Sensitization induces the production of ovalbumin-specific IgE. Upon secondary exposure to aerosolized ovalbumin, sensitized animals develop in vivo airway hyperresponsiveness [21]. The allergen can be delivered by either aerosolization of the allergen or intratracheal or intranasal instillation [2]. As antigen inhalation alone leads to a preferential suppression of antigen-specific IgE antibodies, systemic priming is necessary to induce allergic immune responses before a mucosal antigen is applied [22]. In our study, the mice were sensitized with an intraperitoneal injection of ovalbumin in both models. The models differed, however, in terms of the route used for the repeat allergen challenges and in terms of the doses and frequency of administration. In 1 of the models, ovalbumin was delivered by aerosolization and in the other it was given intranasally for almost the same length of time but at more frequent intervals. The vast majority of animal studies involve short-term exposure to high concentrations of aerosolized antigen (lasting usually no more than 1-2 weeks) but these are not good models of airway remodeling [6]. In both of the asthma models used in this study, recurrent long-term exposure to allergens was provided.

There were some limitations to our study: we were unable to assess cytokine levels (which play an important role in asthma pathogenesis), and we used only a small number of animals. Our aim was to compare chronic structural changes in 2 models of asthma with proven efficacy. Our results show that, in general, both of the methods were successful in establishing a model of asthma despite the fact that mast cell numbers did not vary significantly between the intranasally challenged group and the control group. Goblet cell numbers were found to be significantly lower in the nebulization group than in the intranasal group but significantly higher in both of these groups compared to the control group. Our evaluation of histopathologic changes of asthma in the current study does not allow us to establish whether one of these asthma protocols is superior to the other. To do this, larger histopathologic studies combined with an analysis of cytokine levels are required.

References

1. Tagaya E, Tamaoki J. Mechanisms of airway remodeling in asthma. Allergol Int. 2007;56:331-40.
2. Herz U, Renz H, Wiedermann U. Animal models of type I allergy using recombinant allergens. Methods. 2004 ;32:271-80.
3. Zosky GR, Sly PD. Animal models of asthma.Clin Exp Allergy. 2007;37:973-88.
4. Ramos-Barbón D, Ludwig MS, Martin JG. Airway remodeling: lessons from animal models. Clin Rev Allergy Immunol. 2004;27:3-21.
5. Chang YS, Kim YK, Bahn JW, Kim SH, Park HW, Kim TB, Cho SH, Min KU, Kim YY. Comparison of asthma phenotypes using different sensitizing protocols in mice. Korean J Intern Med. 2005;20:152-8.
6. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax. 1998; 53:849-56.
7. Uzuner N, Kavukçu S, Yilmaz O, Ozkal S, Ilekel H, Karaman O, Soylu A, Kargi A. The role of L-carnitine in treatment of a murine model of asthma. Acta Med Okayama. 2002;56:295-301.
8. Henderson WR Jr, Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, Chi EY. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. Am J Respir Crit Care Med. 2002;165:108-16.
9. Henderson WR Jr, Lewis DB, Albert RK, Zhang Y, Lamm WJE, Chiang GKS, Jones F, Eriksen P, Tien Y, Jonas M, Chi EY. The importance of leukotrienes in airway inflammation in a mouse model of asthma. J Exp Med. 1996;184:1483-94.
10. Pekcetin C, Ergur BU, Kiray M, Bagriyanik A, Tugyan K, Erbil G, Ozogul C. The protective effects of trimetazidine on testicular ischemia and reperfusion injury in rats. Pediatr Surg Int. 2007;23:1113-8.
11. Wilcox, D, Dove, B, McDavid, D, Greer, D' UTHSCSA, Image Tool for Windows. San Antonio, Texas: University of Texas Health Science Center, 1986-2001.
12. Olmez D, Babayigit A, Uzuner N, Erbil G, Karaman O, Yilmaz O, Cetin EO, Ozogul C. Efficacy of sulphasalazine on lung

histopathology in a murine model of chronic asthma. Exp Lung Res. 2008;34:501-11
13. Babayigit A, Olmez D, Karaman O, Bagriyanik A, Yilmaz O, Kivcak B, Erbil G, Uzuner N. Ginseng ameliorates chronic histopathologic changes in a murine model of asthma. Allergy Asthma Proc. 2008;29:493-8. [Epub ahead of print]
14. Lloyd CM. Building better mouse models of asthma. Curr Allergy Asthma Rep. 2007;7:231-6.
15. Cieslewicz G, Tomkinson A, Adler A, Duez C, Schwarze J, Takeda K, Larson KA, Lee JJ, Irvin CG, Gelfand EW. The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. J Clin Invest. 1999;104:301-8.
16. Neuhaus-Steinmetz U, Glaab T, Daser A, Braun A, Lommatzsch M, Herz U, Kips J, Alarie Y, Renz H. Sequential development of airway hyperresponsiveness and acute airway obstruction in a mouse model of allergic inflammation. Int Arch Allergy Immunol. 2000;121:57-67.
17. Kips JC, Pauwels RA. Airway wall remodelling: does it occur and what does it mean? Clin Exp Allergy. 1999;29:1457-66.
18. Masuda T, Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, Nagai H. Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma. Clin Exp Allergy. 2003;33:546-9.
19. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice.J Exp Med. 1997;186:449-54.
20. Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. J Exp Med. 2000 Aug 7;192(3):455-62
21. Kips JC, Anderson GP, Fredberg JJ, Herz U, Inman MD, Jordana M, Kemeny DM, Lötvall J, Pauwels RA, Plopper CG, Schmidt D, Sterk PJ, Van Oosterhout AJ, Vargaftig BB, Chung KF. Murine models of asthma. Eur Respir J. 2003;22:374-82.
22. Holt PG, Britten D, Sedgwick JD. Suppression of IgE responses by antigen inhalation: studies on the role of genetic and environmental factors. Immunology. 1987;60:97-102.

Manuscript received May 3, 2008; accepted for publication September 16, 2008.

Duygu Olmez

Department of Pediatric Allergy
Dokuz Eylul University Hospital
Balcova, Izmir, Turkey.
E-mail: duyguolmez@hotmail.com