

Immune Responses in the Lung and Local Lymph Node of A/J Mice to Intranasal Sensitization and Challenge with Adjuvant-Free Ovalbumin

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ABSTRACT

Pathologic features of IgE-mediated allergic airway diseases include airway infiltration of inflammatory cells (eg, lymphocytes, plasma cells, and eosinophils) and mucous cell metaplasia (MCM) in airway epithelium. CD4⁺ T lymphocytes, specifically those producing a type 2 (Th2) cytokine profile, are necessary for the induction of IgE-mediated allergic airway responses. Most experimental models of IgE-mediated allergic airway disease use systemic (eg, intraperitoneal) administration of an allergen coupled with an adjuvant to sensitize animals. Cytokine changes are measured in a number of ways including in bronchoalveolar lavage fluid (BALF) or lymph node cells stimulated *ex vivo*. The primary objective of this study was to test the hypothesis that intranasal sensitization and challenge of mice with ovalbumin in the absence of an adjuvant will induce the pathologic features that are characteristic of IgE-mediated allergic airway disease. Another objective was to determine if intranasal delivery of this allergen will result in the induction of a profile of cytokine gene expression in the lung and tracheobronchial (TB) lymph node, that is typical of immunologic changes associated with IgE-mediated allergic airway disease. Only mice that were intranasally sensitized and challenged with ovalbumin exhibited pulmonary lesions that included marked MCM in the respiratory epithelium lining the nasal and pulmonary airways, and an associated mixed inflammatory cell influx consisting of lymphocytes, plasma cells and eosinophils. Ovalbumin-treated mice also exhibited enhanced expression of the Th2 cytokine mRNAs IL-4, IL-5, IL-10, and IL-13 in the lung and IL-4 in the TB lymph node, and concurrent increases in ovalbumin-specific IgE in the serum. The results of this study indicate that A/J mice intranasally instilled with ovalbumin without adjuvant have the hallmark histopathologic and immunologic features of IgE-mediated allergic airway disease of humans.

Keywords. Intranasal sensitization and challenge; nasal and pulmonary airways; A/J mice; Th2 cytokines; ovalbumin; adjuvant-free; morphologic features of allergic airway disease; tracheobronchial lymph node.

INTRODUCTION

Ovalbumin is a protein allergen widely used in experimental models of IgE-mediated allergic airway disease that use systemic administration to sensitize animals. Although it is well known that systemic ovalbumin sensitization followed by intranasal challenge will induce features characteristic of IgE-mediated allergic airway disease, it is not clear whether a similar response can be induced by intranasal sensitization and challenge to ovalbumin. The impetus for intranasal administration is that it more closely resembles the natural route of exposure to airborne allergens, ie, inhalation. It is also a simple, inexpensive, and noninvasive method of exposing both the upper and lower respiratory tract of laboratory rodents to allergens. Most experimental models also include an adjuvant (eg, alum) along with the allergen. Adjuvants, like alum, are not part of the natural airborne exposure of allergens and may artificially influence Th1 or Th2 responses in the animal. This is evidenced by the production of vari-

ous immunoglobulin subclasses that may skew the immune response (19).

A large body of evidence supports the critical role of Th2 cytokines in the development and maintenance of IgE-mediated allergic airway disease. For example, interleukin-4 (IL-4) and IL-13 are required for B cells to undergo “class-switching” (ie, from IgM-producing to IgE-producing B cells) (8) and the inhibition of IL-5 prevents antigen-induced airway eosinophilia and late-phase hyperresponsiveness (7, 13). Most experimental models of IgE-mediated allergic airway disease assess changes in cytokine expression by measuring the secreted product of stimulated local draining lymph node cells or by analyzing bronchoalveolar lavage fluid (BALF) for protein or cell associated mRNA expression. An alternative method, described in this report, is the measurement of cytokine gene expression in RNA isolated from the lung tissue itself or from a local draining lymph node.

The primary objective of this study was to determine if intranasal sensitization and challenge of mice with ovalbumin without an adjuvant would effectively sensitize the animals by inducing features characteristic of IgE-mediated allergic airway disease. The second objective of this study was to assess the temporal changes in the expression of multiple cytokine genes in RNA derived from lung tissue and its local draining lymph node using such an exposure regimen. Allergen-induced changes in cytokine gene expression, along with pulmonary and nasal histopathology, and serum IgE levels were assessed in each mouse. This was done to examine the utility of measuring local cytokine gene expression as

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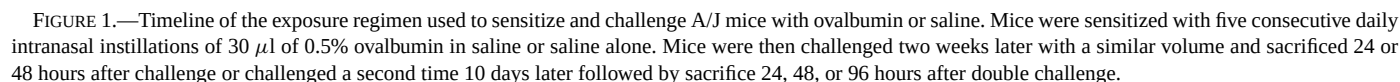
Abbreviations: AB/PAS, Alcian Blue/Periodic Acid-Schiff; BALF, bronchoalveolar lavage fluid; IL, interleukin; IM, intraepithelial mucosubstances; IS, internal standard; MBP, major basic protein; MCM, mucous cell metaplasia; rcRNA, recombinant RNA; RPA, RNase Protection Assay; RT, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; SEM, standard error of the mean; TB, tracheobronchial lymph node; Th1 or Th2, T helper 1 or 2; Vs, volume density.

Mice were anesthetized with 4% halothane and 96% oxygen. Anesthetized mice were treated by intranasal instillation with 30 μ l of 0.5% ovalbumin (Sigma Chemical, St. Louis, MO) in sterile, pyrogen-free saline or 30 μ l of saline alone divided evenly between each naris. The sensitization phase consisted of a single instillation once per day for 5 consecutive days. The animals were challenged once, 14 days after the fifth instillation of the sensitization phase via a single instillation of the same volume and concentration as the instillations during the sensitization phase. Mice were sacrificed 24 or 48 hours after challenge. There were 3 different treatment groups for each sacrifice time. One was sensitized and challenged with saline and a second group was sensitized with ovalbumin and challenged with saline. The last group was sensitized and challenged with ovalbumin. Other mice received a second challenge 10 days after the first challenge again with the same volume and concentration as in the first challenge. These mice were sacrificed 24, 48, or 96 hours after the final challenge. There were 4 different treatment groups for each sacrifice time: 1) sensitization and challenge with saline; 2) sensitization with ovalbumin and challenge with saline; 3) sensitization with saline and challenge with ovalbumin; and 4) both sensitization and challenge with ovalbumin.

Animals and Allergen Sensitization and Challenge

Necropsy, Blood Sample Collection, and Tissue Preparation

Before sacrifice, mice were deeply anesthetized via intraperitoneal injection of 0.1 ml of 12% pentobarbital in saline. Blood samples (0.1–0.5 ml) were taken from the brachial artery and collected in Beckton Dickinson vacutainers. Serum samples were collected after the blood samples were centrifuged to remove cells and the supernatants were frozen and stored at -20°C . The abdominal aorta and renal artery were then severed to exsanguinate the animals.



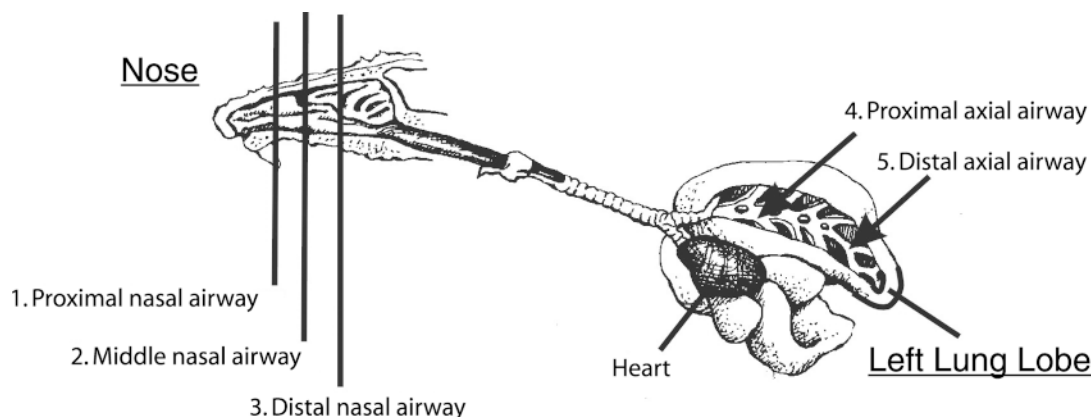


FIGURE 2.—Sites of airway tissue selection for morphometric analysis. The cartoon depicts the lateral wall of one nasal passage with the septum removed and the main axial airway of the left lung lobe. Transverse sections of the nasal cavity were taken 1) immediately posterior to the upper incisor teeth (proximal), 2) at the level of the incisive papilla of the hard palate (middle), and 3) at the level of the second palatal ridge (distal). The left lung lobe was microdissected along the main axial airway, and 2 sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation.

Immediately after death, the trachea was cannulated and the heart/lung block removed. The tracheobronchial (TB) lymph node was removed and placed in 1 ml Tri reagent (Molecular Research Center, Cincinnati, OH). Lymph nodes were pooled 2 or 3 per 1 ml Tri reagent within each group. The lymph nodes were then homogenized and stored at -80°C . The right bronchus was clamped using suture thread. Caudal and medial right lung lobes were placed in 2 ml Tri reagent, homogenized, and stored at -80°C .

After removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant pressure of 30 cm of fixative. After 1 hour, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 24 hours. After fixation, the left lung lobe was microdissected along the main axial airway, and 2 sections were then excised at the level of the fifth (proximal) and eleventh (distal) airway generation (Figure 2), as has been described previously in detail (23). Paraffin sections ($5\ \mu\text{m}$) were stained with Alcian blue (pH 2.5) and periodic acid Schiff's (AB/PAS) reagent, which stain neutral and acidic mucosubstances, respectively. Other paraffin sections were stained separately for eosinophilic major basic protein (MBP, described later).

The head of each mouse was removed from the carcass, and the lower jaw and skin were removed. The heads were then immersed in 10% neutral buffered formalin for 24 hours. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 hours. The nasal cavity of each mouse was transversely sectioned at 3 specific anatomic locations according to a modified method of Young (30) and processed for light microscopy and image analysis. The most proximal nasal section was taken immediately posterior to the upper incisor teeth (proximal); the middle section was taken at the level of the incisive papilla of the hard palate (middle); the most distal nasal section was taken at the level of the second palatal ridge (distal) (Figure 2). Tissue sections were embedded in paraffin, at a thickness of $5\ \mu$, and stained with hematoxylin and eosin for histopathologic assessment. Paraffin sections were also stained with AB/PAS to identify intraepithelial mucosubstances (IM).

Immunocytochemistry in the Lung

Unstained hydrated paraffin sections of the left lung lobe were incubated with the blocking solution, Vectastain ABC-AP Kit Normal Goat Serum (Vector, Burlingame, CA). The sections were then incubated with a polyclonal rabbit antibody directed against mouse eosinophilic MBP generously provided by Drs. Borchers and Lee of the Mayo Clinic of Scottsdale (Scottsdale, Arizona). Treatment with the secondary antibody Vectastain ABC-AP Kit Anti-rabbit IgG followed. Some slides were incubated without the secondary antibody to check for nonspecific binding. Immunoreactive MBP was visualized after treatment with the Vectastain ABC-AP Complex followed by the Vector Red AP Substrate Solution (6). Nasal tissue sections which contained turbinate bone with bone marrow were also stained for MBP. Eosinophils and their cellular precursors within the bone marrow served as positive controls for the MBP immunohistochemistry.

Morphometry of Stored Intraepithelial Mucosubstances in Nasal and Pulmonary Airways

The amount of stored mucosubstances in the respiratory epithelium lining the nasopharyngeal meatus in the most distal nasal section and the respiratory epithelium lining the axial pulmonary airway (airway generation 5) in the left lung lobe was estimated by quantifying the volume density of AB/PAS-stained mucosubstances using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstances was calculated by circumscribing the perimeter of the stained material using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density, V_s) per unit of surface area was estimated using the method described in detail by Harkema et al (11) and was expressed as nl of IM per square mm of basal lamina.

Epithelial and Inflammatory Cell Densities

Numeric cell density of the surface epithelium lining the proximal axial airway (fifth generation) was determined by

counting the total number of epithelial cell nuclei in the surface epithelium and dividing by the length of the underlying basal lamina. The length of the basal lamina was determined as described above. Mucous cell density was also expressed as the number of cells per mm of basal lamina. Eosinophil influx was measured by dividing the total number of eosinophils in the subepithelial interstitium of the airway wall by the length of the corresponding basal lamina. Eosinophils were identified by their morphologic features (ie, slightly larger than neutrophils with bilobed nuclei) and by the positive immunohistochemical staining for MBP in the large cytoplasmic granules (dark pink-red granules).

ELISA for Total and Ovalbumin-Specific Serum IgE

Total serum IgE was measured using a 96-well Immulon ELISA plate (Dynex, Technologies, Chantilly, Virginia) coated with 2 μ g/ml anti-mouse IgE (Purified Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) and incubated overnight at 4°C. For measurement of ovalbumin-specific IgE, a second ELISA plate was coated with 0.5% ovalbumin and incubated overnight at 4°C. After washing, the plates were incubated in 3% Bovine Serum Albumin (3% BSA) at 37°C for 1 hour (BSA, CALBIOCHEM, La Jolla, CA). Serum samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hour. After washing, biotinylated anti-mouse IgE (Biotin-conjugated Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) was then added at 2 μ g/ml and allowed to incubate at 25°C for 1 hour. After washing, 1.5 μ g/ml of streptavidin peroxidase was added followed by incubation at 25°C for 1 hour. After washing, TMB substrate (12.5 ml citric-phosphate buffer +200 μ l of TMB stock solution (6 mg/ml in DMSO)) was added to produce a color reaction. The reaction was terminated by the addition of 6N H₂SO₄. Optical density was determined at 450 nm using an EL-808 microplate reader (Bio-Tek instruments, Winooski, Vermont).

The RNase Protection Assay

Total RNA was isolated from lung tissues and the TB lymph node using the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol and the integrity of the RNA was checked by performing an ethidium bromide diagnostic gel to check for degradation of RNA. The mCK-1 RiboQuant Mouse Cytokine Multi-Probe Template Set (Pharmingen, San Diego, CA) was used to assay mRNA expression of IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, and IFN- γ . The housekeeping genes L32 and GAPDH were included as loading controls. The probes were generated by transcribing with T7 RNA polymerase in the presence of [α -³²P] UTP (3000 Ci/mmol). The probe set was hybridized in excess to RNA isolated from the right lung lobe (50 μ g) or from the TB lymph node (75 μ g) (56°C for 16 hours). Free probe and nonhybridized RNA was then digested with single-stranded RNases using the RiboQuant RNase Protection Assay (RPA) Kit. The remaining RNase-protected probes (only those that have hybridized to target tissue-derived mRNA) were resolved by electrophoresis (3500 V for 1 hour) through a 7M urea –7% acrylamide gel. The gel was dried and specific RNAs were visualized by autoradiography.

Preparation of Internal Standard for IL-4 Quantitative RT-PCR

A recombinant internal standard (IS) was prepared to quantify IL-4 mRNA expression by quantitative/competitive reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (5). Briefly, an artificial/recombinant RNA (rcRNA) was used as an IS containing specific PCR primer sequences for IL-4 that was added to RNA samples in a series of dilutions. A rat β -globin sequence was used as the spacer gene for the IL-4 IS. This method, developed by Vanden Heuvel et al (25), avoids sample-to-sample variation of reference gene expression (eg, β -actin) as well as gene-to-gene differences in amplification efficiency. The IL-4 forward and reverse primer sequences were 5' AAC GAG GTC ACA GGA GAA G 3' and 5' GCT TAT CGA TGA ATC CAG GC 3', respectively. The following was the IS forward primer design from 5' to 3': T7 promoter (TAAT ACGACTCACTATAGG), IL-4 forward primer (as above), and rat β -globin forward primer (AAGCCTGATGCTGTA GAGCC). The IS reverse primer design from 5' to 3' was: (dT)₁₈, IL-4 reverse primer (as above), and rat β -globin reverse primer (AACCTGGATACCAACCTGCC). PCR reaction conditions were performed using 100 ng of rat tail-genomic DNA. PCR-amplified products were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, WI) and transcribed into RNA using Promega's Gemini II In vitro Transcription System. The rcRNA was subsequently treated with RNase-free DNase to remove the DNA template. After quantifying, calculations were made to determine the number of molecules/ μ l of IL-4 IS.

Quantitative IL-4 RT-PCR

Quantitative RT-PCR was performed as described by Gilliland et al (9), except that rcRNA was used as an IS instead of genomic DNA with 10 aliquots of rcRNA from 10⁴ to 10⁸ molecules. Briefly, total RNA (right lung lobe or TB lymph node) and IS rcRNA of known amounts were reverse transcribed into cDNA using oligo(dT)₁₅ as primers using reverse transcriptase (RT) (Promega, Madison WI). To ensure that there was no genomic DNA contamination in the RNA isolates, no-RT controls were employed for all samples. A PCR master mixture consisting of PCR buffer, 4 mM MgCl₂, 6 pmol each of IL-4 forward and reverse primers, and 2.5 U of Taq DNA polymerase was added to the cDNA samples. Samples were then heated to 94°C for 3 minutes and cycled 40 times at (1) 95°C (30 seconds), (2) 58°C (30 seconds), and (3) 72°C (45 seconds). After the completion of 40 cycles, a final elongation step at 72°C (5 minutes) was carried out. PCR products were resolved by electrophoresis in 3% NuSieve 3:1 gel (FMC Bioproducts, Rockland ME) and visualized by ethidium bromide staining. The IL-4 primers produced a 228-bp product from the cellular RNA and a 346-bp product from the IS rcRNA. Quantification was performed using Gel Doc 1000 (BioRad Laboratories, Inc., Hercules, CA) and Multi Analyst Software. The amount of IL-4 mRNA present was determined as described by Gilliland et al (9). Briefly, the ratio of the density of the IS rcRNA to IL-4 mRNA was plotted against the amount of IS rcRNA (in molecules) added to each reaction. The point at which the ratio of IS (rcRNA) to IL-4 mRNA was equal to 1 signified the "cross-over point,"

which represented the amount of IL-4 molecules present in the initial RNA sample.

Statistics

The data obtained from each experimental group were expressed as a mean group value $\pm$ the standard error of the mean (SEM). The differences among groups were determined by 1-way and 2-way analysis of variance (ANOVA) and an All Pairwise Comparison Test (Tukey), using SigmaStat software from Jandel Scientific Co. (San Rafael, CA).

RESULTS

Pulmonary Histopathology

The exposure regimen used to sensitize and challenge mice with ovalbumin resulted in distinct pulmonary lesions that were restricted to mice that were intranasally sensitized and challenged with ovalbumin. The principal morphologic alterations in the lungs of these mice were marked MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils and mononuclear cells (mainly lymphocytes and plasma cells) in the interstitial tissue surrounding the airways (Figure 3). Airway lesions were more severe in the main axial airways and pre-terminal bronchioles, but were also present to a lesser degree in the terminal bronchioles. The interstitial tissue surrounding large blood

vessels in the alveolar parenchyma (pulmonary veins) and along the conducting airways (pulmonary arteries) exhibited a similar inflammatory response. Ovalbumin-sensitized mice that received 2 intranasal challenges of ovalbumin had more severe airway epithelial and inflammatory lesions than ovalbumin-sensitized mice that received only 1 intranasal challenge of ovalbumin. The inflammatory responses in all of these mice were principally present in the pulmonary airways and large vessels of the lung. No significant ovalbumin-induced inflammatory responses were evident in the alveolar parenchyma.

Treatment-associated changes were absent in the lungs of the control mice that received intranasal sensitization and challenges of saline. The only histologic change in the airways of mice that were sensitized with ovalbumin and received saline during the challenge phase was a minimal MCM limited to the proximal axial airways. This airway epithelial change was more conspicuous in mice that received two saline challenges rather than one saline challenge. No airway inflammation was associated with this minimal epithelial change.

The effect of the sensitization phase was examined by sacrificing mice after the sensitization phase alone. Five consecutive one-day instillations of ovalbumin caused a small increase in IM with no accompanying airway inflammation as assessed on Day 6, indicating that the animals developed the described changes only after challenge.

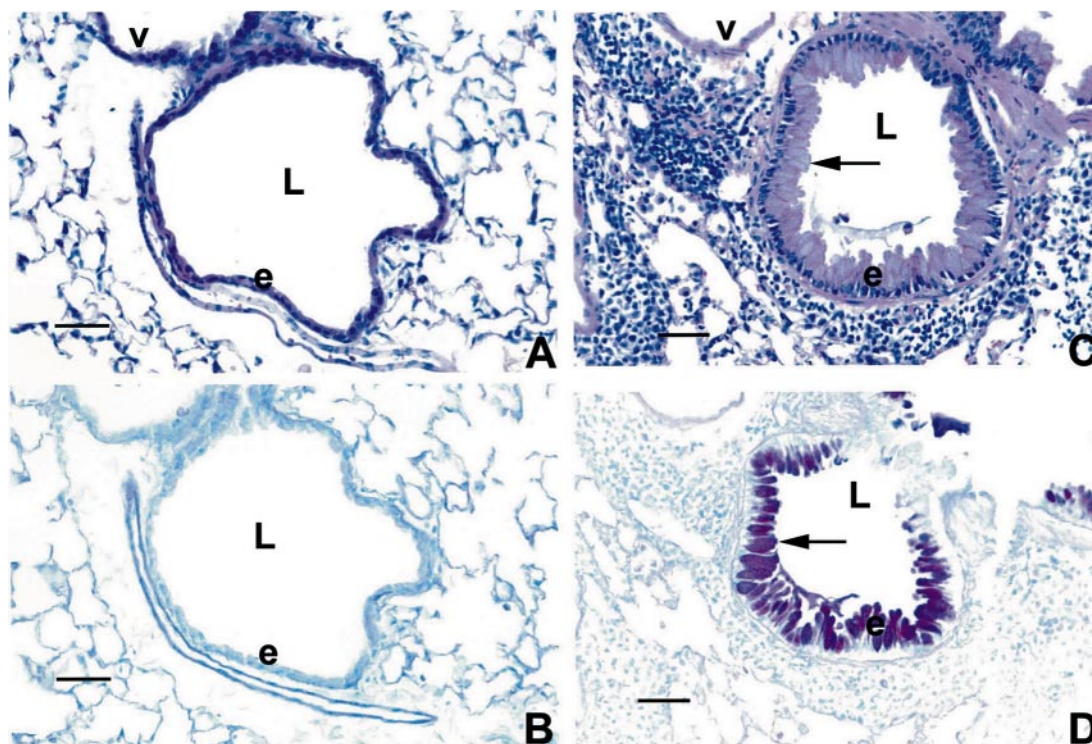


FIGURE 3.—Light photomicrographs of conducting airways of the left lung from mice intranasally sensitized and challenged twice with saline (A, B) or 0.5% ovalbumin (C, D) and sacrificed 96 hours after challenge. Tissues were stained with hematoxylin and eosin (A, C) for morphological assessment and Alcian Blue (pH = 2.5)/Periodic Acid Schiff's Sequence (AB/PAS) for mucosubstances in the airway epithelium (B, D). Numerous mucous cells (arrow) with AB/PAS-stained mucosubstances are present only in the airway epithelium (e) of the ovalbumin-sensitized and -challenged mouse (C, D). A marked inflammatory cell influx consisting primarily of lymphocytes, plasma cells and eosinophils is also present in the interstitial tissue surrounding the airway of this mouse (C). v = blood vessel; L = airway lumen; Bars = 50 μ .

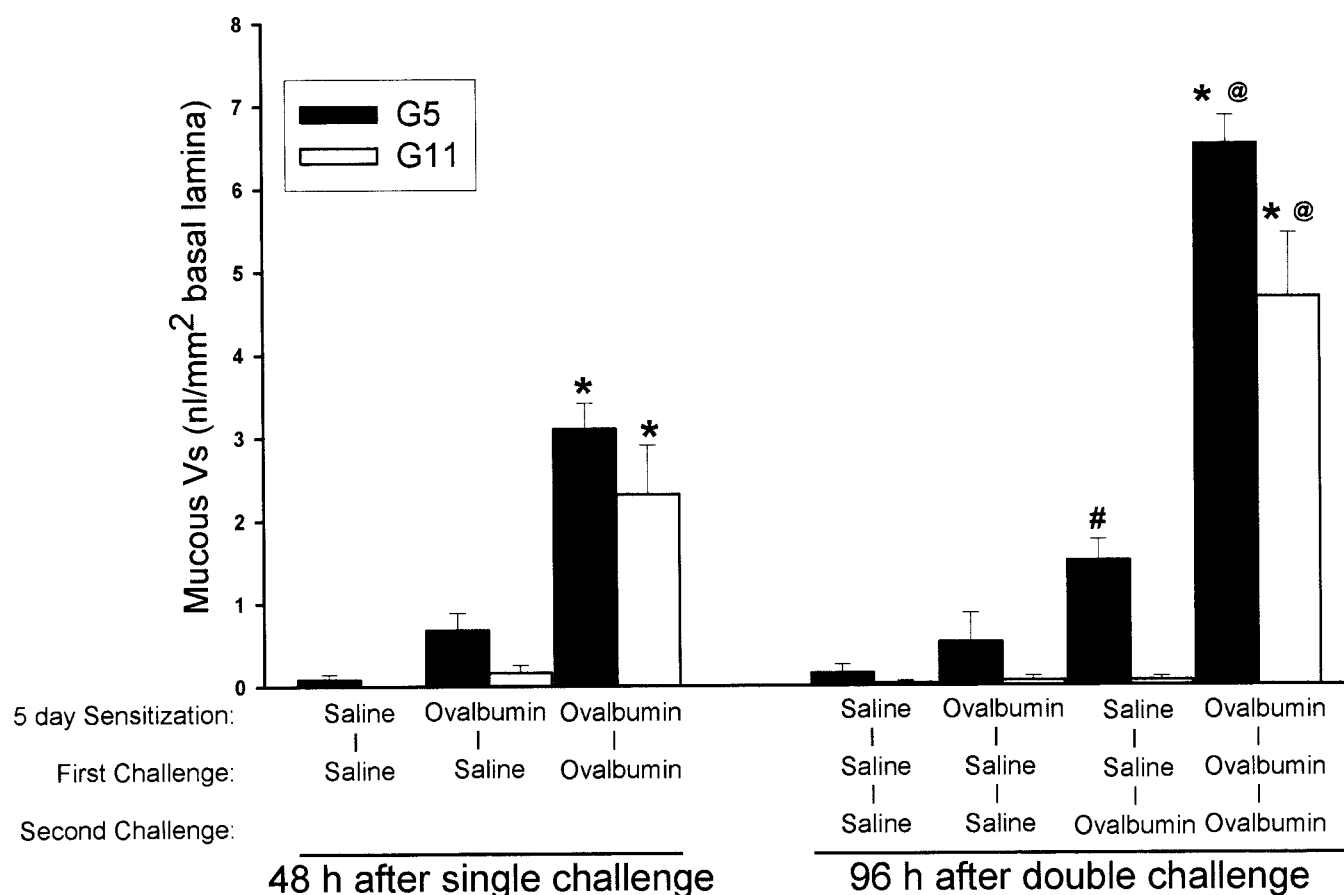


FIGURE 4.—Morphometric quantification of AB/PAS-stained material in the surface epithelium lining mouse pulmonary airways (generations 5 (proximal) and 11 (distal)) 48 hours after single challenge or 96 hours after double challenge with ovalbumin. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and sacrificed 48 hours after challenge or challenged a second time 10 days later followed by sacrifice 96 hours after double challenge. Bars represent the Volume Density (Vs) of intraepithelial mucosubstances $\pm$ standard error of the mean ($n = 5$ /group). * = significantly greater than groups challenged similarly ($p < 0.05$). # = significantly greater than groups instilled with saline at same airway generation ($p < 0.05$). @ = significantly greater than similar group challenged once ($p < 0.05$).

Stored Intraepithelial Mucosubstances in Pulmonary Airways

The amount of AB/PAS-stained IM in the epithelium lining the main axial airway at the fifth (proximal airway) and eleventh generations (distal airway) increased most in mice that were intranasally sensitized and challenged with ovalbumin (Figure 4). Mice that were instilled with saline during the sensitization phase and for the first challenge and with ovalbumin for the second challenge exhibited a 10-fold increase in IM at the fifth generation. This increase, however, was markedly lower than that exhibited by the lungs of mice that were sensitized and challenged with ovalbumin. The mice had a 42-fold increase in the amount of IM compared to the saline-instilled controls. It is notable that although a single ovalbumin challenge was sufficient to induce a significant increase in IM in ovalbumin-sensitized mice, two ovalbumin challenges resulted in a doubling of the amount of IM as compared to the lungs of mice that received a single challenge (Figure 4). Also, the increase in IM was greater in the axial airway at the fifth generation (proximal airway) than at the eleventh generation (distal airway).

Numeric Cell Densities of Epithelial Cells in Pulmonary Airways

A morphometric analysis of the surface epithelium lining the proximal axial airway was performed in mice that were sensitized and challenged twice to estimate the metaplastic transformation of epithelial cells from nonsecretory to mucus-secreting goblet cells (Figure 5). A 28-fold increase in mucous cell density was observed in mice that received ovalbumin during both sensitization and challenge phases as compared to the saline-only control group. No effect was observed in mice that received ovalbumin only during the sensitization phase or only as the second challenge. It is also notable that there was no evidence of hyperplasia in the main axial airway of the left lung lobe in any of the treatment groups as evidenced by the absence of changes in total epithelial cell density between the various treatment groups.

Pulmonary Airway Eosinophilia

As described above, recruitment of inflammatory cells, specifically eosinophils, into and around subepithelial airway tissues is a hallmark of the allergen-induced airway response. Eosinophils per mm basal lamina were enumerated in the

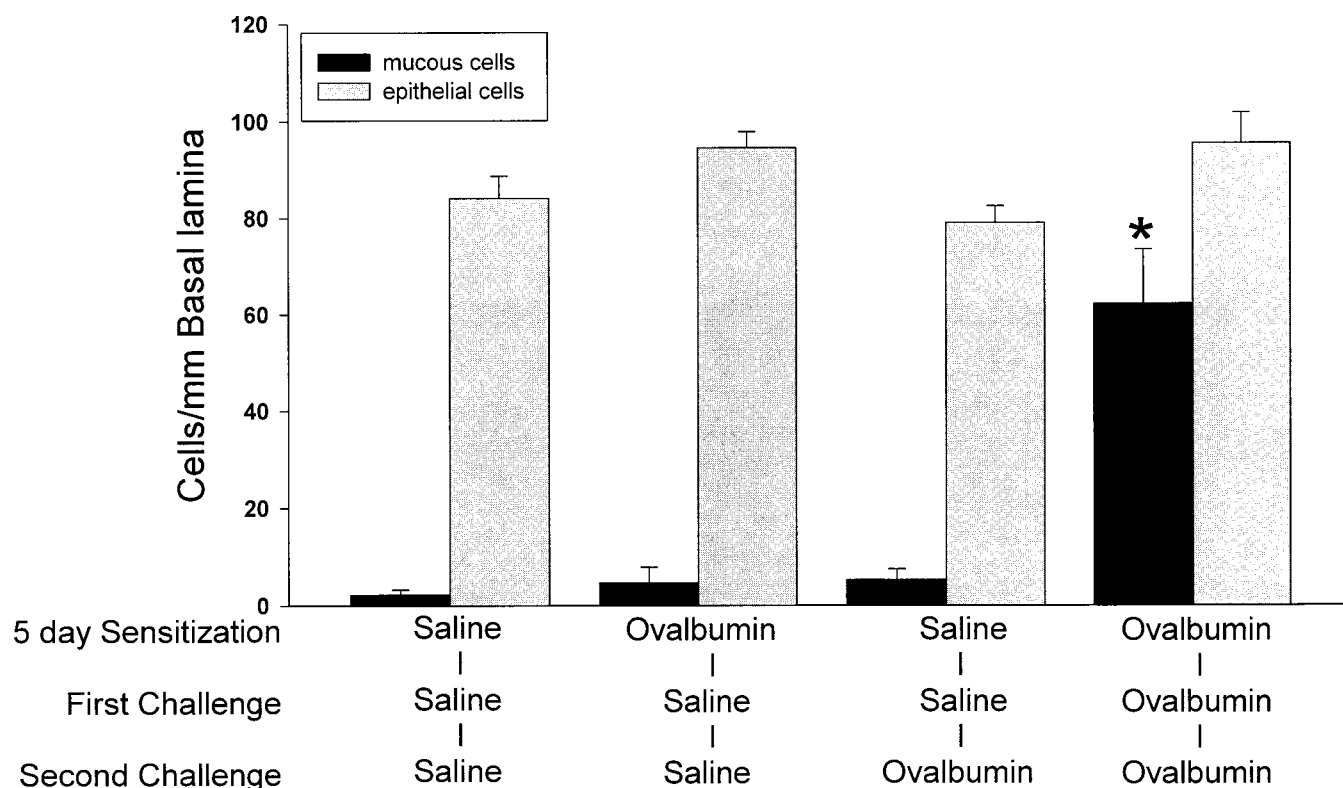


FIGURE 5.—Quantification of mucous and epithelial (total) cell density in the surface epithelium lining mouse pulmonary airways (generation 5) 96 hours after double challenge with ovalbumin. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hours after double challenge. Bars represent the number of cells per mm basal lamina $\pm$ standard error of the mean ($n = 5/\text{group}$). * = significantly greater than all other groups of this cell type ($p < 0.05$).

interstitium of the 5th (proximal) and 11th generation (distal) axial airway of mice that were sensitized and then challenged twice with either saline or ovalbumin. Mice that were sensitized and challenged with ovalbumin showed a 27-fold and 24-fold increase in the number of eosinophils present in the proximal (generation 5) and distal (generation 11) axial airways of the lung lobe, respectively, as compared to the saline controls (Figure 6).

Nasal Histopathology

The exposure regimen used to sensitize and challenge mice with ovalbumin resulted in distinct nasal lesions that were restricted to mice that were intranasally sensitized and challenged with ovalbumin. The principal morphologic alterations in the nasal airways of these mice were MCM in the surface epithelium lining the nasal pharyngeal meatus and an associated mild inflammatory cell influx consisting of eosinophils, lymphocytes and plasma cells in the interstitial tissue surrounding the airways (Figure 7).

Stored Intraepithelial Mucosubstances in Nasal Airways

The amount of IM in the airway epithelium lining the nasopharyngeal meatus was also estimated using image analysis and standard morphometric techniques. Ovalbumin sensitization and challenge resulted in an increase in the amount of IM in the respiratory epithelium that lines the nasopharyngeal meatus as compared to the saline controls (Figure 8).

Serum IgE

None of the treatment groups resulted in a significant increase in serum levels of total IgE (Figure 9). Conversely, mice sensitized and challenged with ovalbumin exhibited a marked increase, ie, a 10-fold increase in ovalbumin-specific serum IgE as compared to saline only controls (Figure 9). Mice that received only ovalbumin sensitizations or only an ovalbumin challenge did not exhibit significant increases in ovalbumin-specific serum IgE.

Cytokine Gene Expression

RNase Protection Assay: Mice that were intranasally sensitized and challenged twice with ovalbumin exhibited an increase in the expression of the Th2 cytokine mRNAs IL-4, IL-5, and IL-13, and the cytokine mRNA of IL-10 in the lung that, with time after challenge, decreased in magnitude as evidenced by less intense bands (Figure 10, lanes 6, 10, and 14). The saline controls exhibited no such increase in Th2 cytokine mRNA expression (Figure 10, lanes 3–5, 7–9, and 11–13). It is notable that IL-15 and IL-9 mRNA and the Th1 cytokine mRNA IFN- γ were constitutively expressed and that neither ovalbumin nor saline treatments influenced the expression of these cytokine mRNAs in the lung tissue.

There were no differences in cytokine mRNA expression in RNA from the TB lymph node as detected using the RPA (data not shown).

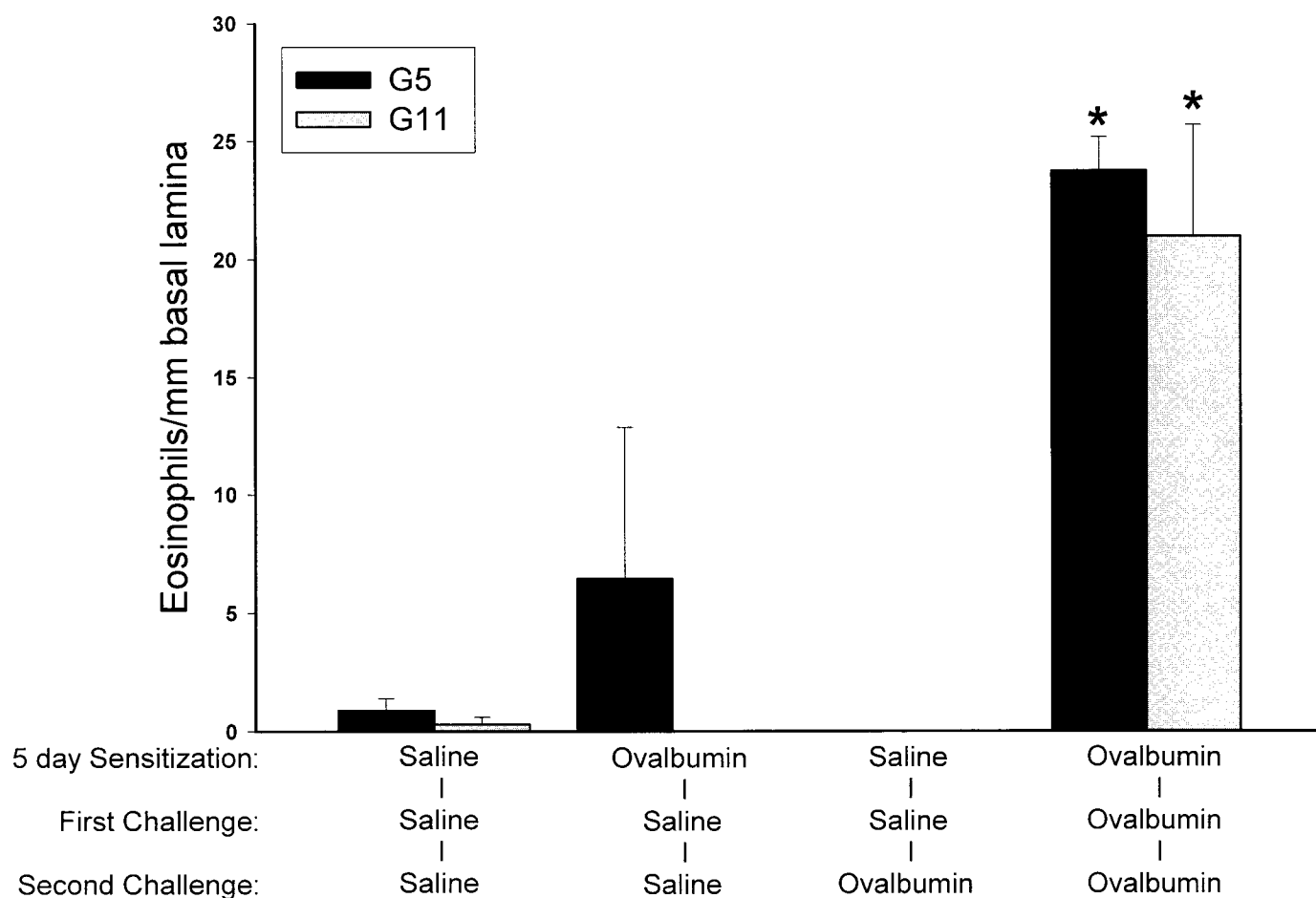


FIGURE 6.—Morphometric quantification of eosinophils immunohistochemically stained for major basic protein in mouse airway interstitium (generations 5 and 11) 96 hours after double challenge with ovalbumin. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hours after double challenge. Bars represent the number of eosinophils per mm basal lamina $\pm$ standard error of the mean ($n = 4$ /group). * = significantly greater than all groups at similar airway region ($p < 0.05$).

RT-PCR: After establishing the profile of Th2 cytokine mRNAs that were expressed using the RPA, RT-PCR was performed on right lung lobe RNA from mice that were sensitized and then challenged once or twice with either ovalbumin or saline. This was done to determine the quantitative differences in the expression of the critical Th2 cytokine mRNA IL-4. Mice that were sensitized and then challenged once with ovalbumin exhibited an approximate 5-fold increase in IL-4 mRNA expression 24 hours after challenge as compared to the saline-instilled controls (Figure 11). The magnitude of this increase persisted at that level for at least 48 hours after single challenge. Ovalbumin-sensitized mice that received 2 ovalbumin challenges exhibited an approximate 40-fold increase in IL-4 mRNA expression 24 hours after the second challenge as compared to controls (Figure 12). IL-4 mRNA levels after double challenge were similar 48 hours after exposure but waned in magnitude by 96 hours postexposure, ie, IL-4 mRNA levels decreased to approximately 16-times those of the saline controls. Mice that received only ovalbumin sensitizations or only an ovalbumin challenge did not exhibit significant increases in IL-4 mRNA expression in the lung.

IL-4 RT-PCR was also performed on RNA isolated from the TB lymph node from mice that were sensitized and then challenged twice with ovalbumin. Ovalbumin-sensitized and challenged mice exhibited a 4-fold increase in IL-4 mRNA expression at 24 hours after challenge compared to saline controls that persisted at that level at least until 96 hours (Figure 13).

DISCUSSION

The primary objective of this study was to determine if the pathologic and immunologic features of IgE-mediated allergic airway disease could be induced in mice by intranasal sensitization and challenge with adjuvant-free ovalbumin. Morphologic changes in the pulmonary airways were correlated with changes in cytokine gene expression in the lung and the TB lymph node after ovalbumin treatment to determine if local cytokine gene expression may be used as a biomarker for the respiratory allergenicity of selected proteins. Intranasal sensitization and challenge with ovalbumin in the absence of an adjuvant elicited the characteristic histopathologic and immunologic features of IgE-mediated allergic airway disease in murine lung. Our results suggest that cytokine gene

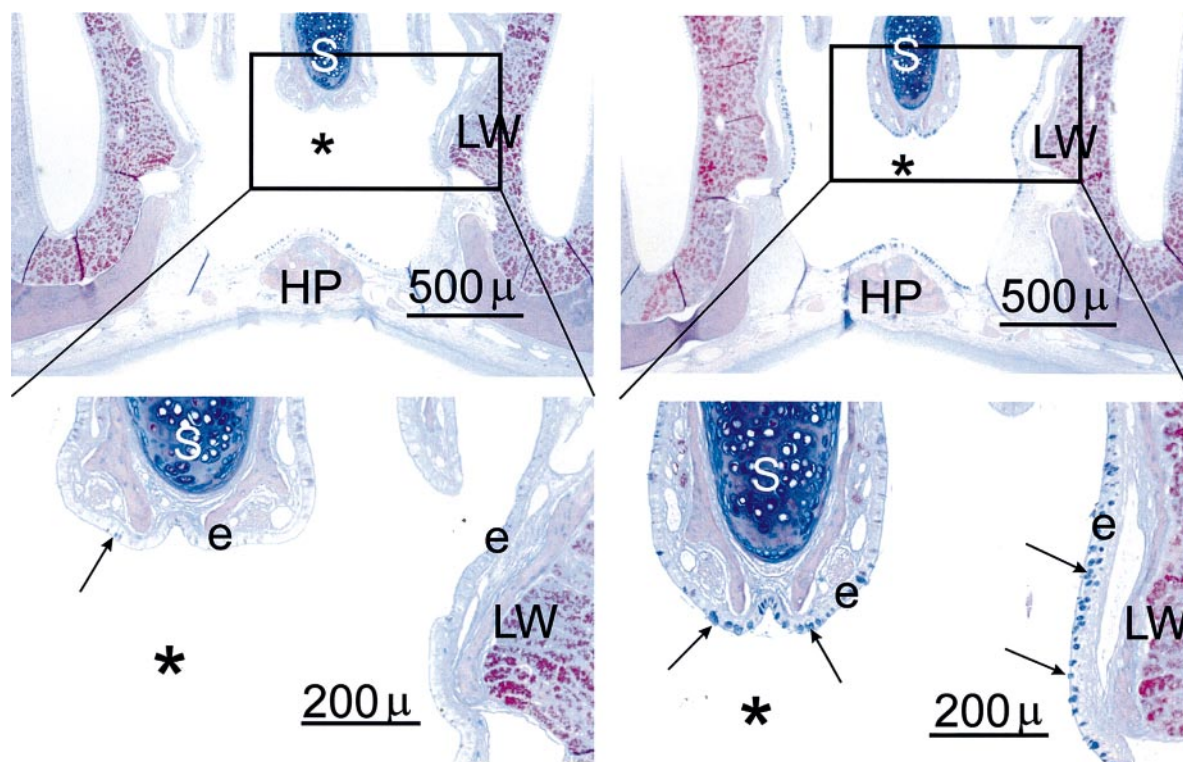


FIGURE 7.—Light photomicrographs of the distal region of the nasal cavity at the level of the second palatal ridge of mice 96 hours after sensitization and 2 challenges to saline (left panels) or ovalbumin (right panels). Tissue sections were histochemically stained with AB/PAS for identification of stored mucosubstances (arrows) in the epithelium lining the nasopharyngeal meatus. The lower panels are enlargements of the boxed regions in the panels shown above. S—nasal septum, HP—hard palate, LW—lateral wall, e—respiratory epithelium.

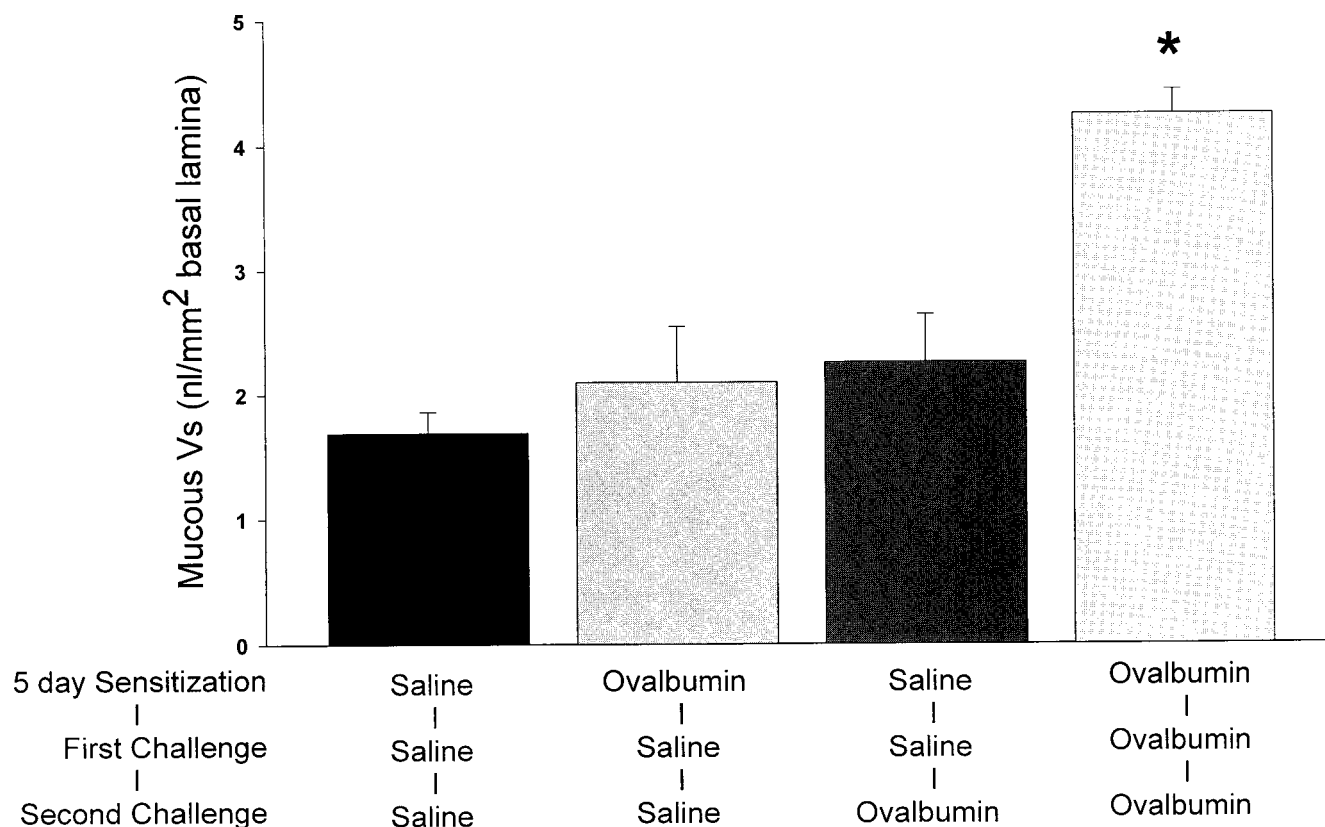


FIGURE 8.—Morphometric quantification of AB/PAS-stained material in the surface epithelium lining the nasopharyngeal meatus of mice 96 hours after double challenge to ovalbumin or saline. Mice were sensitized and then challenged twice with ovalbumin or saline. Bars represent the volumetric density (Vs) of intraepithelial mucosubstances $\pm$ the standard error of the mean ($n = 5/\text{group}$). *—significantly greater than all other groups ($p < 0.05$).

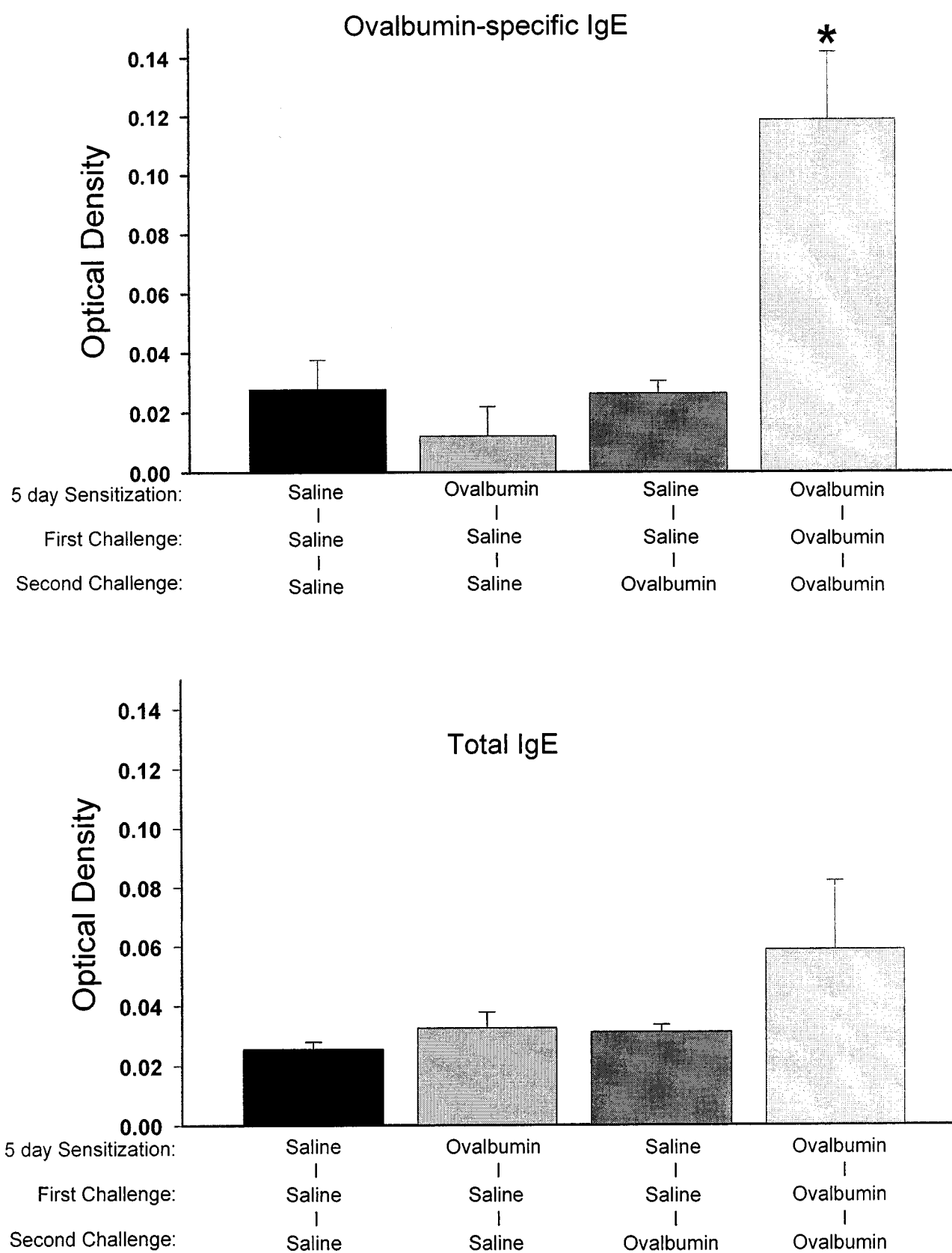


FIGURE 9.—Ovalbumin-specific (upper panel) and Total IgE (lower panel) levels in mouse serum isolated 96 hours after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hours after double challenge. Bars represent average optical density $\pm$ standard error of the mean ($n = 5$ /group). * = significantly greater than all other groups.

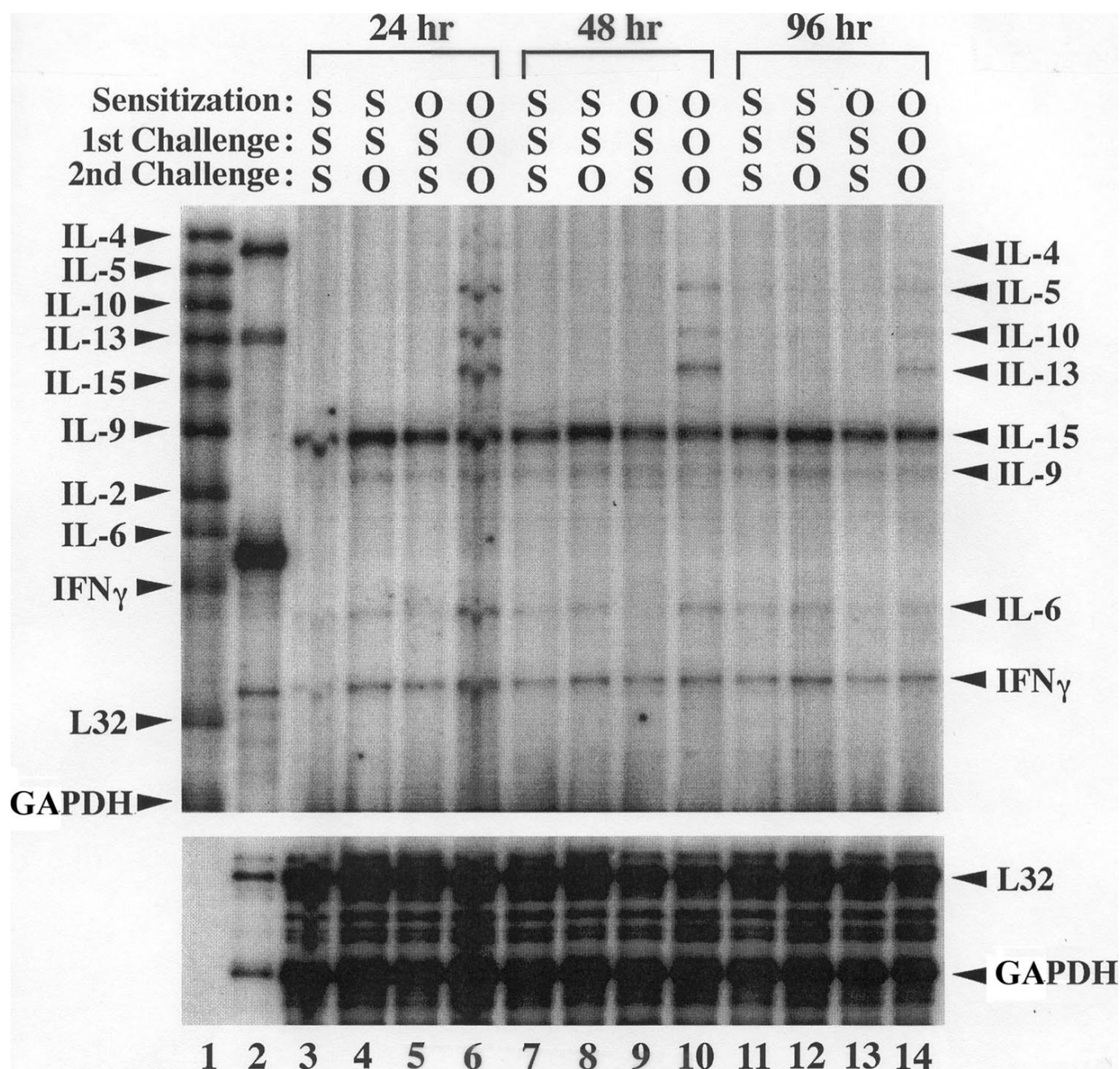


FIGURE 10.—Autoradiograph of electrophoretic gel depicting cytokine mRNA expression from RNase Protection Assay performed using RNA isolated from the cranial and medial right lung lobes of mice 24, 48, or 96 hours after double challenge with ovalbumin. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline (O) or saline alone (S). Mice were then challenged 2 weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24, 48, or 96 hours after double challenge. Lane 1 = undigested probes for IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, INF- γ , and the housekeeping genes L32 and GAPDH. Lane 2 = mouse control RNA IL-4, IL-10, IL-2, INF- γ , L32, and GAPDH that each hybridized with their corresponding probe followed by digestion with RNase thus resulting in the small shift in migration. Lanes 3–6 = RNA from animals sacrificed 24 hours after double challenge. Lanes 7–10 = RNA from animals sacrificed 48 hours after double challenge. Lanes 11–14 = RNA from animals sacrificed 96 hours after double challenge. The last lane of each time-point (6, 10, and 14) depicts RNA from animals that were sensitized and challenged to ovalbumin.

expression in lung tissue and in a locally draining lymph node of the lung may be a sensitive biomarker for identifying respiratory allergens. In addition, similar histopathologic changes were detected in the nasal airway suggesting that this intranasal exposure method may be used to study allergic rhinitis.

The most common methods typically used to sensitize mice to protein allergens in murine models of IgE-mediated allergic airway disease include intraperitoneal and intra-airway administration. After sensitization, the animals are then challenged via the airways to induce features of IgE-mediated allergic airway disease such as airway hyperresponsiveness

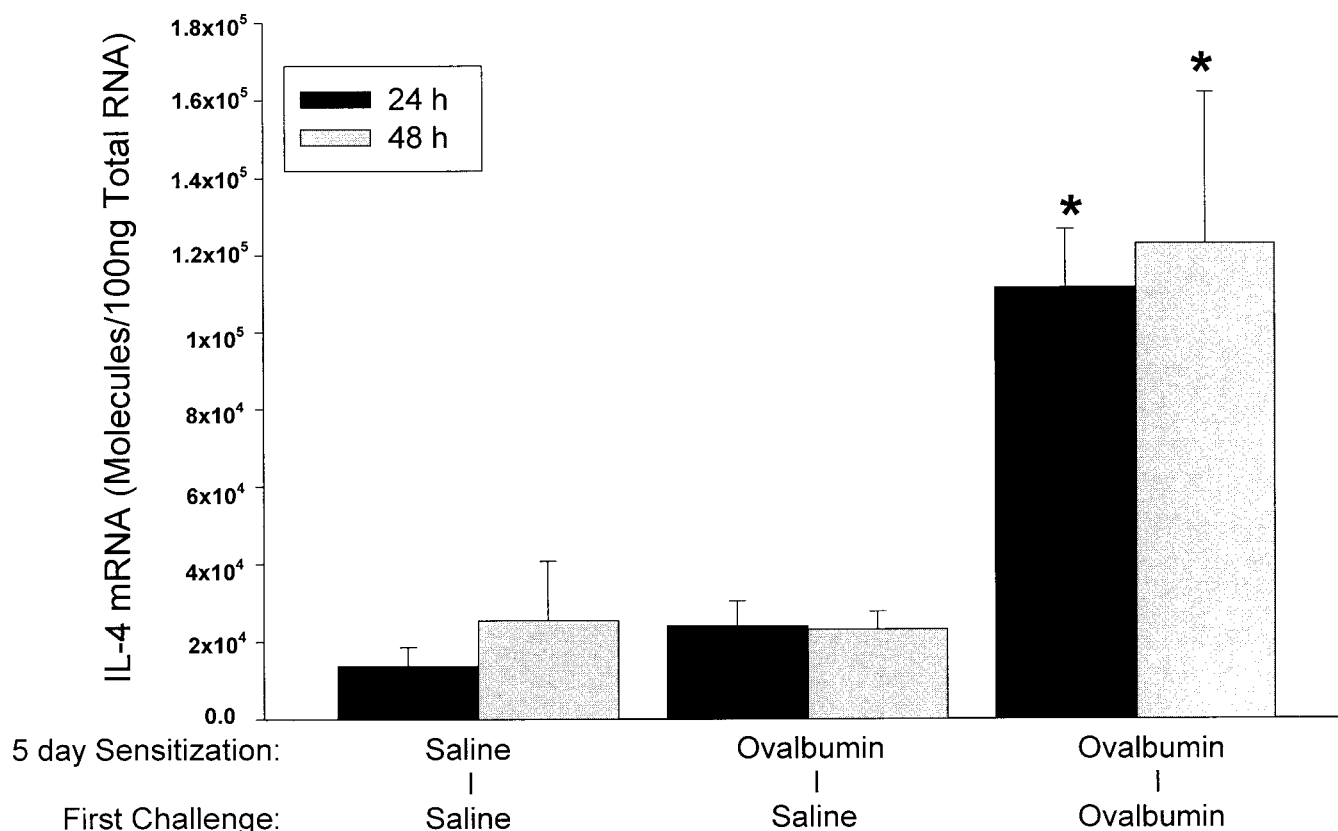


FIGURE 11.—Quantification of IL-4 mRNA in cranial and medial right lung lobes 24 or 48 hours after single challenge with ovalbumin using RT-PCR. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and sacrificed 24 or 48 hours after challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated $\pm$ standard error of the mean ($n = 5/\text{group}$). * = significantly greater than all other groups at similar time-point ($p < 0.05$).

and airway inflammation. The intraperitoneal administration of protein allergens is an unnatural route of exposure to aeroallergens. Also, when antigens are intraperitoneally administered in experimental models, they are often coupled with an adjuvant. An adjuvant, used to potentiate an immune response, can influence the Th1/Th2 cytokine profile and therefore may artificially alter downstream immunologic responses (eg, immunoglobulin isotypes secreted by B cells) (19).

Mice may also be sensitized to allergens via intra-airway administration. One method of intra-airway administration used to sensitize mice to protein allergens is intratracheal instillation. Intratracheal sensitization of various protein allergens including keyhole limpet hemocyanin (21) and latex proteins (28) has been shown to successfully induce IgE-mediated allergic airway disease. In addition to intratracheal instillation, some models of IgE-mediated allergic airway disease use aerosolized antigen to sensitize mice to a specific antigen. There are conflicting reports regarding the effectiveness of aerosolized antigen as a method of sensitization. While successful sensitization has been demonstrated with some experimental models (10), in others, the development of airway inflammation after challenge was not observed (22).

Very few reported models, however, have used intranasal instillation of antigen as a method of intra-airway administration to sensitize animals. It has been unclear whether a

strong immunologic and pathologic response characteristic of IgE-mediated allergic airway disease in the pulmonary airways can be induced by intranasal sensitization (and challenge) to ovalbumin. The intranasal instillation of allergens (ie, *Schistosoma mansoni* egg antigen, *Aspergillus fumigatus*) has been used to successfully induce features of allergic rhinitis (18, 24). In addition, the intranasal instillation of allergens has been used to elicit antibody responses for the identification of the respiratory sensitization potential of detergent enzymes in the mouse intranasal test (MINT) (3, 20). Our exposure regimen differed from the MINT protocol in the schedule and duration of allergen dosing. The MINT protocol includes instillations on days 1, 3, and 10 followed by sacrifice on day 15 (3, 20). Our study consisted of 5 intranasal instillations during a distinct sensitization phase followed by the first challenge (single intranasal instillation) 2 weeks later and a second challenge 10 days after the first challenge followed by sacrifice from 24 to 96 hours after final instillation.

We found that the intranasal instillation of ovalbumin was a simple, inexpensive, and noninvasive method of inducing IgE-mediated allergic airway disease in the upper and lower respiratory tract of the A/J mouse. Adjuvant was not necessary to effectively sensitize the nasal or pulmonary airways to the allergen. Not all of the intranasally inhaled allergen was delivered to the respiratory tract. Some of it was undoubtedly delivered to the esophagus and upper gastrointestinal tract.

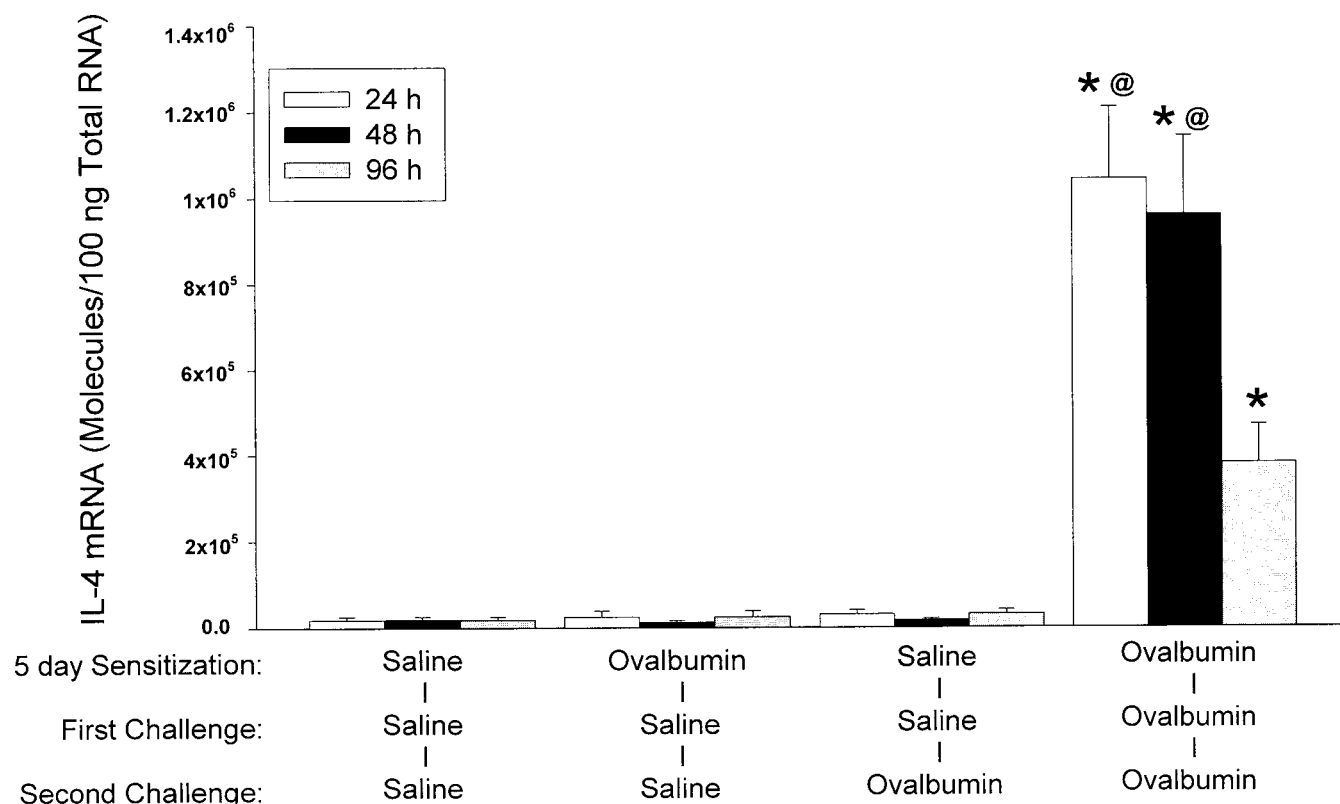


FIGURE 12.—Quantification of IL-4 mRNA in cranial and medial right lung lobes 24, 48, or 96 hours after double challenge with ovalbumin using RT-PCR. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24, 48, or 96 hours after double challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated $\pm$ standard error of the mean ($n = 5$ /group). * = significantly greater than all other groups at same time-point ($p < 0.05$). @ = significantly greater than similarly-challenged group sacrificed 96 hours after challenge ($p < 0.05$).

Our experimental design, however, did not allow us to determine the amount of instillate that was delivered to the gut. In addition, our study was not designed to determine the contribution of the gastrointestinal tract to the overall sensitization of the mouse to ovalbumin.

Many animal models of IgE-mediated allergic airway disease employ continuous administration of the allergen by various routes without any significant lag period between initial sensitization and subsequent challenge to the allergen. This may increase the risk of making the animal tolerant to the antigen. The exposure regimen used in the present study included a distinct sensitization phase after which no significant immune effect was observed, and a challenge phase that consisted of one or two intranasal instillations. This regimen is somewhat similar to human exposures to many proteins and low molecular weight chemicals in the workplace that ultimately result in occupational asthma. Sensitization to a specific allergen in such cases of occupational asthma usually takes place some time before re-exposure and the subsequent elicitation phase (1).

The airways of asthmatics are characterized by the presence of chronic inflammation with infiltration of lymphocytes, plasma cells, eosinophils, and mast cells in the bronchial mucosa and epithelial changes that include MCM (12, 26). The lung lesions resulting from intranasal sensitization and challenge of mice with ovalbumin included marked

MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils, lymphocytes, and plasma cells in the interstitial tissue surrounding the airways. Two ovalbumin challenges in sensitized mice induced a more substantial increase in the number of mucus-secreting cells than a single challenge indicating that the 2-challenge model elicited a more robust response.

There was also a modest increase in the amount of IM in the absence of a cellular or cytokine immune response observed in mice that received ovalbumin only once before sacrifice. These minimal changes in IM may have been due to some irritating properties of the ovalbumin solution rather than an immune-mediated response. The effect of the sensitization phase was also examined by sacrificing mice after the sensitization phase alone. Five consecutive 1-day instillations of ovalbumin caused a similar minimal increase in IM with no accompanying airway inflammation. This minor increase in IM was also likely due to some irritating effect of the inhaled solution on the airway epithelium that resulted in a minimal increase in IM. This response was nowhere near the severity of the MCM induced in the mice that were also challenged with the ovalbumin solution. In addition, only the mice that were both sensitized and challenged with ovalbumin had an inflammatory cell infiltrate accompanying the marked MCM indicating an immune-mediated response. These histologic

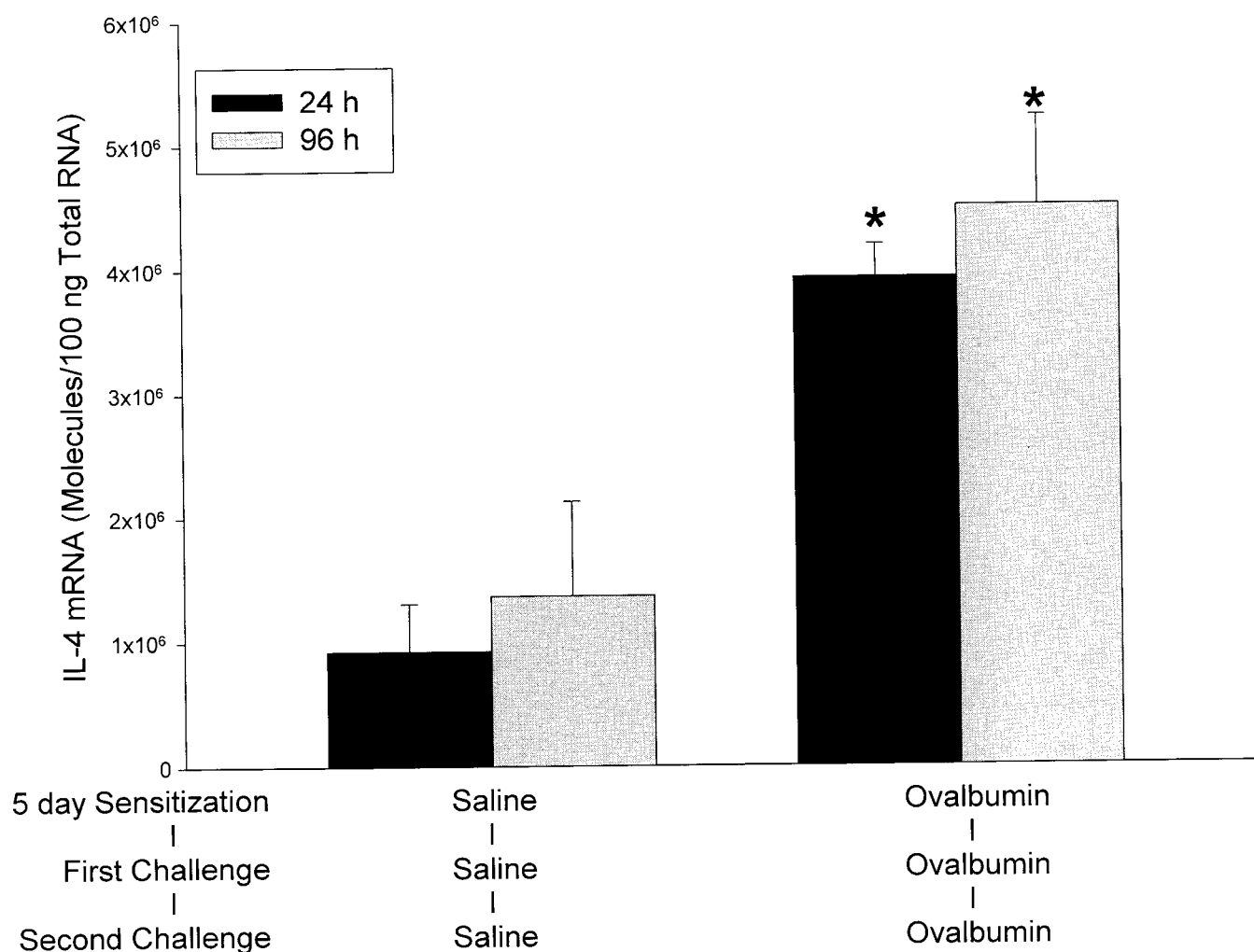


FIGURE 13.—Quantification of IL-4 mRNA in tracheobronchial lymph node 24 or 96 hours after double challenge with ovalbumin using RT-PCR. Mice were sensitized with 5 consecutive daily intranasal instillations of 30 μ l of 0.5% ovalbumin in saline or saline alone. Mice were then challenged 2 weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 or 96 hours after double challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated $\pm$ standard error of the mean ($n = 5$ /group). * = significantly greater than all other groups at same time-point ($p < 0.05$).

features were not present in mice that received only ovalbumin sensitization or only ovalbumin challenge.

The single-most predisposing factor for the development of asthma in humans is atopy (14). Individuals with atopy have a hereditary predisposition to produce an IgE-mediated response against common environmental allergens (14). The primary immunoglobulin isotype associated with immediate-type hypersensitivity responses in mice is also IgE (15). Terminations of antigen-specific IgE in the serum of mice that were intranasally sensitized and challenged with ovalbumin showed an increase in ovalbumin-specific IgE. In our study, there appeared to be a requirement for an elicitation phase in order to induce an increase in ovalbumin-specific IgE.

The Th2 subtype of the CD4⁺ T cell produces a unique profile of cytokines that has been linked to the pathogenesis of IgE-mediated allergic airway disease. Th2 cells produce IL-4, IL-5, and IL-13 (31). This Th2 cytokine profile has been consistently found in both bronchoalveolar lavage fluid and biopsies of allergic asthmatics (26). There are many models of IgE-mediated allergic airway disease that vary in the method

used for cytokine analysis with some focusing on changes in BALF-derived cytokine content while others analyze local draining lymph node cells that have been re-stimulated with a mitogen in vitro. In the present study, we determined the kinetics of Th2 cytokine gene expression in RNA isolated from the lung and the TB lymph node. Intranasal sensitization and challenge of mice to ovalbumin increased lung-derived mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13. In addition, there was an increase in IL-10 mRNA. The magnitude of the increases peaked at the earliest measured time, ie, 24 hours after double challenge, and quickly decreased in magnitude by 96 hours after double challenge. The kinetics suggests that cytokine gene expression peaks soon after allergen reexposure and wanes in a short period of time thereafter. The magnitude of IL-4 mRNA expression was several-fold greater after 2 ovalbumin challenges than after a single challenge. Thus, two ovalbumin challenges produce a more robust cytokine mRNA response than a single challenge. Also, once IL-4 mRNA expression was elevated, it remained elevated for at least 48 hours after challenge.

Intranasal sensitization and challenge of mice to ovalbumin also increased TB lymph node-derived mRNA expression of IL-4 24 hours after challenge and persisted at that level at least until 96 hours. Interestingly, the RNase Protection Assay was not sufficiently sensitive to detect this difference in IL-4 mRNA expression in the TB lymph node. The lack of sensitivity in detecting the increase in IL-4 mRNA is likely due to the high background expression of IL-4 mRNA in the lymph node that may have prevented the detection of an increase in expression in the treated groups. In addition, the fold-increase in IL-4 mRNA expression in the lymph node as determined by PCR was much smaller than that exhibited by the lungs of these animals and suggests that this assay may be unable to detect small increases in expression.

The increase in IL-10 mRNA in this model coincides with the development of the airway pathology suggesting that it may play a role in the induction of the pathologic changes as some murine models suggest (16, 17, 29). However, the role of IL-10 induction has been somewhat controversial leading some to speculate that its primary role is inhibitory, serving to mitigate the severity of the immune response (2). The exact role of IL-10 in this model is unclear.

Individuals afflicted with asthma often suffer from concurrent IgE-mediated allergic airway diseases of the upper airways such as IgE-mediated allergic rhinitis. The main symptoms of IgE-mediated allergic rhinitis are itching, sneezing, watery discharge and obstructed nose (4). The pathologic features of this nasal airway disease include plasma exudation, hypersecretion of mucus, and cellular infiltrates consisting of T- and B-lymphocytes, mast cells, eosinophils, and plasma cells in the nasal airway (4). The mechanism of IgE-mediated allergic rhinitis, such as asthma, is dependent on Th2 cytokines (4). In the present study, we found that intranasal sensitization and challenge with ovalbumin induced in A/J mice features similar to that of IgE-mediated allergic rhinitis in people. A histopathologic assessment of the nasal cavity of our A/J mice revealed that intranasal sensitization and challenge to ovalbumin caused an increase in the number of mucus-secreting cells in the respiratory epithelium that lines the nasal cavity. This epithelial change was associated with an inflammatory influx consisting of eosinophils and lymphocytes similar to that observed in the lung. Therefore, our exposure regimen elicited some of the characteristic features of IgE-mediated allergic rhinitis and may be useful in the study of allergic rhinitis.

The present study illustrated that intranasal sensitization and challenge of mice to ovalbumin in the absence of an adjuvant successfully induced many of the histopathologic, morphologic and immunologic features associated with IgE-mediated allergic airway disease. In addition, the assessment of local cytokine changes in the lung and its draining lymph node is an effective method for determining changes in cytokine gene expression after intranasal sensitization and challenge to an allergen. The analysis of local cytokine gene expression after intranasal exposure may prove useful in the identification of other allergenic proteins and low molecular weight chemicals.

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