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OVALBUMIN AEROALLERGEN EXPOSURE-RESPONSE IN BROWN NORWAY RATS

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A major route of exposure to allergens is through the respiratory tract. Comparatively few animal studies have used aerosolized high-molecular-weight allergens for sensitization, and in these studies, proper characterization of the aeroallergen exposure was usually missing. The purpose of this study was to profile the exposure-response relationship in Brown Norway rats (BNR) to well-characterized ovalbumin (OVA) aerosols. Rats were exposed 30 min/wk × 6 wk to respirable OVA aerosols from <1 mg/m^3 to 64 mg/m^3 air. Ovalbumin-specific circulating immunoglobulin (Ig)E, IgG, and IgA were measured throughout the study period. Rats were sacrificed 1 day after the last exposure. Pulmonary tissue was processed for histopathological and histochemical analysis. Tracheas were isolated, perfused, and assessed for in vitro responsiveness to methacholine. Serum concentrations of OVA-specific antibodies increased with both exposure concentration and number of exposures. The number of BNR with measurable titers also increased with both dose and time. Pulmonary inflammatory changes were noted only in BNR exposed to higher OVA concentrations (15 and 64 mg/m^3 air). Increased tracheal reactivity to methacholine was not found in any of the sensitized BNR. In summary, sustained aeroallergen concentration-dependent changes in specific antibody responses and pulmonary inflammation have been demonstrated.

Allergic sensitization was at one time thought not to exhibit the typical sigmoid dose-response relationship. Studies of allergic sensitization rarely employ multiple sensitization dosage regimens. The relevance of injected dose has been demonstrated in studies examining the production of reagenic antibodies in mice, where sensitization with low doses of antigen (0.1 μg ovalbumin with alum) produced prolonged synthesis of reagins, while high-dose antigen (100 μg ovalbumin with alum) caused an early, but transient, reagenic response (Vaz et al., 1971). A similar phenomenon has been reported in guinea pigs. Andersson (1980) demonstrated that low-dose antigen causes production of both immunoglobulin (Ig)E and IgG antibodies in the guinea pig, while only IgG_1 was produced when guinea pigs were immunized with larger amounts of antigen.

Repeated exposure to ovalbumin (OVA) has been reported to both produce immunological tolerance (Sedgwick & Holt, 1983; McMenamin

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et al., 1992) and enhance IgE production in the Brown Norway rat (BNR) (Siegel et al., 1997). Similar discrepancies have been reported with airway reactivity. Airway reactivity following repeated exposure of the BNR to OVA has been reported to increase (Elwood et al., 1991) and also to be decreased (Kips et al., 1992). The state of allergic pulmonary inflammation during repeated inhalation OVA challenges is also unclear. BAL neutrophil and lymphocyte increases have been reported after single and repeated OVA exposures (Haczku et al., 1994).

Allergic sensitization to high-molecular-weight (MW) antigens has more commonly been accomplished by injection of antigen along with adjuvant in all animal models. Antigen aerosol challenges are then given to the sensitized BNR. Allergic sensitization via the pulmonary route has also been accomplished in several animal models, including BNR. The majority of the studies employing aerosolized antigen for challenge and/or sensitization lack measurement of the antigen concentration to which the animals were exposed. The lack of exposure characterization adds an additional unknown variable component that may be critical in explaining discrepancies found in the literature.

Previous pulmonary allergic sensitization studies reported from our laboratory employed weekly, 30 min/wk × 6 wk OVA aerosol exposures of 18 mg/m^3 to BNR (Siegel et al., 1997). This exposure paradigm produced long-lasting production of specific IgE, IgG, and IgA. The purpose of the present study was to test the hypothesis that an antigenic exposure-response relationship can be demonstrated to OVA in BNR. Specific antibodies were measured periodically throughout the study. In vitro airway reactivity and histological and immunohistological analysis were evaluated 1 day after the last aerosol challenge.

MATERIALS AND METHODS

Animals

Male Brown Norway rats (200–225 g) were purchased from Harlan Laboratories (Indianapolis, IN). The animals were housed in polycarbonate cages containing wire-bottom inserts to prevent exposure to feces and bedding. Cages were held in laminar-flow hoods. Food and water were provided ad libitum.

Antigen Exposures

Ovalbumin (OVA, Sigma Chemical, St. Louis, MO) was used as the antigen/allergen. An aerosolized solution of OVA in saline, from 0% to 5% (w/v), was generated using an Ultra-Neb 99 ultrasonic nebulizer (DeVilbiss, Somerset, PA). Both OVA and saline were checked for and found to be free of endotoxin. Filtered air was passed through the nebulizer, and then the aerosolized OVA was further diluted with filtered air before entering the

exposure chamber. After 15 min an additional 5 ml of OVA/saline was added to the generator. The size distribution of the aerosol was determined using a TSI model 3300 aerodynamic particle analyzer (TSI Incorporated, St. Paul, MN). Chamber OVA aerosol concentrations were determined by collecting samples in an impinger containing saline, and subsequent protein analysis was conducted using Coomassie blue dye reagent (Bio-Rad Laboratories, Hercules, CA). Rats were exposed to the OVA for 30 min, 1×/wk, for 6 wk. The first exposure day was day 0. Air controls were also assessed to evaluate any possible effect of saline exposure. No significant differences were found between the air and saline controls for all parameters measured.

Serum Ovalbumin Specific IgE, IgG, and IgA Measurements

Blood was collected from the tail vein at intervals 2 days after OVA exposure or from the vena cava at sacrifice, 1 day after the sixth antigen exposure. All rats were anesthetized with brevitol (50 mg/kg) prior to vena puncture. Animals were given an overdose of phenobarbital (60 mg) prior to terminal tissue collection. Three sera dilutions with 5% horse serum albumin (HOSA)/phosphate-buffered saline (PBS) of 1/100, 1/1000, and 1/10,000 were analyzed for specific IgE and IgA. Specific IgG determinations were done on sera dilutions of 1/1000, 1/10,000 and 1/100,000. Diluted sera (100 µl) was added to a 96-well plate (catalog number 76-381-04, ICN Pharmaceuticals, Inc., Costa Mesa, CA) that had been previously coated with 200 µl of 10 mg OVA/ml carbonate coating buffer and blocked with 5% HOSA/coating buffer. All enzyme-linked immunosorbent assay (ELISA) buffers were prepared according to Voller and Bidwell (1986). Plates were washed three times after each step with PBS/Tween buffer. The plates were incubated overnight at 4°C. Next, sheep IgG, anti-rat IgE (100 µl, 1/500 dilution in HOSA/PBS; catalog number 64-325-1, ICN Pharmaceuticals, Inc.), followed by horseradish peroxidase-bound donkey IgG, anti-sheep IgG (100 µl, 1/1000 dilution in HOSA/PBS; catalog number 67-253-1, ICN Pharmaceuticals, Inc.), were sequentially incubated in the plate for 2 h at room temperature. The plates were washed three times after each incubation. The plates were developed using the substrate tetramethylbenzidine (TMB, Sigma Chemical Co.) and read at 630 nm.

Both OVA-specific IgG and IgA measurements were made using the same protocol but substituting appropriate detection antibodies (Sigma Chemical) for each assay. Goat IgG, anti-rat IgG (catalog number R2129, 1/500 dilution), goat IgG, anti-rat IgA (catalog number R9630, 1/500 dilution), and peroxidase labeled rabbit IgG anti-goat IgG (catalog number A3540, 1/14,000 dilution) were the detection antibodies used for these measurements. The serum from one animal exposed to OVA was more extensively titered and concentration/response curves were obtained. This reference serum was assigned a value of 100. Ovalbumin-specific IgE and IgG data are reported as relative titers using these curves. ELISA IgA absorbance

values obtained at the 1/1000 dilution are reported. An absorbance reading >2.5 times background was considered a positive titer for all isotypes when determining prevalence of sensitization.

Pathology

After cutting open the chest and exposing the lungs, two sutures were tied around the left bronchus and the left lobe was removed by cutting between the sutures. The left lung lobe was preserved by airway perfusion with 2.5 ml 10% neutral buffered formalin. Tissues were routinely processed overnight and embedded in paraffin the morning after necropsies. Slides were stained with hematoxylin and eosin and read in a blinded fashion by a board-certified veterinary pathologist.

Immunohistochemistry

Tissue Preparation The trachea was exposed, a slit was cut just above the bifurcation, and an 18-gauge needle covered with polyethylene tubing) was inserted into the right bronchus and tied in place with a suture. The right lobes were infused with cold optimal cutting temperature (OCT, Tissue-Tek® O.C.T., Sakura Finetek US, Inc., Torrance, CA):PBS (1:1) at a pressure of 30 cm H_2O. The filled right diaphragmatic lobe was removed, cut into pieces, covered with OCT on labeled cork circles, and snap-frozen in isopentane cooled in liquid nitrogen. Frozen samples were kept at –80°C.

Tissue sections, 6 μm in thickness (Hacker-Bright Micro Cryostat 2122, Hacker Instruments, Inc., Fairfield, NJ), were collected on Vectabond-coated slides (Vector Laboratories, Inc., Burlingame, CA) and air dried overnight. After fixing for 10 min in cold acetone, sections were air dried, washed briefly in PBS, and incubated for 30 min with 200 μl of one of the following monoclonal antibodies from Harlan Bioproducts for Science (Indianapolis, IN): mouse anti-Ia (MCA 46G, clone MRC OX-6); mouse anti-rat CD4 (MCA 55G, clone W3/25); or mouse anti-rat macrophages (MCA 342, Clone ED2). The secondary antibody was a rat-absorbed, rabbit anti-mouse immunoglobulin polyclonal antibody (DAKO Z456, Carpinteria, CA), followed by alkaline phosphatase conjugated monoclonal antibody (APAAP) (DAKO D651), with a fast red chromagen (DAKO kit K670; contains levamisole) used for localization. Tissues were counterstained in Gill's III hematoxylin (Poly Scientific, Bay Shore, NY) and mounted in Crystal/Mount (Biomedia Corp., Foster City, CA).

Eosinophil Detection A histochemical method for cyanide-resistant eosinophil peroxidase activity was used to stain for eosinophils present within the lung tissue (Yam et al., 1971; Zucker-Franklin & Grusky, 1976). Slides were incubated in 0.5 mg/ml diaminobenzidine (DAB) containing 0.015% H_2O_2 and 0.01 *M* KCN in Tris-buffered saline, pH 7.6, for 5 min at room temperature. After two washes with H_2O, sections were counterstained with Harris hematoxylin (Polysciences, Inc., Warrington, PA) for

30 s. Consecutive sections were stained with hematoxylin and eosin to confirm cell types, and although a few of the nuclei were trilobed or circular, most of the DAB-positive cells corresponded to eosinophils.

Image Analysis Slides were coded and read in a "blind" fashion. Positive cells in bronchioles were counted with a 40× objective. Airways in the tissues were less than 1 mm in diameter and no cartilage was present. Subdivisions of the airway walls were as recommended by Bai et al. (1994), with the mucosa being the region that includes the epithelium, basement membrane, and lamina propria, and with the submucosa extending from the lamina propria to the paraenchyma. Operationally, regions were selected that included both the mucosa and submucosa, excluding the smooth muscle and damaged areas, for cell counts. Regions of interest were drawn on the image, and positive cells within these regions were enumerated using the Optimas image analysis system (Optimas Corp., Edmonds, WA). For the Ia-positive cells, counts were made within selected regions of the mucosa and submucosa (excluding muscle regions and disrupted epithelium). For eosinophils, DAB-positive cells were counted in the lamina propria and submucosa; the epithelium was excluded since few positive cells were ever seen in that location. At least four different regions were counted on each slide, and the total number of positive cells was divided by the total region of interest area on a given slide. These results were expressed as number of positive cells per square millimeter.

Physiological Assessment

The preparation of tracheas for perfusion has been described in detail (Fedan & Frazer, 1992). Briefly, a tracheal segment was removed and cleaned in modified Krebs–Henseleit solution [MKH solution, containing (m*M*): NaCl, 113.0; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; and glucose, 5.7; pH 7.4, 37°C bubbled with 95% O_2–5% CO_2). The segment was mounted to a perfusion holder (modified from those used previously for guinea pig tracheas), which contained side-hole catheters that became inserted into the lumen from each end. The holder was placed into a 25-ml bath of gassed 37°C MKH solution (the extraluminal, or EL, bath). The inlet and outlet ends of the indwelling catheters were connected to the positive and negative sides, respectively, of a differential pressure transducer. The trachea was perfused at a rate of 25 ml/min (unless otherwise indicated) with gassed, recirculating MKH solution (37°C) from a separate, 30-ml bath (the intraluminal, or IL, bath) with transmural pressure adjusted to zero. Responses were measured as changes in the inlet minus outlet pressure difference (ΔP, in cm H_2O). The preparations were equilibrated for 1 h before experimental manipulations were begun, during which time they were washed at 15-min intervals by changing the extraluminal (EL) and intraluminal (IL) MKH solution.

Concentration-Response Curves Stepwise-increasing, cumulative additions of agonists were made to the baths to generate concentration-

response relationships for EL and IL additions of methacholine (Sigma Chemical Co., St. Louis, MO); the EL curve preceded the IL curve as previously described (Fedan & Frazer, 1992). After the maximum response of the EL curve was attained, the preparations were washed at 15-min intervals for 1.5 h, and a concentration-response relationship was generated for IL methacholine additions.

Analysis of Results

The results were analyzed for differences using one-way or two-way analysis of variance or Student's *t*-test for nonpaired and paired comparisons, as appropriate. Linear regression analysis was performed to access dose-response relationships between exposure dose, exposure time, and OVA-specific antibodies. The strength of the correlation was tested by calculating the t_r values (Sokal & Rohlf, 1969). Responses of isolated, perfused tracheas were quantified as ΔP in cm H_2O. Geometric mean EC50 values were derived from least-squares analysis of a logit curve fit (SigmaPlot). Statistical comparisons were done using normally distributed –log EC50 values.

RESULTS

Exposure Characterization

Impingers were run for each entire 30-min exposure period to collect OVA aerosol samples. Protein content was quantified. A second impinger used in series with the first was employed during methods development to assess sampling efficiency. Protein was below detectable levels (<12.5 μg/ml ovalbumin) in the second impinger. Table 1 lists the concentration of OVA/saline used in the nebulizer and the average ± SEM aerosol concentration generated at each concentration. The ultrasonic nebulizer produced a bimodal aerosol mass distribution. Ovalbumin aerosol of ≤3 μm diameter constituted 95.6 to 99.1% of the number of particles, but only 22.1 to 34.2% of the mass. Figure 1 is the OVA aerosol size distribution by count and mass for the two higher aerosol concentrations. The aerosol characteristics of mass median aerodynamic diameters, count median aerodynamic diameters, and percent of the aerosol ≤3 μm are also included in Table 1.

It was not possible to use an OVA concentration greater than 50 mg/ml, because the viscosity interfered with aerosol generation. At 50 mg/ml the nebulizer energy had to be increased to maintain a similar aerosol size distribution to that of the other ovalbumin aerosol clouds generated. The increased viscosity of 50 mg/ml OVA after increasing the nebulizer output energy caused only a slight shift of the aerosol size distribution. The average particle size by count increased by approximately 0.5 μm. The mass distribution was still bimodal.

TABLE 1. Ovalbumin aerosol characteristics

Nebulizer ovalbumin concentration (mg/ml saline)	Chamber ovalbumin concentration (mg/m^3 ± SEM)	Aerodynamic median diameter (μm)		Percent ≤3 μm	
		Mass	Count (σ_g)	Mass	Count
0.1	<1	8.1	0.93 (1.26)	28.3	99.1
1	3.3 ± 0.88	8.1	0.96 (1.15)	27.2	99.3
10	15.4 ± 2.95	8.7	0.98 (1.29)	22.1	98.7
50[a]	64.1 ± 11.10	7.4	1.39 (1.52)	34.2	95.6

[a]Ultrasonic nebulizer energy setting was increased to compensate for increased viscosity of OVA solution.

Anti-Ovalbumin-Specific Antibody Responses

Figures 2–4 are the IgE, IgG anti-OVA titers and IgA absorbances, respectively. Blood was drawn at days 16, 23, 30, and 36 following the first antigen exposure (day 0). Specific IgE, IgG, and IgA antibody titers were followed in individual animals over time. Ovalbumin-specific antibody titers consistently increased over the 36 days of the study in rats from all OVA exposure groups. The level of circulating OVA-specific antibodies was also highly dose dependent.

The prevalence of rats with detectable specific antibodies increased with both time and exposure concentration (Table 2). A sera was considered to be positive for specific antibody if the ELISA absorbance reading at the lowest dilution was >2.5 times nonspecific binding absorbance obtained from control seras. Specific IgG and IgE antibody prevalence was 100% by day 36 for rats exposed to ≥15.4 mg/m^3 OVA. Half of the rats exposed to the lowest level (<1 mg/m^3 OVA) had measurable specific IgE by day 36, while an additional two rats had detectable specific IgG without concomitant specific IgE.

Histology

Semiquantitative assessment of the distribution, severity, and type of inflammatory changes and perivascular cuffing in pulmonary histological sections was performed on tissue from five to six rats per exposure and control groups. Focal to multifocal mild eosinophilic perivascular cuffing was seen in three of five saline control rats and from our experience seems to be a normal feature of the BNR. The severity or distribution was not different in the BNR exposed to OVA. Multifocal perivascular cuffing was noted in all the rats exposed to 15.4 or 64.1 mg/m^3 OVA, and the presence of neutrophils was noted in half of these animals. No granulomatous or histiocytic pulmonary inflammatory changes were noted in control rats. There were no significant changes from saline controls in rats exposed to <1 or 3.3 mg/m^3 OVA. All rats in the 15.4- and 64.1-mg/m^3 OVA exposure groups had multifocal minimal to moderate eosinophilic

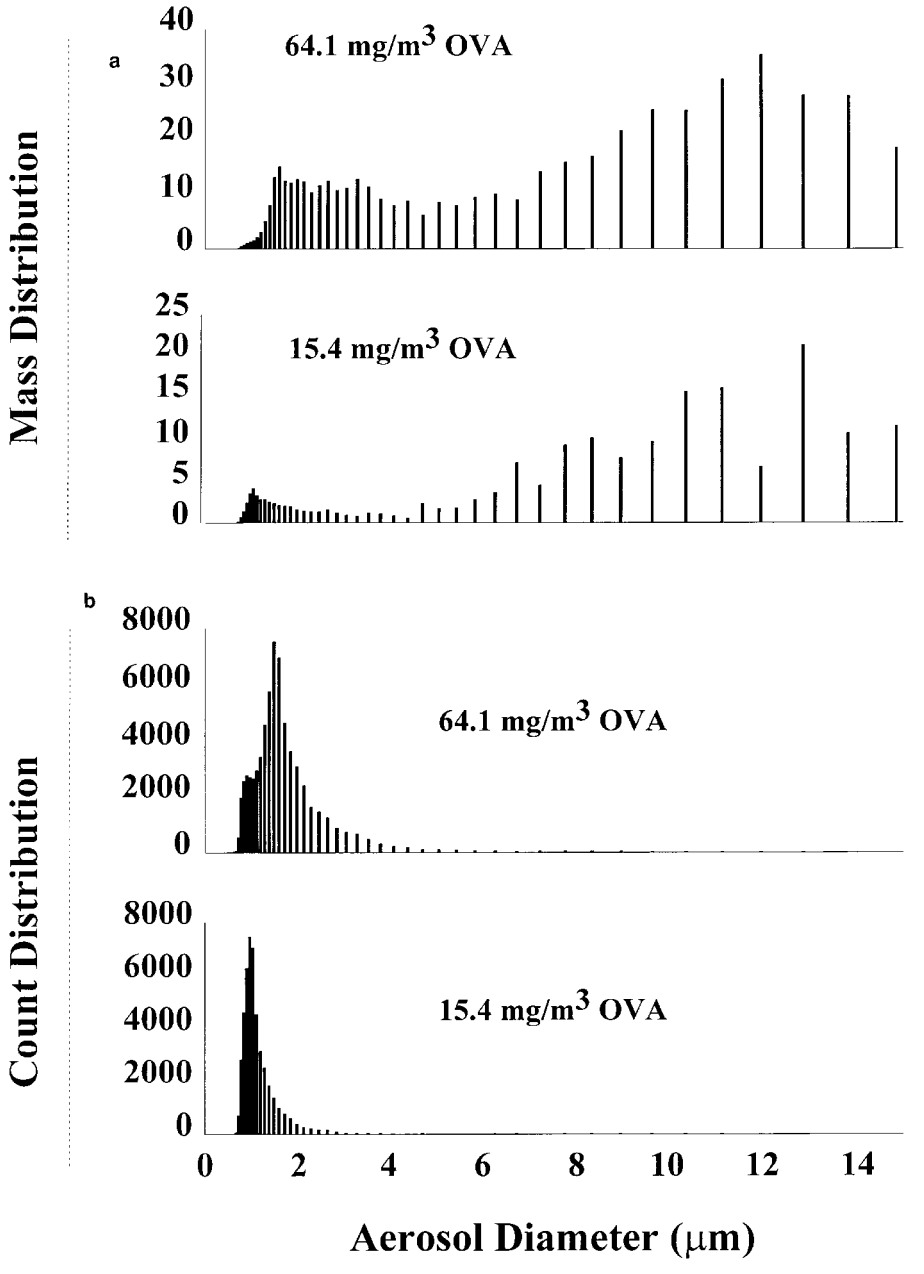

FIGURE 1. Ovalbumin aerosol characterization: (a) aerosol size distribution mass and (b) particle number for 64.1 and 15.4 mg/m^3 OVA. The particle number distribution was unimodal, but the size distribution by mass was bimodal. Specific aerosol characteristics for all the concentrations used are reported in Table 1.

or pleocellular perivascular cuffing. Mild to moderate, multifocal histiocytic alveolitis was noted in 3 of 5 and 4 of 5 rats exposed to 15.4 and 64.1 mg/m^3 OVA, respectively. Eosinophilic granules were commonly noted in these lesions.

Immunochemical Cellular Localization

Ia-Positive Cells The antibody used was specific for Ia antigen present on B lymphocytes, dendritic cells, and some macrophages. In BNR lung tissue, many of the alveolar macrophages were positive. Positive cells were also located in the interstitium and throughout the bronchial-associated lymphoid tissue (BALT), especially in regions that were negative with the T-cell antibody. There were many positive cells with long processes located in the epithelium, lamina propria, and submucosa. In fortuitous tangential sections of larger bronchioles, a network of positive

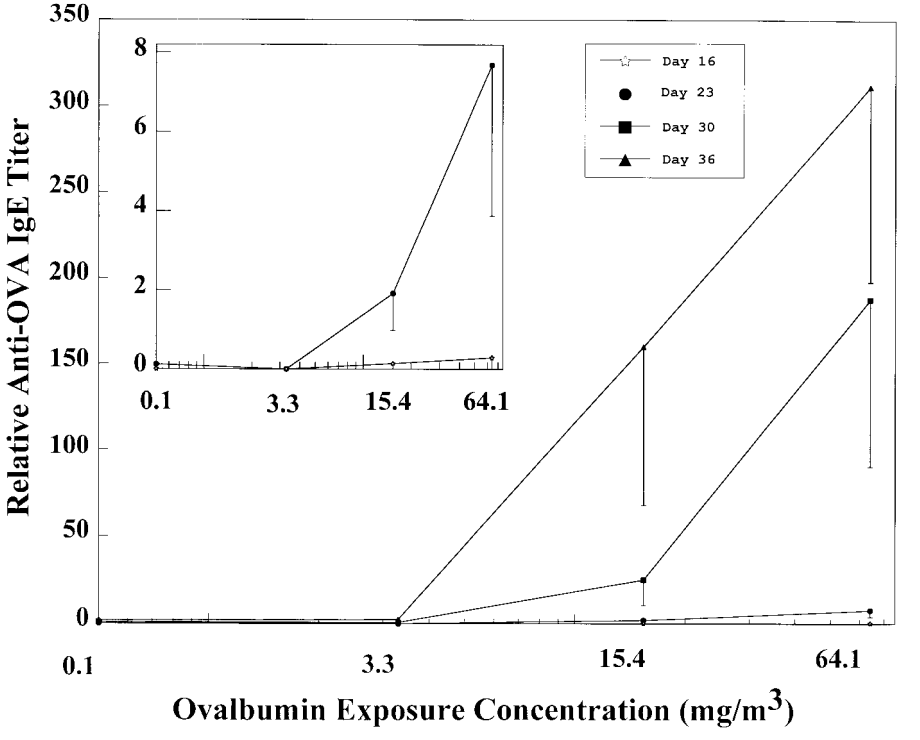

FIGURE 2. Aerosolized OVA concentration–anti-OVA IgE response curve. Brown Norway rats were exposed weekly to 30-min aerosols of OVA from <1 to 64.1 mg/m^3. The insert is a magnification of sera anti-OVA IgE levels measured at the earlier time points of day 16 and 23. The strength of the relationship between exposure concentration and OVA-specific IgE at days 23, 30, and 36 was $p <$ .0001, .001, and .05, respectively. The exposure time–log anti-OVA IgE (at 64.1 mg/m^3 OVA) correlation was significant ($p < .001$). Each point is the average of $n = 5$ or 6 ± SEM.

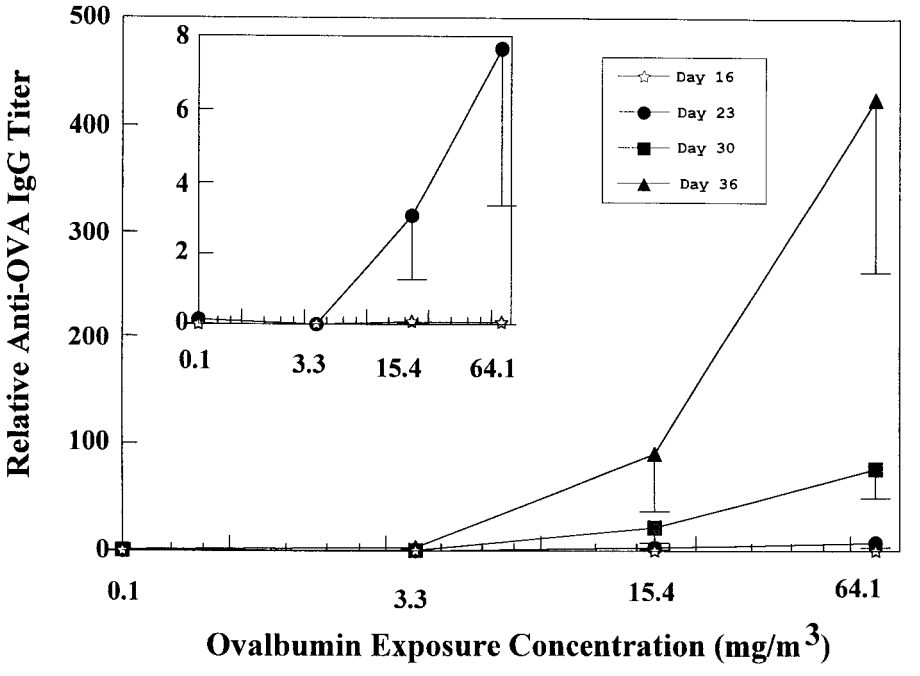

FIGURE 3. Aerosolized OVA concentration–anti-OVA IgG response curve. Brown Norway rats were exposed weekly to 30-min aerosols of OVA from <1 to 64.1 mg/m^3. The insert is a magnification of sera anti-OVA IgG levels measured at the earlier time points of days 16 and 23. The strength of the relationship between exposure concentration and OVA-specific IgG at days 23, 30, and 36 was $p <$ .001, .001, and .0001, respectively. The exposure time–log anti-OVA IgG (at 64.1 mg/m^3 OVA) correlation was significant ($p <$ .001). Each point is the average of $n = 5$ or 6 ± SEM.

cells was visible. There was no significant difference between control and ovalbumin-treated animals in the number of positive cells in the mucosa and submucosa.

Macrophages The anti-ED2 antibody is specific for tissue macrophages; alveolar macrophages and dendritic cells are not labeled. In our sections, positive cells were dendritic in appearance and were plentiful in the airways. Like that seen with the Ia antibody, cells were located primarily in the lamina propria and submucosa; however, they were not found in the epithelium. No positive cells were found in the BALT or the parenchyma. No obvious differences were seen between controls and experimental animals.

T Cells The antibody used labels the rat T-helper subset and macrophages. Many positive cells were seen in distinct regions of the BALT; they were rounded cells with large nuclei. There were also many positive cells with long processes in the lamina propria, mucosa, and submucosa, very similar to the population seen with the macrophage antibody. No positive cells were located in the epithelium. No obvious differences were seen between controls and experimental animals.

DAB (Eosinophil Peroxidase)-Positive Cells No DAB-positive cells were present in the epithelium. When infiltration occurred, DAB-positive cells were located primarily in the submucosa and lamina propria, with a very few in the parenchyma. The average ± SEM numbers of DAB positive cells/μm^3 for saline controls and treatment with <1, 3.3, 15.4, and 64.1 mg/m^3 OVA were 60 ± 23, 160 ± 38, 89 ± 0.18, 259 ± 47, and 655 ± 234, respectively. The 15.4- and 64.1-mg/m^3 OVA exposed rat had a significant increase in pulmonary DAB-positive cells as compared to saline controls ($p < .05$).

In Vitro Nonspecific Tracheal Reactivity

Tracheas used for physiological assessment of airway reactivity to methacholine were taken from the same rats used for serological, histological, and immunohistochemical analysis. Whole tracheas were isolated and perfused as described in the methods section. Table 3 list the IL and EL -logEC50 values to methacholine for isolated perfused trachea. Figure 5 illustrates that the IL methacholine concentration-response curves were displaced significantly to the right of the EL curves. This effect, seen in all treatment groups, reflects the modulatory role of the epithelium on reactivity that has been observed repeatedly in guinea pig airways (Fedan &

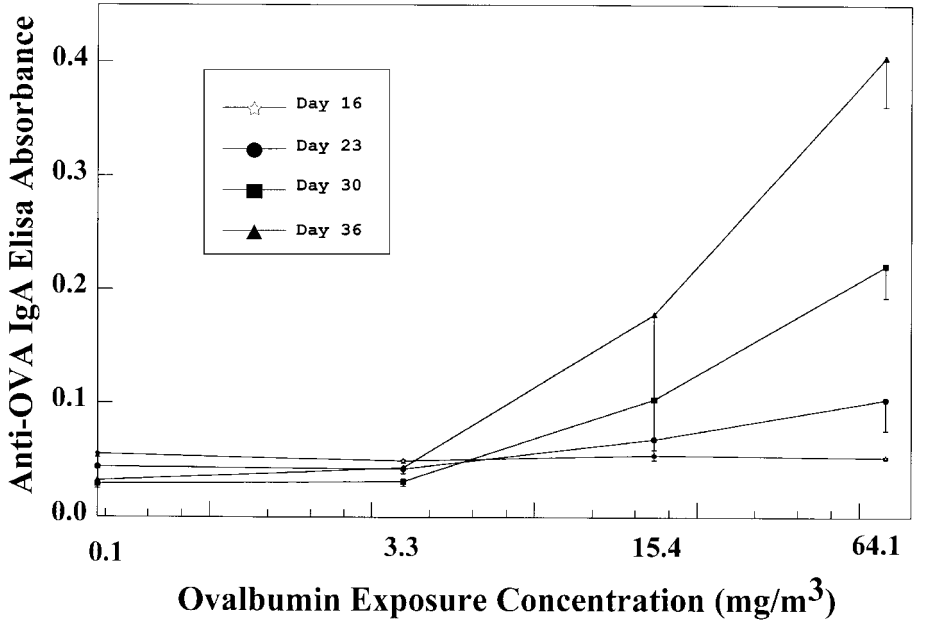

FIGURE 4. Aerosolized OVA concentration–anti-OVA IgA response curve. Brown Norway rats were exposed weekly to 30-min aerosols of OVA from <1 to 64.1 mg/m^3. The strength of the relationship between exposure concentration and OVA-specific IgE at days 23, 30, and 36 was $p < .001$ for each. The exposure time–log anti-OVA IgA (at 64.1 mg/m^3 OVA) correlation was significant ($p < .0001$). Each point is the average of $n = 5$ or 6 ± SEM.

TABLE 2. Dose-dependent anti-OVA antibody prevalence

Conc. (mg/m^3)	n	Anti-OVA IgE[a]				Anti-Ova IgG[a]				Anti-Ova IgA[a]			
		Day 16	Day 23	Day 30	Day 36	Day 16	Day 23	Day 30	Day 36	Day 16	Day 23	Day 30	Day 36
<1	6	0	1	2	3	0	2	4	5	0	1	1	1
3.3	6	0	0	3	3	0	0	3	4	0	0	1	1
15.4	5	1	3	3	5	2	3	5	5	1	3	2	4
64.1	6	1	5	6	6	1	5	6	6	0	5	6	6

Note. Blood from each rat was drawn from the tail vein on days 16, 23, and 30 or from the vena cava at sacrifice for assessment of specific antibodies. An ELISA absorbance reading for the lowest dilution of a sera of >2.5× nonspecific binding was considered positive. Conc, concentration.

[a]Number of rats (from $n = 5$ or 6) with a positive titer.

TABLE 3. Reactivity of isolated, perfused rat trachea from control and ovalbumin-treated Brown Norway rats to methacholine applied to the extraluminal and intraluminal baths

	–log EC50 (*M*) ± SEM	
Treatment	Extraluminal	Intraluminal
Air control	5.65 ± 0.13	3.42 ± 0.11[a]
Saline control	5.80 ± 0.10	3.67 ± 0.06[a]
0.1 mg Ovalbumin	5.54 ± 0.19	3.32 ± 0.09[a]
1 mg Ovalbumin	5.78 ± 0.05	3.47 ± 0.05[a]
10 mg Ovalbumin	5.73 ± 0.08	3.57 ± 0.08[a]
50 mg Ovalbumin	5.79 ± 0.06	3.50 ± 0.10[a]

[a]Significantly less than the extraluminal value. There were no significant differences in the extraluminal or intraluminal –log EC50 values between the treatment groups.

Frazer, 1992; Munakata et al., 1989). The maximum response to IL methacholine was significantly less than the EL maximum response after treatment with <1, 3.3, and 64.1 mg/m^3 OVA as compared to either air or saline control rat tracheas. Nevertheless, neither the EL or IL EC50 values were affected by OVA treatment. There were no differences in the relaxation responses obtained upon application of IL NaCl (120 m*M*), which suggests that the release of epithelium-derived relaxing factor was not altered by OVA (data not shown.)

DISCUSSION

The present study employed weekly 30-min ovalbumin exposures of concentrations up to 64 mg/m^3 to sensitize Brown Norway rats. Occupational exposure and sensitization to egg proteins can occur in the food processing industry. Ovalbumin concentrations as high as 360 μm/m^3 have been measured in egg processing (NIOSH, 1988). Thus, egg processing workers are exposed for 8 h or more daily to significant egg protein levels. The OVA environmental concentrations used in this study are an order of magnitude greater than that measured at the workplace; however, the total exposure dose is in a comparable range.

Specific circulating antibody levels increased in BNR exposed to OVA throughout the study period. Renz et al. (1992) reported persistent OVA-specific IgE and IgG production in mice exposed to 10-day periods of 20-min, daily OVA aerosols separated by 20-day nonexposure intervals. The antibody production in the mice did plateau. In contrast to the persistent or increasing antibody production in the mouse and BNR repeatedly exposed to OVA, development of immune tolerance has also been reported in BNR following repeated OVA exposure (Sedgwick & Holt, 1983; McMenamin et al., 1992). The cause of this discrepancy is not known. One possible differ-

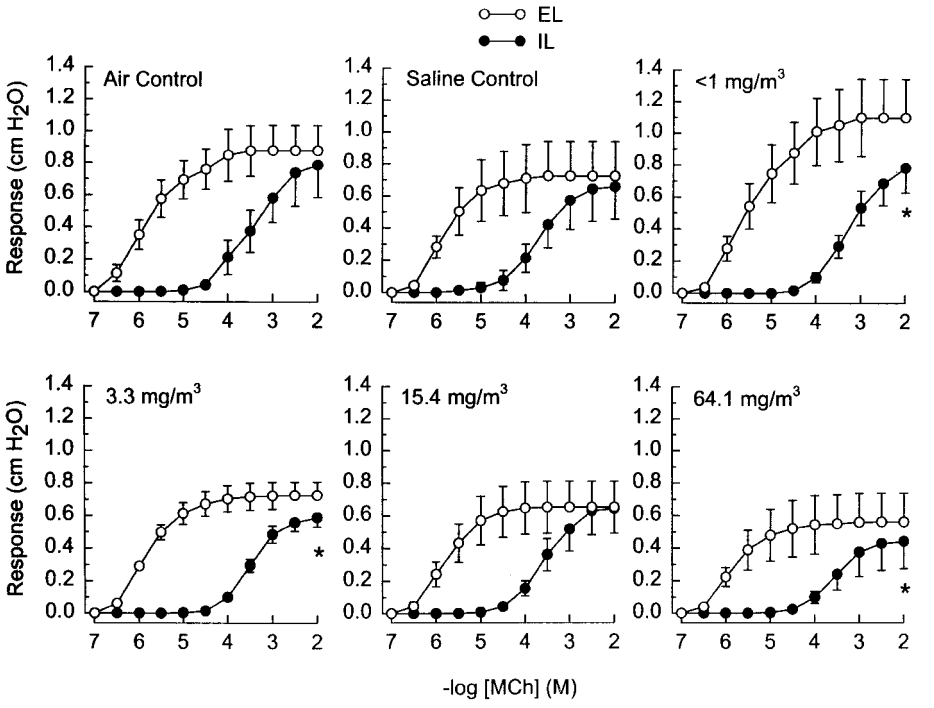

FIGURE 5. EL and IL methacholine concentration-response curves from control and OVA-treated Brown Norway rats. Asterisk indicates that the maximum response to IL methacholine was significantly less than the EL maximum response after treatment with <1, 3.3, and 64.1 mg/m^3 OVA as compared to either air or saline control rat tracheas ($p < .05$).

ence could be that of dose, but the lack of exposure characterization in these published studies prevents comparison of exposure levels. Differences in nebulizers, air flows, and inhalation exposure system surface area amount and types can cause vastly different exposure concentrations. The exposure characterization described here provides important exposure data. Ultrasonic nebulizers can produce a mist with >99% of the aerosol particulate ≤3μm; however, the remaining 1% of the aerosol generated may contain >70% of the total mass. The aerosol-size-dependent airway deposition pattern for the rat has been reported (Wolf & Dorato, 1993). The vast majority of the OVA aerosol used in the present study (approximately 75% of the mass) will be deposited in the head airways, while <2% will deposit in the pulmonary compartment. While both the rat nose and lung have associated lymphoid tissue, the rapid mucocilliary clearance of the upper airways would greatly reduce the deposited aerosol's availability for local antigen processing. The lymph nodes draining the lower respiratory tract have been identified as the primary site for initiation of the IgE response to inhaled antigen; however, the contribution of nasal associated lymphoid tissue was not evaluated (McMenamin et al., 1992).

To our knowledge, there have been no previously reported inhalation dose–IgE response studies. The importance of injected antigen dose on IgE production is well known. Vaz et al. (1971) reported that ip injection of 100 μg OVA plus alum resulted in an early and transient peak of specific IgE in mice, while the IgG_1 titers continued to increase with time. Low-dose OVA (0.1–1 μg OVA plus alum) produced a persistent specific IgE response. Approximately 5 μg OVA would be deposited in the lower lung by the 64-mg/m^3 exposure, assuming a minute volume of 150 ml and deposition curves similar to that reported for the rat in the literature (Wolf & Dorato, 1993). The relative amount of OVA introduced into the lower lung by inhalation (without the use of adjuvant) in the present study is similar to those used when sensitization is accomplished via injection.

Lung tissues were taken 1 day after the last exposure. Assessments of the degree of inflammation by pathology and immunohistochemical analysis were done independently and provided similar results. There were no significant inflammatory changes in pulmonary tissues from animals exposed to <1 or 3.3 μg/m^3 OVA. The distribution of perivascular cuffing in the rats exposed to the higher concentrations of OVA, however, was consistently multifocal and tended to contain neutrophils, as well as eosinophils. This pleocellular nature was also noted when perivascular cuffing was present in BNR exposed to <1 or 3.3 mg/m^3 OVA. The presence of perivascular neutrophils, suggesting migration to the lung, may be a more sensitive indicator of an inflammatory response to OVA than the presence of histocytic alveolitis or increase in the number of eosinophils in the alveoli.

Cells with dendritic processes in the airways were labeled by all three of the antibodies we used. These were probably macrophages, although some could have been dendritic cells. Because of the limits of the methods using frozen sections, it was not possible to identify dendritic cells unambiguously. Cells in the epithelium, which were positive only for the Ia antibody, were probably dendritic cells. This would be in agreement with Holt et al. (1990), who reported a network of dendritic cells in the rat tracheal epithelium. We were not able to quantitate the epithelial Ia-positive cells, however, because of damage to the epithelium from the freezing process.

Most of the DAB-positive cells were eosinophils, though a few may have been neutrophils, or macrophages that had engulfed eosinophils. Most of the DAB-positive cells were located in the lamina propria and submucosa. This is a different pattern from that seen in guinea pigs, where a large influx of DAB-positive cells was found in the epithelium after OVA sensitization (Lawrence et al., 1997). It is not known if this difference is a species difference or caused by differing methodologies in exposure.

Airway hyperreactivity to methacholine in the perfused trachea experiments was not evident on day 36 of the study. This is consistent with the

findings reported by others (Kips et al., 1992; Haczku et al., 1994). These previous studies sensitized the BNR by antigen-adjuvant injection and then gave single or multiple aerosol challenges of OVA. After 8 wk of OVA challenges, the airways were not hyperreactive. The present findings indicated that after <1, 3.3, and 64.1 mg/m^3 OVA, but not 15.4 mg/m^3 OVA, the perfused trachea was *less* reactive to methacholine added to the mucosal surface—that is, to the IL bath—as reflected in a significant decrease in the maximum response obtained relative to that obtained after adding the drug to the serosal surface, that is, to the EL bath. On the other hand, the EC50 values were not affected in either bath. The fact that the small decrease in reactivity occurred in relation to mucosally applied methacholine suggests that the modulatory, inhibitory role of the epithelium was enhanced after OVA. It is not clear, however, why this effect was not seen at the 15.4 mg/m^3 OVA dose in relation to the other measurements that were made. We have observed this increase in the inhibitory effects of epithelium after challenge of OVA-sensitized guinea pigs with OVA (Warner et al., 1996), and after challenge with a conjugate of toluene diisocyanate (TDI)–albumin of TDI-sensitized guinea pigs (Huang et al., 1998). The present findings have extended such changes in epithelial function in sensitized and challenged animals to rats. However, it is well to note that challenge of guinea pigs with OVA (Lawrence et al., 1997), but not TDI–albumin conjugate (Huang et al., 1998), led to upregulation of dendritic cells. Upregulation of dendritic cells was not evoked by OVA in BNR in the present study.

The present study has demonstrated that pulmonary allergic sensitization is dose dependent. The cumulative exposure dose may also be important. The amount of circulating anti-OVA IgE increased with both chamber concentration and cumulative exposure dose. The prevalence of rats with measurable anti-OVA IgE also increased with time and dose. There was no discernable relationship between the amount of specific circulating IgE and the intensity of pulmonary allergic inflammation. A threshold of exposure to >3.3 μg/m^3 OVA did seem to exist for the development of detectable pulmonary allergic inflammation; however, it is not known if this was directly due to the level of antigen exposure or related to the presence of a threshold level of allergic antibody. Allergic sensitization of the airways in humans is thought to come about through aerosol exposure to low levels of environmental antigens. Relevant exposure dose should be an important consideration in modeling pulmonary allergy and asthma.

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