

Ovalbumin-Induced Airway Inflammation and Fibrosis in Mice Also Exposed to Ozone

Jerold A. Last, Rachel Ward, Lisa Temple, Nicholas J. Kenyon

Pulmonary and Critical Care Medicine, School of Medicine, University of California, Davis, California, USA

A murine model of allergen-induced airway inflammation was used to examine the effects of exposure to ozone on airway inflammation and remodeling. Sensitized BALB/c mice were exposed to ovalbumin aerosol for 4 wk before and after 2 wk of exposure to either 0.2 ppm or 0.5 ppm ozone. Other groups of mice were exposed to ovalbumin aerosol for 6 wk with continuous concurrent exposure to ozone during wk 1–6, or during intermittent concurrent exposure to ozone. Lung inflammation was measured by quantitative differential evaluation of lung lavage cells and by histological evaluation of stained lung sections. Alterations in lung structure (airway fibrosis) were evaluated by quantitative biochemical analysis of microdissected airways. The same total number of cells was observed in lavage fluid from animals exposed for 4 wk to ovalbumin alone or to ovalbumin for 4 wk immediately before or after exposure to 2 wk of 0.2 or 0.5 ppm ozone. Mice exposed to ovalbumin for 6 wk with concurrent exposure to either 0.2 ppm or 0.5 ppm ozone during wk 3–6 had a significant decrease in the total number of cells recovered by lavage. Values as low as 7% of the cell number found in mice exposed to ovalbumin aerosol alone were observed in the mice exposed to ovalbumin plus 0.2 ppm ozone during wk 3–6. There were significant differences in the cell differential counts in the lavage fluid from mice exposed to ovalbumin alone as compared with values from mice exposed to ovalbumin and ozone under all of the protocols studied. When ozone was given for 2 wk prior to ovalbumin exposure (Experiment 1), there were a high percentage of macrophages and low percentages of lymphocytes and eosinophils in the lung lavage. When ozone was given for 2 wk after ovalbumin exposure (Experiment 2), there were a moderate percentage of macrophages, a low percentage of eosinophils, and a high percentage of lymphocytes in the lung lavage. When ozone and ovalbumin were given simultaneously (Experiments 3 and 4), there were a high percentage of macrophages in the lavage with 0.2 ppm ozone and a high percentage of eosinophils. Ozone appears to antagonize the specific inflammatory effects of ovalbumin exposure, especially when given before or during exposure to ovalbumin. Airway remodeling was examined by two different quantitative methods. None of

the groups exposed concurrently to ovalbumin and ozone had a significant increase in airway collagen content as compared to the matched groups of mice exposed to ovalbumin alone. The findings were consistent with an additive response of mice to simultaneous exposure to ovalbumin and ozone. Ozone exposure alone for 6 wk did not affect the number of goblet cells in the airways, while mice exposed to ovalbumin aerosol alone for 6 wk had about 25% goblet cells in their conducting airways. Concurrent exposure to ovalbumin and 0.2 ppm ozone caused significant increases in goblet cells (to 43% of total cells) in the conducting airways of the exposed mice. We conclude that when mice with allergen-induced airway inflammation induced by ovalbumin are also exposed to ozone, the lung inflammatory response may be modified, but that this altered response is dependent on the sequence of exposure and the concentration of ozone to which they are exposed. At the concentrations of ozone tested, we did not see changes in airway fibrosis. However, goblet-cell hyperplasia appeared to be increased in mice exposed concurrently to ovalbumin and 0.2 ppm ozone.

Several studies appear to link exacerbations of severe asthma with ambient ozone levels (Weisel et al., 1995; Stieb et al., 1996; McConnell et al., 2001; Douwes et al., 2002). Despite the frequently demonstrated association between asthma attacks and ozone exposure, a mechanistic basis for understanding such an association has not been forthcoming. Hence, there is a need for studying the effects of ozone exposure on suitable animal models of asthma (more correctly, allergen-induced airway inflammation) to facilitate doing controlled experiments that will allow for mechanistic insights and rigorous tests of hypothesis. A few groups have previously attempted to do so. Neuhaus-Steinmetz and coworkers (2000) exposed mice either to ozone alone or simultaneously to combinations of ozone and ovalbumin aerosol. They found increased lymphocytes and eosinophils in lavage fluid, increased airway reactivity to methacholine challenge, and other changes. They interpreted their findings to suggest that concurrent, intermittent ozone exposure (0.25 ppm) increased the immune responses to ovalbumin and shifted the responses towards a more Th-2-like response pattern. Similar results were reported by Depuydt et al. (2002). Sumitomo et al. (1990) reported that guinea pigs exposed to 5 ppm ozone for 2 h prior to sensitization (via the airways) required less ovalbumin for

Received 15 July 2003; sent for revision 22 August 2003; accepted 12 September 2003.

This work was supported in part by grants from the Philip Morris Research Management Group and from NIEHS (ES05707).

Address correspondence to Jerold Last, Toxic Substances Program, 1131 Surge I, University of California, Davis, CA 95616-8723, USA. E-mail: jalast@ucdavis.edu

sensitization or for provocation. Similar conclusions were also drawn by Sun et al. (1997) and by Schlesinger and coworkers (2002), who measured bronchial responsiveness and inflammation in ovalbumin-sensitized guinea pigs given 1 ppm ozone. Comparable findings in mice were reported by Yamauchi et al. (2002). Thus, prior or simultaneous exposure to ozone appears to increase the response to ovalbumin in mice and guinea pigs. None of these experiments were designed to ask whether such increased responses were greater than or less than additive.

Ozone exposure causes oxidant stress in the lung, which may directly or indirectly contribute to the release of mediators and cytokines in the conducting airways. In addition, ozone exposure probably causes the normally very tight junctions of the airway epithelium to become more open, resulting in edema and movement of proteins across the epithelium (Hu et al., 1982; Guth et al., 1986). Thus, presentation of an antigen such as ovalbumin to the airways during or immediately after ozone exposure might be expected to enhance the dose of ovalbumin to the lung at a given exposure concentration. This suggestion is in accord with the frequently observed relationship between exposure to ozone and increased response to inhaled allergens in human subjects with asthma (Drazen & Beier, 1997). Were this putative mechanism to be correct, then the temporal sequence of exposure to ozone and ovalbumin, with ozone before, during, or after ovalbumin exposure, could give very different results, even if total exposures to each of these agents were the same. Thus, we designed a set of experiments to test whether the sequence of exposure of mice to the same total amounts of ovalbumin aerosol and ozone affected the observed response of the animals.

Clearly, there are an almost infinite number of combinations and permutations of animal models, duration and concentration of ozone exposure and ovalbumin (or other antigen) exposure, and sequence of exposure to the ozone and ovalbumin aerosols possible. Comparisons between studies using different animal models, exposure regimens, and assays for effect are often contradictory. In the present study, we systematically tried to examine the interactions between ozone and ovalbumin exposure under several different experimental conditions in a single, well-controlled animal model of ovalbumin exposure that we had previously characterized thoroughly (Kenyon et al., 2003). We were especially interested in examining different sequences of exposure (ozone before, during, and after ovalbumin aerosol challenge) and two different concentrations of ozone (0.2 and 0.5 ppm). These ozone concentrations were chosen to model peak hourly ambient concentrations actually observed in summer months in the South Coast Air Basin of California and in polluted cities in Latin America such as Santiago, Chile, and Mexico City (0.2 ppm, the action level for a Stage 1 alert), and the amount that would precipitate a stage 3 smog alert, 0.5 ppm. We have recently observed that there is a significant increase in collagen content, which can be localized to the airway compartment, in the lungs of previously sensitized BALB/c mice exposed to ovalbumin aerosol for 4 wk at the concentration we used in the present study (Kenyon et al., 2003). We have also

observed a characteristic temporal pattern of inflammatory cell recruitment to the lung, as measured by differential cell counts of lavage fluid, and increased airway reactivity to methacholine challenge in mice exposed to ovalbumin aerosol (Kenyon et al., 2003). Thus, for the present study, mice were exposed to ovalbumin for 4 wk either before or after 2 wk of exposure to ozone, or to ovalbumin aerosol for 6 wk with concurrent exposure to ozone during wk 1–6, 3–6, or 2, 4, and 6. We analyzed (1) lung inflammation, as measured by quantitative evaluation of cells recovered by lung lavage and by histologic evaluation, and (2) alterations in lung structure (airway fibrosis), as measured by quantitative biochemical analysis of microdissected airways.

METHODS

Animals

Specific-pathogen-free BALB/c mice aged about 6 wk (16–20 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed until used in filtered Bio-Clean facilities in the Animal Resources Center at our facility on a 12-h light, 12-h dark cycle. The mice were provided standard feed (Purina rat chow) and water ad libitum for several days prior to any experimental procedures to acclimatize to their new surroundings. All procedures were performed under an IUCAC-approved protocol.

Procedures for sensitization and exposure of mice to ovalbumin aerosol have been described in detail elsewhere (Kenyon et al., 2003). Briefly, mice were systemically sensitized to ovalbumin by the intraperitoneal (ip) route. Two ip injections were administered, on days 1 and 15 of an experiment, of 10 $\mu\text{g}/0.1$ ml chicken egg albumin (ovalbumin, grade V, $\geq 98\%$ pure, Sigma, St. Louis, MO). Exposures to ovalbumin aerosol, 10 ml of a 10-mg/ml (1%) solution, were begun on day 28 after the first ip injection of ovalbumin. Thus, for Experiment 1, mice were exposed to ozone during the second 2 wk of the sensitization period. Mice were exposed for up to 60 min 3 times per week for the duration of a given experiment.

Methods for exposure of mice to ozone have been described in detail elsewhere (Kenyon et al., 2002). Briefly, mice were exposed for 8 h per night (midnight to 8:00 a.m.) to either 0.2 ppm ($400 \mu\text{g}/\text{m}^3$) or 0.5 ppm ($1000 \mu\text{g}/\text{m}^3$) of ozone. This 8 h/night protocol gives a response similar to exposure to the same total dose of ozone for 24 h (Gelzleichter et al., 1992). Four independent experiments with separate durations of exposure and analyses were performed, as illustrated in Figure 1. A 2-wk ozone exposure preceded 4 wk of ovalbumin aerosol exposure in the first experiment (Experiment 1), and followed the ovalbumin exposure in the second experiment (Experiment 2). Ozone exposures were timed so as to maintain the initiation of ovalbumin aerosol exposure on day 28 after the initiation of systemic sensitization. In the third and fourth experiments (Experiments 3 and 4), ovalbumin aerosol was administered for 6 wk with simultaneous exposure to ozone (6 wk) or intermittent exposure to ozone during wk 2, 4, and 6 or during wk 3–6. Actual

Experiment #1

0-----1-----2-----3-----4-----5-----6 Weeks
 I-----ovalbumin aerosol exposure-----I
 I----0.2 or 0.5 ppm ozone---I

Experiment #2

0-----1-----2-----3-----4-----5-----6 Weeks
 I-----ovalbumin aerosol exposure-----I
 I-----0.2 or 0.5 ppm ozone---I

Experiment #3

0-----1-----2-----3-----4-----5-----6 Weeks
 I-----ovalbumin aerosol exposure-----I
 I-----0.2 ppm of ozone or 0.5 ppm of ozone -----I

Experiment #4

0-----1-----2-----3-----4-----5-----6 Weeks
 I-----ovalbumin aerosol exposure-----I
 (OR) I-----ozone-----I I-----0.2 or 0.5 ppm ozone-----I I-----ozone---I

FIG. 1. Exposure protocols used in experiments 1–4 presented in this article.

measured exposure concentrations are given in Table 1. Control animals were treated identically to those ozone-exposed and/or ovalbumin exposed, except the exposure chambers contained only filtered air.

Lung Isolation and Fixation

Mice were killed with an overdose of pentobarbital/dilantin one hour after cessation of the last aerosol exposure, their tracheas were cannulated, and their lungs were lavaged twice with 1-ml portions of phosphate-buffered saline, pH 7.6 (PBS). The pooled lavage fluid was centrifuged in a bench-top unit at 1200 rpm for 10 min and the resulting pellet was again suspended in PBS. One hundred microliters of this suspension was used to prepare slides, which were stained with Diff-Quick (International Reagent Corp., Kobe, Japan), using a cytocentrifuge. Lungs were fixed for histological evaluation at 30 cm pressure with 1% paraformaldehyde for at least 24 h, then either processed for light microscopy in paraffin or stored in 70% ethanol for dissection and analysis of airways. Other lung preparations were dissected without fixation to prepare airways, which were frozen and stored at -80°C until used for extraction of RNA for polymerase chain reaction (PCR) analysis.

Tissue Staining and Evaluation of Fibrosis

After fixation, the right lung was placed in 70% ethanol and prepared for paraffin embedding in a standard fashion. Lung sections of 5 μm thickness were made with special attention to cutting through the larger lobar bronchi in parallel. Sections were then dried in a standard fashion and prepared for staining. Lung sections were stained with either hematoxylin and eosin (H&E) or PAS-Schiff stains for analysis by light microscopy using 400 $\times$ magnification.

Preparation of Airway Homogenates for Assay

Lobar bronchi and daughter airways were dissected from either the right lung or both lungs under a dissecting microscope.

Lungs were bluntly dissected using two sets of forceps to obtain a preparation of the larger airways separated from adhering parenchyma and attached to the remaining stub of trachea after cannulation. The tracheal stub was discarded prior to assay. The dissected airways were stored at 4°C in 70% ethanol until assayed (fixed lungs) or snap frozen on dry ice and stored at -80°C . For hydroxyproline and total protein assays, half of the airway preparation (generally the right lung airways minus the right upper lobe, which was used for histology) was used. The airway was homogenized in water with a Branson sonifier 250 (VWR Scientific, San Francisco, CA; duty cycle 40, output control 4) until the tissue was completely homogenized.

Protein Assay

The Micro BCA protein assay reagent kit (Pierce, Rockford, IL) was used, following the manufacturer's instructions. Standard curves using bovine serum albumin were linear between 0 and 20 μg .

Hydroxyproline Assay

The colorimetric method of Woessner (1961) was used. Aliquots of 50 and 100 μL of the neutralized hydrolysate (6 *N* HCl, 24 h, 121°C) were routinely assayed, but smaller and larger aliquots were used when necessary to adjust samples to add a constant amount of protein to each vial for hydrolysis. Standard curves were linear between 0 and 32 μg hydroxyproline.

PCR Analysis of Airway Tissue

These methods have been described in detail elsewhere (Kenyon et al., 2003). RNA was isolated by extraction into Trizol following the manufacturer's instructions. Quantitative PCR assay was performed with gene-specific double-labeled fluorescent probes and sets of specific primers in an ABI PRISM 7700 Sequence detection system (PE Applied Bio-systems). Each primer and probe was used at a final concentration of 1000 and 200 nmol/L, respectively. Reverse transcriptase reactions were incubated at 50°C for 30 min, and after inactivation of reverse transcriptase at 95°C for 12 min, 45 cycles of amplification were carried out with denaturation at 95°C for 15 s each and both annealing and extension at 60°C for 1 min each. To compensate for differences in cell number and/or RNA recovery, the copy number of each of the collagen mRNAs was determined relative to β -actin mRNA determined simultaneously.

Statistical Analysis of Data

Instat 2.0 (GraphPad Software, San Diego, CA) was used for data analysis. For parametric analysis of data, a *t*-test with appropriate correction for multiple comparisons and, where appropriate, unequal standard deviations between groups was used.

RESULTS

We performed experiments with four different protocols: (1) ozone preceding or (2) ozone following exposure to ovalbumin

TABLE 1
Differential cell counts in lung lavage from mice exposed to ovalbumin $\pm$ ozone

Group	Total cells ($\times 10^{-3}$)	Eosinophils (%)	Macrophages (%)	Lymphocyte (%)	PMNs (%)
Air controls (20)	223 $\pm$ 29	0.1 $\pm$ 0.1	98 $\pm$ 1	0 $\pm$ 0	0.6 $\pm$ 0.5
Experiment 1					
OVA (3)	311 $\pm$ 59 ^a	9.0 $\pm$ 3.0 ^a	38.0 $\pm$ 5.7 ^a	42.0 $\pm$ 1.5 ^a	1.4 $\pm$ 0.5
0.20 ppm O ₃ (4)	266 $\pm$ 14	0	91.2 $\pm$ 2.4	7.8 $\pm$ 1.9 ^a	1.0 $\pm$ 0.6
OVA + O ₃ (4)	291 $\pm$ 19 ^a	0.9 $\pm$ 0.4	89.5 $\pm$ 1.8 ^a	8.0 $\pm$ 0.9 ^a	0 $\pm$ 0
Experiment 2					
OVA (4)	282 $\pm$ 36 ^a	14.0 $\pm$ 3.0 ^a	73.5 $\pm$ 3.2 ^a	11.0 $\pm$ 4.6 ^a	1.2 $\pm$ 0.4
0.20 ppm O ₃ (4)	297 $\pm$ 56	0	91.0 $\pm$ 4.5	1.8 $\pm$ 0.9	1.6 $\pm$ 1.2
OVA + 0.2 ppm O ₃ (6)	310 $\pm$ 27 ^a	3.3 $\pm$ 0.8 ^a	51.2 $\pm$ 2.0 ^a	38.8 $\pm$ 1.6 ^a	1.6 $\pm$ 0.7
OVA + 0.5 ppm O ₃ (6)	342 $\pm$ 72 ^a	5.8 $\pm$ 1.5 ^a	51.0 $\pm$ 3.7 ^a	39.7 $\pm$ 2.4 ^a	4.0 $\pm$ 0.7 ^a
Experiment 3					
OVA (4)	652 $\pm$ 146 ^a	14.0 $\pm$ 3.1 ^a	73.5 $\pm$ 3.2 ^a	11.0 $\pm$ 4.6 ^a	1.2 $\pm$ 0.4
0.2 ppm O ₃ (4)	290 $\pm$ 10	2.2 $\pm$ 1.1	94.5 $\pm$ 0.5	0.8 $\pm$ 0.5	2.5 $\pm$ 1.2
OVA + 0.2 ppm O ₃ (4)	338 $\pm$ 52 ^{a,b}	4.4 $\pm$ 1.1 ^a	92.0 $\pm$ 2.1	3.2 $\pm$ 1.4 ^a	0.3 $\pm$ 0.2
0.5 ppm O ₃ (3)	373 $\pm$ 39	4.0 $\pm$ 1.2	90.7 $\pm$ 4.9	1.3 $\pm$ 0.3	4.2 $\pm$ 3.4
OVA + 0.5 ppm O ₃ (3)	262 $\pm$ 29 ^b	18.7 $\pm$ 5.8 ^a	75.7 $\pm$ 4.3 ^a	4.3 $\pm$ 1.7 ^a	1.2 $\pm$ 1.1
Experiment 4					
0.2 ppm O ₃ , wk 3–6 (4)	9 $\pm$ 2	2.9 $\pm$ 0.6	94.0 $\pm$ 1.4	1.2 $\pm$ 0.2	2.2 $\pm$ 1.5
OVA + 0.2 ppm O ₃ , wk 3–6 (3)	63 $\pm$ 25 ^{a,b}	20 $\pm$ 7 ^a	76 $\pm$ 6 ^a	3 $\pm$ 1	0.1 $\pm$ 0.1
OVA + 0.5 ppm O ₃ , wk 3–6 (4)	48 $\pm$ 6.6 ^{a,b}	12 $\pm$ 4 ^a	80 $\pm$ 5 ^a	7 $\pm$ 2 ^a	1.2 $\pm$ 0.7

Note. Data are presented as mean value $\pm$ SEM, with *n*, the number of mice, given in parentheses and the total number of weeks exposed to ovalbumin (3 exposures per week) indicated in Fig. 1. The data for total cells are the actual number counted on the slide used for determining the cell differential, while the data for differential cell types are given as percentages of the total cell population for the specific cell types enumerated: macrophages, lymphocytes, neutrophils (PMNs), and eosinophils. The sum of the individual cell types may not exactly equal 100% due to rounding errors and the presence of sloughed epithelial cells in the lavage fluid. However, more than 95% of the cells actually counted in a field were assigned to one or another category of the specific cell types indicated in the table (i.e., "other" cells always comprised less than 5% of the total cells counted). Ozone concentrations (mean ppm $\pm$ 1SD) measured in the chambers, with *n*, the number of determinations, given in parentheses were as follows: Experiment 1: 0.201 $\pm$ 0.017 (826), 0.503 $\pm$ 0.020 (827); Experiment 2: 0.205 $\pm$ 0.014 (826), 0.501 $\pm$ 0.018 (823); Experiment 3 and 4: 0.201 $\pm$ 0.010 (4963), 0.501 $\pm$ 0.013 (4843).

^aSignificantly different from the air control group.

^bSignificantly different from the ovalbumin aerosol alone (OVA) group.

aerosol, (3) ozone simultaneously with ovalbumin, and (4) ozone intermittently with ovalbumin aerosol.

Experiment 1, Ozone Exposure for 2 wk Followed by Ovalbumin Aerosol for 4 wk

The normal lung lavage from a healthy mouse contains more than 90% alveolar macrophages (Hamada et al., 2000), and our observations were consistent with this finding. Control mice exposed only to filtered air contained 223,200 $\pm$ 28,800 total cells per lavage sample, of which 98 $\pm$ 1% were pulmonary alveolar macrophages. Over a period of 4 wk (12 exposures to ovalbumin aerosol), the total cell number recovered by lavage from these BALB/c mice significantly increased by about 1.5-fold to 311,400 $\pm$ 59,400 (Figure 2A). Of these cells, 42 $\pm$ 2% were lymphocytes, 38 $\pm$ 6% were macrophages, and 9 $\pm$ 3% were eosinophils. There was no significant difference between the total cell number in the lavage fluid from mice exposed to ovalbumin and in those exposed to the combination of ovalbumin

and 0.2 ppm ozone. However, the cell differentials were significantly different when mice were exposed to ovalbumin as compared to the findings when mice were exposed to the combination of ovalbumin and ozone. For the combined exposure group we found (values for ozone alone are given in parentheses) the following: 8 $\pm$ 1% lymphocytes (8 $\pm$ 2%), 90 $\pm$ 2% macrophages (91 $\pm$ 2%), and 1 $\pm$ 0.4% eosinophils (0%).

Collagen content of microdissected airways was analyzed from the exposed mice and from their age-matched control mice exposed only to filtered air. Figure 3A illustrates the airway collagen content in mice exposed to ovalbumin aerosol $\pm$ 0.2 or 0.5 ppm ozone and their appropriate controls. Control animals exposed only to filtered air had 13 $\pm$ 1.2 μ g hydroxyproline (*n* = 14) in their airways. There was a significant increase, to 21.8 $\pm$ 3.2 μ g hydroxyproline in the collagen content of the airways from the mice exposed to ovalbumin. There was also a significant increase (*p* = .049) in the airway collagen content of the mice exposed to the combination of ovalbumin and

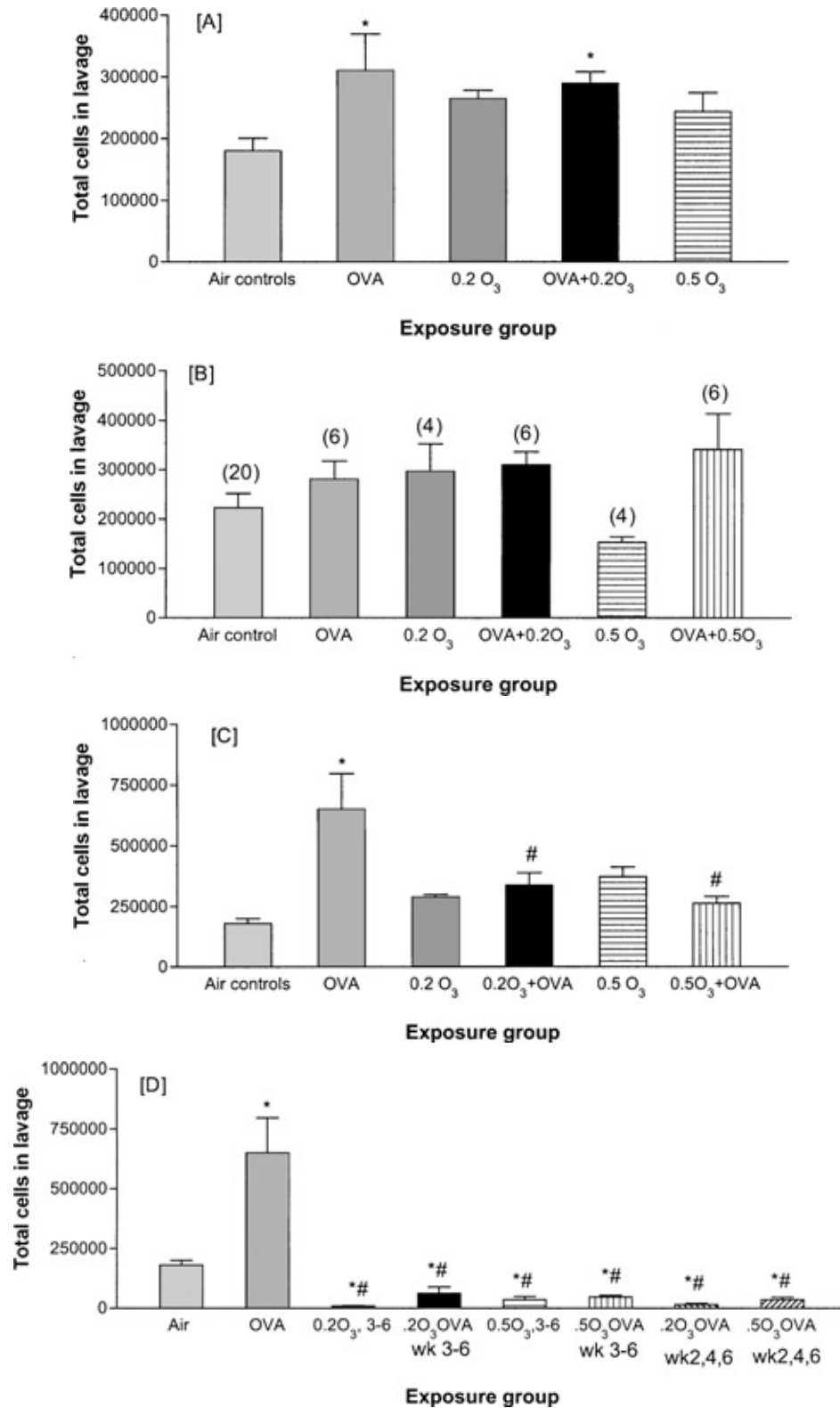


FIG. 2. Total cells recovered by lung lavage from mice exposed to ovalbumin aerosols with and without additional exposure to ozone. Data are mean values $\pm$ SEM, with n , the number of mice examined in each group, in parentheses; 5 fields ($200\times$ magnification) were counted from each of the cytocentrifuge slides. (A) Ozone exposure for 2 wk followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk followed by ozone exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and ozone for 6 wk. (D) Exposure to ovalbumin for 6 wk, with intermittent exposure to ozone during wk 3–6 (4 wk total) or wk 2, 4, and 6 (3 wk total). Asterisk indicates, significantly different from the air control group; #, significantly different from the ovalbumin aerosol alone (OVA) group.

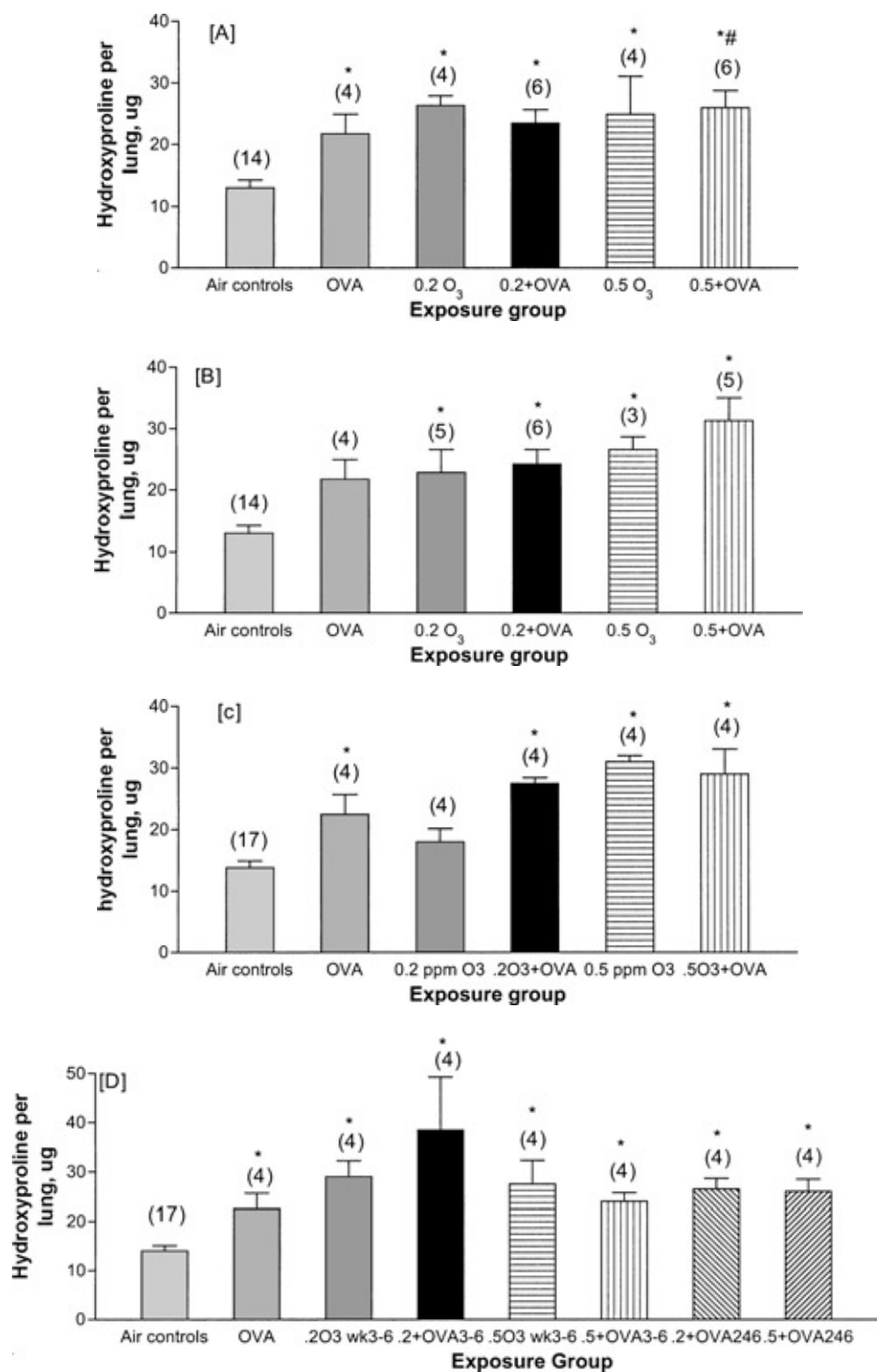


FIG. 3. Airway collagen content from mice exposed to ovalbumin aerosol three times per week for the indicated number of weeks, with or without additional exposure to ozone. Aliquots of microdissected airways were hydrolyzed and analyzed by a colorimetric method for their content of 4-hydroxyproline. Data are presented as micrograms of 4-hydroxyproline per right lung $\pm$ SEM for lungs from the number of mice indicated in parentheses. (A) Ozone exposure for 2 wk, followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk, followed by ozone exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and ozone for 6 wk. (D) Exposure to ovalbumin for 6 wk, with intermittent exposure to ozone during wk 3–6 (4 wk total) or wk 2, 4, and 6 (3 wk total). Asterisk, significantly different from the air control group; #, significantly different from the ovalbumin aerosol alone (OVA) group.

0.5 ppm ozone as compared with mice exposed to ovalbumin alone.

Experiment 2, Ovalbumin Aerosol for 4 wk Followed by Ozone Exposure for 2 wk

In our second set of experiments, we reversed the order of exposure so that the mice received 4 wk of exposure to ovalbumin prior to exposure to ozone for 2 wk. Because of this difference in experimental design, the group exposed to ovalbumin alone had a recovery period of 2 wk breathing filtered air prior to being killed and analyzed. In this experiment (Figure 2B) we found values comparable to the first experiment, $223,200 \pm 29,000$ cells per lavage sample in the control animals exposed only to filtered air, and $281,700 \pm 36,000$ cells in the group exposed to ovalbumin alone, a value that was not significantly different from the controls. In the latter group, $11 \pm 5\%$ were lymphocytes, $74 \pm 3\%$ were macrophages, and $14 \pm 3\%$ were eosinophils. There was no significant difference between the total cell number in the lavage fluid from mice exposed to ovalbumin and those exposed to the combination of ovalbumin and 0.2 ppm ozone. However, the cell differentials were significantly different between mice exposed to ovalbumin and mice exposed to the combination of ovalbumin and 0.2 ppm ozone. For the combined exposure group we found (values for ozone alone are given in parentheses) the following: $39 \pm 2\%$ lymphocytes ($2 \pm 0.9\%$), $51 \pm 2\%$ macrophages ($91 \pm 4\%$), and $3 \pm 0.8\%$ eosinophils (0%). When mice exposed to a combination of ovalbumin and 0.5 ppm ozone (or to 0.5 ppm ozone alone) were examined in this experiment, we found an essentially identical cell differential, $40 \pm 2\%$ lymphocytes ($3 \pm 1\%$), $51 \pm 4\%$ macrophages ($96 \pm 1\%$), and $6 \pm 2\%$ eosinophils (0%).

There was a significant increase in the airway collagen content in the mice exposed to ovalbumin as compared with those exposed only to air, consistent with what we had observed in our first experiment (Figure 3B). The apparent differences in airway collagen content between the mice exposed to ovalbumin alone and the mice exposed to ovalbumin combined with ozone were not significant.

Experiment 3, Ovalbumin Aerosol for 6 wk Concurrently With Ozone Exposure

In our third set of experiments, mice were exposed concurrently to ozone and ovalbumin. In this experiment (Figure 2C) we found $261,900 \pm 117,900$ cells per lavage sample in the control animals exposed to air, and $651,600 \pm 145,800$ in the group exposed for 6 wk to ovalbumin alone, a value that was significantly ($p = .0009$) greater than the controls. Of these cells, $11 \pm 5\%$ were lymphocytes, $74 \pm 3\%$ were macrophages, and $14 \pm 3\%$ were eosinophils. There were significantly more total cells in the lavage fluid from mice exposed to ovalbumin than from those exposed to the combination of ovalbumin and 0.2 ppm ozone ($p = .0444$). In addition, the cell differentials were significantly different between mice exposed to ovalbu-

min and mice exposed to the combination of ovalbumin and 0.2 ppm ozone. For the combined exposure to ovalbumin and 0.2 ppm ozone for 6 wk, we found $3 \pm 1\%$ were lymphocytes, $92 \pm 2\%$ were macrophages, and $4 \pm 1\%$ were eosinophils. Mice exposed to 0.2 or 0.5 ppm ozone alone for 6 wk had $94.5 \pm 0.5\%$ and $91 \pm 5\%$ macrophages in their lavage fluid, respectively. For combined exposure to ovalbumin and 0.5 ppm ozone for 6 wk we found $4 \pm 2\%$ lymphocytes, $94 \pm 1\%$ macrophages, and $3 \pm 0.6\%$ eosinophils, values not significantly different than those found for mice exposed to ovalbumin and 0.2 ppm ozone.

Exposure of sensitized mice to ovalbumin aerosol gave rise to changes in the lungs that were consistent with those previously observed by others. We have previously reported evidence of airway inflammation, mucous-cell hyperplasia and hypertrophy, and marked airway submucosal fibrosis in BALB/c mice exposed to ovalbumin aerosol as the lesions evolved over time (Kenyon et al., 2003). We examined the number of goblet cells per field (as an indicator of mucous cell hyperplasia and hypertrophy) by PAS staining of sections of lung that contained clearly demarcated large bronchi. We found no goblet cells in airways from control animals that had breathed only room air. We also found no goblet cells in the airways from mice exposed to either 0.2 or 0.5 ppm ozone for 6 wk, or for wk 3–6 of a 6-wk exposure interval (see later discussion). Mice exposed to ovalbumin alone had $24.4 \pm 1.6\%$ goblet cells per 6 high-power fields evaluated (Table 2), while the animals exposed to ovalbumin + 0.2 ppm ozone for 6 wk had a significantly higher ($p = .0205$) percentage of goblet cells, 43.2 ± 2.2 .

TABLE 2
Goblet cell counts in conducting airways of mice exposed to ovalbumin $\pm$ ozone

Exposure group	Goblet cells in airways (%)
Air	0 ± 0
Ovalbumin $\times$ 6 wk	24.4 ± 1.6^a
Ovalbumin + 0.2 ppm ozone $\times$ 6 wk	$43.2 \pm 2.2^{a,b}$
0.2 ppm ozone $\times$ 6 wk	0 ± 0
0.5 ppm ozone $\times$ 6 wk	0 ± 0
Ovalbumin + 0.5 ppm ozone $\times$ 6 wk	16.4 ± 2.2^a
0.2 ppm ozone wk 3–6	0 ± 0
0.5 ppm ozone wk 3–6	0 ± 0
Ovalbumin + 0.2 ppm ozone wk 3–6	$33.5 \pm 2.5^{a,b}$
Ovalbumin + 0.5 ppm ozone wk 3–6	22.2 ± 2.8^a
Ovalbumin + 0.2 ppm ozone wk 2, 4, 6	18.5 ± 1.5^a
Ovalbumin + 0.5 ppm ozone wk 2, 4, 6	21.8 ± 0.8^a

Note. Data are presented as mean value $\pm$ SEM with the total number of weeks exposed to ovalbumin (3 exposures per week) indicated. The data for total cells are the actual number of goblet cells counted in 6 microscope fields, divided by the total cell number in the 6 fields.

^aSignificantly different from the air control group.

^bSignificantly different from the ovalbumin aerosol alone (OVA) group.

Figure 3C illustrates the collagen content of isolated airway tissue from mice exposed to ozone and ovalbumin simultaneously. We found a significant increase (63%) in the amount of collagen in the airways of the animals exposed to ovalbumin as compared to the sensitized control animals breathing filtered air, a finding consistent with the earlier experiments. There was no significant difference in airway collagen content between the mice exposed to 0.2 ppm ozone and those exposed to filtered air. Airways from mice exposed to 0.2 ppm ozone + ovalbumin contained significantly more collagen than those from mice exposed only to 0.2 ppm ozone; however, the apparent increase in airway collagen content as compared with mice exposed to ovalbumin alone seen in Figure 3C was not significant. The airway collagen content in mice exposed to 0.5 ppm ozone alone was significantly higher than in the animals exposed to air, while there was no difference between airway collagen content in mice exposed to 0.5 ppm ozone alone or to 0.5 ppm ozone + ovalbumin.

Experiment 4, Ovalbumin Aerosol for 6 wk With Intermittent Ozone Exposure

Several permutations of continuous exposure to ovalbumin with intermittent exposure to ozone were also examined. Animals were exposed to ovalbumin alone for 2 wk and to the combination of 0.2 or 0.5 ppm ozone and ovalbumin for the remaining 4 wk of a 6-wk exposure. Other mice were exposed to ovalbumin alone for 6 wk and to either 0.2 or 0.5 ppm ozone and ovalbumin during wk 2, 4, and 6 of a 6-wk exposure. Control animals received the appropriate concentration of ozone with air instead of ovalbumin for each of these protocols. Total cell counts for lavages from these animals are shown in Figure 2D. Differential cell counts for lavages of some of these animals are presented in Table 1. The profound decrease in total lavagable cells associated with these intermittent exposure regimens is particularly striking. Exposure of mice to ovalbumin for 6 wk with concurrent exposure to either 0.2 or 0.5 ppm ozone during the last 4 wk of exposure resulted in a reduction in total cells recovered by lavage of about 90%. The relative percentages of eosinophils, lymphocytes, and macrophages were not changed as compared with the cell differentials in the mice exposed only to ovalbumin; only the total cell number was decreased. Also noteworthy is the striking decrease in the total number of cells recovered by lavage from the mice exposed only to 0.2 ppm ozone for wk 3–6 of a 6-wk exposure. It should be noted that these mice had been sensitized intraperitoneally with ovalbumin even though they were not exposed to ovalbumin aerosol. Two explanations of this observation seem apparent. Either the cells were not in the airways (either dead, retained within the tissue, or not originally recruited to the airway), or they were “sticky” and were retained in the airway lumen rather than being removed by lavage. We examined both of these possibilities by histological evaluation of lung sections.

Hematoxylin and eosin (H&E) stained sections of airways were examined for the presence of eosinophils, and 10 representative fields per lung section were counted from repre-

sentative animals from each of the groups in our third experiment, simultaneous exposure to ozone and ovalbumin aerosol. We saw no evidence for accumulation of eosinophils (or any other kind of leukocytes) on the luminal side of the airways, ruling out the possibility that “sticky” cells had resisted lavage by adhering to the airway epithelial surface. We enumerated eosinophils under the airways (in and adjacent to the submucosa). We found 1.5 ± 0.5 eosinophils per 10 fields ($400\times$) in the sensitized animals exposed to air, 17 ± 0 in those exposed to ovalbumin aerosol alone, and 8 ± 0 and 2.5 ± 1.5 in those exposed to 0.2 or 0.5 ppm ozone alone, respectively. Values for the mice exposed to the various combinations of ovalbumin and ozone studied ranged from 18.5 ± 0.5 to 27 ± 0 , consistent with an additive (or more than additive) response. It is impossible to equate numbers of eosinophils recovered by lavage and number of eosinophils counted in sections in any quantitative way. However, our results are consistent with at least some, and perhaps all, of the eosinophils “missing” from the lavage fluid in the combined exposures to combinations of ovalbumin and ozone during wk 3–6 or wk 2, 4, and 6 (Figure 2C) being “found” inside the airway tissue of these mice.

Figure 3D shows the collagen content of isolated airway tissue from mice exposed simultaneously to ovalbumin and to ozone on an intermittent schedule. We found a significant increase (63%) in the amount of collagen in the airways of the animals exposed to ovalbumin as compared to the sensitized control animals breathing filtered air; these were the same animals as shown in Figure 3C. There was also a significant increase in airway collagen content between the mice exposed to 0.2 ppm ozone and filtered air. Airways from mice exposed to 0.2 ppm ozone + ovalbumin apparently contained more collagen than those from mice exposed only to 0.2 ppm ozone or to ovalbumin alone; however, the apparent increase in airway collagen content as compared with mice exposed to ovalbumin alone or to 0.2 ppm ozone alone seen in Figure 3D was not significant. These observed results are consistent with an additive response to exposure to the mixture of ovalbumin and 0.2 ppm ozone during wk 3–6 of the exposure to ovalbumin. The airway collagen content in mice exposed to 0.5 ppm ozone alone during wk 3–6 of ovalbumin exposure was also significantly higher than in the animals exposed to air; however, there was no significant difference between airway collagen content in mice exposed to 0.5 ppm ozone alone or to 0.5 ppm ozone + ovalbumin using this exposure protocol. This apparent trend toward increased content of collagen in the airways of the animals exposed to ovalbumin and ozone during wk 3–6 (Figure 3D) may be related to the observation of increased numbers of eosinophils in the airway submucosal region of these animals.

Finally, we saw no difference between airway collagen content between mice exposed to either ovalbumin alone or 0.2 ppm ozone alone for a total of 3 wk and mice exposed to the combination of ovalbumin for 6 wk and 0.2 ppm ozone intermittently during wk 2, 4, and 6.

Reverse-transcription PCR (RT-PCR) was performed on isolated airways from several of the subgroups in the simultaneous exposure protocol. Relative ratios of the mRNAs for type I and type III collagens [$\alpha(I)1$ and $\alpha(III)1$] were not significantly different in mice exposed to 0.5 ppm ozone with and without ovalbumin aerosol for 6 wk or for wk 3–6 or for wk 2, 4, and 6. However, there was a significant difference ($p = .0486$, t -test, two-tailed) between the mice exposed to 0.5 ppm ozone alone, who had an $\alpha(I)1$: β -actin mRNA ratio of 3.7, and those exposed to 0.5 ppm ozone + ovalbumin for 6 wk, in which we found a ratio of 0.7, suggesting elevated collagen mRNA expression levels in the mice exposed to ozone alone relative to those exposed to the ozone simultaneously with ovalbumin aerosol.

DISCUSSION AND CONCLUSIONS

We have previously (Kenyon et al., 2003) demonstrated several changes in the lungs of sensitized BALB/*c* mice exposed to ovalbumin aerosols. We observed a sequence of cellular inflammatory changes in the lungs, airway fibrosis, increased airway collagen content, and increased airway reactivity. In the present study, we analyzed (1) cells recovered by lung lavage as an index of lung inflammation and (2) collagen content of microdissected airways as an index of airway fibrosis. We hypothesized that various temporal combinations of ozone and ovalbumin might give additive or antagonistic responses, depending on the assay chosen. We further hypothesized that such a duality of response might be due to the potential for the inflammatory response to be either antagonized or not affected by a combination of agents that elicit a Th-2-type response (ovalbumin) and a Th-1-type response (ozone).

We observed the same total number of cells in lavage fluid from animals exposed to ovalbumin alone or to the combinations of ovalbumin and ozone (at both 0.2 and 0.5 ppm ozone) regardless of whether the mice were exposed to ozone before or after the ovalbumin. However, simultaneous exposure to ovalbumin and ozone resulted in significant decreases in the total number of cells recovered by lavage. Values as low as 7% of the number found in mice exposed to ovalbumin aerosol alone (and as low as 18% of the values found in the mice exposed to air) were observed (Table 1).

More dramatic differences were found when the cell differentials in the lavage fluid from mice exposed to ovalbumin were compared with values from mice exposed to ovalbumin and ozone. We quantified the relative numbers of alveolar macrophages, neutrophils, lymphocytes, and eosinophils lavaged from the airways over time, as shown in Table 1. Exposure to ozone alone was associated with high percentages of macrophages in the lavage fluid, measurable numbers of neutrophils, and low percentages of lymphocytes and eosinophils, a response that we call "ozone-like." Exposure to ovalbumin aerosol gave the opposite result, a response we call "ovalbumin-like." Our most noteworthy observations were probably the following: (1) When ozone was given prior to ovalbumin (Exper-

iment 1), the cell differential in the combined exposure group seemed to be ozone-like, not ovalbumin-like. (2) When ozone was given after ovalbumin (Experiment 2), the cell differential in the combined exposure groups seemed intermediate between the two extremes (moderate percentage of macrophages, low percentage of eosinophils, high percentage of lymphocytes). (3) When ozone and ovalbumin were given simultaneously for 6 wk, the response was ozone-like at 0.2 ppm, and intermediate (high eosinophils, moderate macrophages) at 0.5 ppm. (4) The response was also intermediate (despite the drastically reduced total cell content) when ozone was only given during the last 4 wk of exposure to ovalbumin.

In Experiment 1, where animals were sacrificed immediately after the cessation of 4 wk of ovalbumin exposure, a high relative percentage (42%) of lymphocytes was seen in the ovalbumin alone exposure group, with only 8% lymphocytes in the combined exposure group. Airway collagen content was similar in both groups. In Experiment 2, where animals were sacrificed 2 wk after the cessation of their 4 wk of ovalbumin exposure, a relatively low (11%) percentage of lymphocytes was seen in the ovalbumin alone exposure group, with about 40% lymphocytes in the combined exposure groups. Airway collagen content trended toward higher values in the combined exposure groups.

In Experiments 3 and 4, where animals were sacrificed immediately after the cessation of 6 wk of ovalbumin exposure, low percentages of lymphocytes were observed in all exposure groups (11% in ovalbumin alone, 1–7% in the combined exposure groups). Airway collagen content was similar in all exposed groups. There was no obvious correlation across all four experiments between either percentage eosinophil or lymphocyte content of lung lavage fluid and airway collagen content. It is possible that exposure to ovalbumin plus intermittent ozone resulted in increased migration of eosinophils from the airway lumen to the airway submucosal space, and enhanced the fibrogenic response in the airways.

A central role has been postulated for T cells, especially Th-2 cells, in the pathogenesis of allergen-induced airway changes in mice (Kips et al., 2000). Th-2 cells accumulate in the airways in response to allergen challenge by inhalation. A sequence of subsequent pathophysiological events has been suggested (Renauld, 2001; Douwes et al., 2002) wherein lymphocytes release IL-5 and other Th-2-derived cytokines that attract eosinophils to the lung, and specifically to the airway mucosa. These eosinophils, in turn, are suggested to be one major source of transforming growth factor (TGF)- β (Ohno et al., 1996; Tanaka et al., 2001), which stimulates the differentiation of airway submucosal fibroblasts to myofibroblasts (Hashimoto et al., 2001). Myofibroblasts are thought to synthesize and secrete the collagen into the airway submucosal space that results in airway fibrosis (Zhang & Phan, 1996). Our results with sensitized BALB/*c* mice exposed to ovalbumin aerosol are consistent with this postulated pathway. We find large numbers of lymphocytes and eosinophils in lung lavage fluid after 2 wk of exposure to ovalbumin, and the elevated lymphocyte content persists with

continued exposure to ovalbumin in this model (Kenyon et al., 2003, and the present study).

BALB/c mice are known to readily mount a Th-2 cytokine response to challenge (Srikiatkachorn et al., 1999), and are appropriately referred to as "Th-2 high responder mice" (Neuhaus-Steinmetz et al., 2000). BALB/c mice may thus be viewed as a strain susceptible to ovalbumin aerosol. Other studies have suggested that ozone-induced lung inflammation and increased airway reactivity are dependent on a pathway involving TNF- α (Kleeberger et al., 1997; Cho et al., 2001). Since TNF- α is a quintessential component of the Th-1 response (Mosmann & Coffman, 1989), these findings suggest that ozone elicits a Th-1 response in the lung. It has been suggested by many investigators that an imbalance between Th-1 and Th-2 cytokine responses may underlie not only the pathogenesis of asthma, but also fibrosis in the lung and other tissues (Sime & O'Reilly, 2001). Therefore, we were interested in comparing both the inflammatory and the fibrogenic responses to ovalbumin aerosol that we observed in BALB/c mice to the same responses in mice exposed to combinations of ovalbumin and ozone. We used relatively low concentrations of ozone (0.2 and 0.5 ppm) in these experiments to try to examine responses at concentrations of ozone that might have environmental relevance. Somewhat paradoxically, Neuhaus-Steinmetz et al. (2000) reported that concurrent exposure to similar concentrations (0.25 ppm) of ozone caused a shift in the immune response to ovalbumin toward a Th-2 pattern in BALB/c mice, as measured by antibody titers, selected cytokine and leukotriene levels in lung lavage fluid, and airway hyperresponsiveness. However, ozone exposure patterns and details of sensitization to ovalbumin differed in their exposure protocols from ours. Since the Th-1 and Th-2 phenotypes are ultimately defined by cytokine profiles in the lung, future studies with fluorescent activated cell sorting or ELISA methods to better define cytokine profiles in the airways of mice exposed to mixtures of ovalbumin and ozone seem warranted.

In our previous studies, we have reported that BALB/c mice demonstrate significant increases in accumulation of airway collagen after 4 wk of exposure to ovalbumin aerosol under conditions identical to those used in the current study (Kenyon et al., 2003). We therefore measured the collagen content of microdissected airways from mice exposed to ovalbumin aerosol, with and without ozone. Consistent with our earlier observations, mice exposed to ovalbumin for 4 wk exhibited a significant increase in their airway collagen content (Figure 3A). There was no significant effect of 2 wk of exposure to ozone before or after the exposure to ovalbumin aerosol on airway collagen content. We also examined airway collagen content in mice exposed simultaneously to ozone and ovalbumin aerosol. In these experiments, the apparent increase in airway collagen content in mice exposed to ovalbumin aerosol was not significantly greater than in those exposed to air (Figure 3C). However, the values for the mice exposed to 0.5 ppm ozone alone, and to all of the combinations, were significantly greater than the air controls. The

RT-PCR data suggested that the majority of the newly synthesized collagen was type I, consistent with our earlier findings in mice exposed to ovalbumin aerosols without ozone (Kenyon et al., 2003). None of the combinations showed a significant increase as compared to ovalbumin exposure alone. These results are consistent with an additive response to combined simultaneous exposure to ovalbumin and ozone. Thus, by the quantitative evaluation of airway fibrosis, an important marker of lung structure, we conclude that exposure of BALB/c mice to 0.2 or 0.5 ppm ozone before, during, or after exposure to ovalbumin aerosol causes additive effects when airway collagen content is measured.

We examined one other aspect of airway remodeling in the lungs of the mice from Experiment 3, goblet-cell hyperplasia in the airway epithelium (Table 2). Mice exposed only to filtered air or to ozone alone had no detectable goblet cells. We found $24 \pm 2\%$ goblet cells in mice exposed to ovalbumin aerosol, $43 \pm 2\%$ in mice exposed to ovalbumin + 0.2 ppm ozone, and $34 \pm 2\%$ in mice exposed to ovalbumin + 0.2 ppm ozone during wk 3–6 of exposure. None of the other combined exposure groups differed significantly from mice exposed to ovalbumin aerosol alone. These results suggest increased goblet-cell hyperplasia in mice exposed to ovalbumin and 0.2 ppm ozone as compared to ovalbumin alone. However, when ozone concentration is increased to 0.5 ppm we do not see such an increase over the results with ovalbumin alone, suggesting that the simultaneous presence of ozone with ovalbumin might affect the inflammatory-cell populations in the airways and thus, alter cytokine signaling in the lungs in a concentration-dependent manner.

In summary, we had several goals in the present study. We wanted to compare both the lung inflammatory responses and the airway remodeling responses of BALB/c mice to ovalbumin aerosol alone with the responses to combinations of ovalbumin and ozone. Ovalbumin aerosol is known to provoke a Th-2 response in the lungs of experimental animals, while ozone elicits a Th-1 response in the lung. We hypothesized that ozone would antagonize the effects of ovalbumin exposure on the BALB/c strain, especially with regard to influencing the specific inflammatory cells accumulating in the airways during and after exposure. Our results are consistent with this hypothesis, especially with respect to the accumulation of inflammatory cells as evaluated by differential cell counts of whole lung lavage fluid, most notably with the simultaneous exposure protocols (Experiments 3 and 4). We found differences in cell differentials between animals exposed to ovalbumin aerosol alone and those exposed to ovalbumin plus ozone in all of these experiments. Significant differences in total cell number (as well as cell differentials) were observed only in the third and fourth experiments, when ovalbumin and ozone were administered simultaneously. There is an alternative explanation of our findings of decreased numbers of inflammatory cells in the lung lavage fluid from mice exposed to combinations of ovalbumin and ozone, especially the protocols using intermittent exposure to ozone in Experiment 4.

The decreased accumulation of inflammatory cells in the lung lavage fluid may be a consequence of increased migration of these cells into the airway submucosal space, which would not necessarily be interpreted as antagonism of ovalbumin effects by ozone. Airway remodeling, as evaluated either as goblet-cell hyperplasia or as airway fibrosis, showed additive or less than additive effects for most of the exposure conditions studied. There is a suggestion that simultaneous exposure to ovalbumin plus 0.2 ppm ozone might have elicited greater responses than exposure to the combination of ovalbumin plus 0.5 ppm ozone in some of these assays. Further studies are necessary to determine whether this is indeed the case.

REFERENCES

- Cho, H.-Y., Zhang, L.-Y., and Kleeberger, S. R. 2001. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor- α receptors. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280:L537-L546.
- Depuydt, P. O., Lambrecht, B. N., Joos, G. F., and Pauwels, R. A. 2002. Effect of ozone exposure on allergic sensitization and airway inflammation induced by dendritic cells. *Clin. Exp. Allergy* 32:391-396.
- Douwes, J., Gibson, P., Pekkanen, J., and Pearce, N. 2002. Non-eosinophilic asthma: Importance and possible mechanisms. *Thorax* 57:643-648.
- Drazen, J. M., and Beier, D. R. 1997. The genetics of air pollution. *Nature (Genet.)* 17:365-366.
- Gelzleichter, T. R., Witschi, H. P., and Last, J. A. 1992. Concentration-response relationships of rat lungs to exposure to oxidant air pollutants: A critical test of Haber's Law for ozone and nitrogen dioxide. *Toxicol. Appl. Pharmacol.* 112:73-80.
- Guth, D. J., Warren, D. L., and Last, J. A. 1986. Comparative sensitivity measurements of lung damage made by bronchoalveolar lavage after short-term exposure of rats to ozone. *Toxicology* 40:131-143.
- Hamada, K., Goldsmith, C., Goldman, A., and Kobzik, L. 2000. Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air pollutant aerosol. *Am. J. Respir. Crit. Care Med.* 161:1285-1293.
- Hashimoto, S., Gon, Y., Takeshita, I., Matsumoto, K., Maruoka, S., and Horie, T. 2001. Transforming growth factor- β_1 induces phenotypic modulation of human lung fibroblasts to myofibroblast through a c-jun-NH₂-terminal kinase-dependent pathway. *Am. J. Respir. Crit. Care Med.* 163:152-157.
- Hu, P. C., Miller, F. J., Daniels, M. J., Hatch, G. E., Graham, J. A., Gardner, D. E., and Selgrade, M. K. 1982. Protein accumulation in lung lavage fluid following ozone exposure. *Environ. Res.* 29:377-388.
- Kenyon, N. J., van der Vliet, A., Schock, B. C., Okamoto, T., McGrew, G. M., and Last, J. A. 2002. Susceptibility to ozone-induced acute lung injury in iNOS-deficient mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282:L540-545.
- Kenyon, N. J., Ward, R. W., and Last, J. A. 2003. Airway fibrosis in a mouse model of airway inflammation. *Toxicol. Appl. Pharmacol.* 186:90-100.
- Kips, J. C., Tournoy, K. G., and Pauwels, R. A. 2000. Gene knockout models of asthma. *Am. J. Respir. Crit. Care Med.* 162:S66-70.
- Kleeberger, S. R., Levitt, R. C., Zhang, L.-Y., Longphre, M., Harkema, J., Jedlicka, A., Eleff, S. M., DiSilvestre, D., and Holroyd, K. J. 1997. Linkage analysis of susceptibility to ozone-induced lung inflammation in mice. *Nature Genet.* 17:475-478.
- McConnell, R., Berhane, K., Gilliland, F., London, S. J., Islam, T., Gauderman, W. J., Avol, E., Margolis, H. G., and Peters, J. M. 2002. Asthma in exercising children exposed to ozone: A cohort study. *Lancet* 359:386-391.
- Mosmann, T. R., and Coffman, R. L. 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145-173.
- Neuhaus-Steinmetz, U., Uffhausen, F., Herz, U., and Renz, H. 2000. Priming of allergic immune responses by repeated ozone exposure in mice. *Am. J. Respir. Cell Mol. Biol.* 23:228-233.
- Ohno, I., Nitta, Y., Yamauchi, K., Hoshi, H., Honma, M., Woolley, K., O'Byrne, P., Tamura, G., Jordana, M., and Shirato, K. 1996. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 15:404-409.
- Renauld, J.-C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54:577-589.
- Schlesinger, R. B., Cohen, M., Gordon, T., Nadziejko, C., Zelikoff, J. T., Sisco, M., Regal, J. F., and Menache, M. G. 2002. Ozone-induced modulation of airway hyperresponsiveness in guinea pigs. *Res. Rep. Health. Effects Inst.* 109:1-40.
- Sime, P. J., and O'Reilly, K. M. A. 2001. Fibrosis of the lung and other tissues: New concepts in pathogenesis and treatment. *Clin. Immunol.* 99:308-319.
- Srikiatkachorn, A., Chang, W., and Braciale, T. J. 1999. Induction of Th-1 and Th-2 responses by respiratory syncytial virus attachment glycoprotein is epitope and major histocompatibility complex independent. *J. Virol.* 73:6590-6597.
- Stieb, D. M., Burnett, R. T., Beveridge, R. C., and Brook, J. R. 1996. Association between ozone and asthma emergency department visits in Saint John, New Brunswick, Canada. *Environ. Health Perspect.* 104:1354-1360.
- Sumitomo, M., Nishikawa, M., Fukuda, T., Kaneko, T., Ikeda, H., Suzuki, S., and Okubo, T. 1990. Effects of ozone exposure on experimental asthma in guinea pigs sensitized with ovalbumin through the airway. *Int. Arch. Allergy Appl. Immunol.* 93:139-147.
- Sun, J., Koto, H., and Chung, K. F. 1996. Interaction of ozone and allergen challenges on bronchial responsiveness and inflammation in sensitized guinea pigs. *Int. Arch. Allergy Immunol.* 112:191-195.
- Tanaka, H., Masuda, T., Tokuoka, S., Komai, M., Nagao, K., Takahashi, Y., and Nagai, H. 2001. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm. Res.* 50:616-624.
- Weisel, C. P., Cody, R. P., and Liroy, P. J. 1995. Relationship between summertime ambient ozone levels and emergency department visits for asthma in central New Jersey. *Environ. Health Perspect.* 103:97-102.
- Woessner, J. F., Jr. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* 93:440-447.
- Yamauchi, T., Shima, M., Kuwaki, T., Ando, M., Ohmichi, M., Fukuda, Y., and Adachi, M. 2002. Acute effects of ozone exposure on lung function in mice sensitized to ovalbumin. *Toxicology* 172:69-78.
- Zhang, H. Y., Gharaee-Kermani, M., Zhang, K., Karmiol, S., and Phan S. H. 1996. Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am. J. Pathol.* 148:527-537.