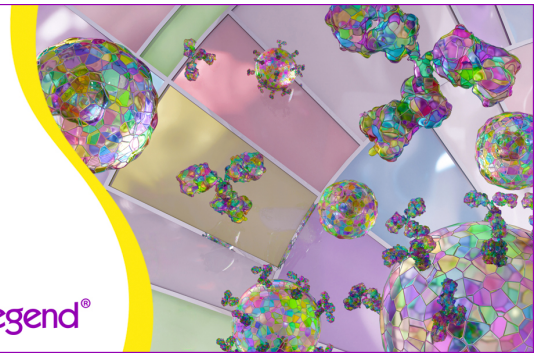


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# Secondhand Smoke Induces Allergic Sensitization in Mice<sup>1</sup>

Robert Rumold,\* Minna Jyrala<sup>†</sup>, and David Diaz-Sanchez<sup>2\*</sup>

Epidemiological studies have suggested increased prevalence of atopy in children of maternal smokers. Although secondhand smoke or environmental tobacco smoke (ETS) has been shown to augment allergic responses, its role in atopic sensitization is still controversial. We studied whether ETS could initiate a Th2 response and thus induce primary allergic sensitization. Mice were exposed for 10 consecutive days to either 1% aerosolized OVA, ETS (5 cigarettes), or both ETS and OVA. C57BL/6 mice receiving both ETS and OVA developed OVA-specific IgE and IgG1, 12, 14, and 25 days after the initial exposure, whereas those receiving OVA alone did not. Thirty days after the initial challenge (20 days after its completion), mice were re-exposed to OVA. Bronchoalveolar lavage performed 24 h later revealed an influx of eosinophils in the group initially challenged with both ETS and OVA, but not in those exposed to ETS alone or OVA alone. Increases in IL-5, GM-CSF, and IL-2 were observed in bronchoalveolar lavage from this OVA/ETS-exposed group, whereas IFN- $\gamma$  levels were significantly inhibited. These results suggest that ETS can induce allergic sensitization to a normally harmless Ag, and they may explain why secondhand smoke is a major risk factor for the development of allergy in children. *The Journal of Immunology*, 2001, 167: 4765–4770.

The worldwide prevalence and severity of allergic disease is increasing in the general population (1–3). Although this increase is probably the result of several factors, epidemiological studies have implicated both a decrease in childhood infections and an increase in environmental pollution as risk factors (reviewed in Ref. 4). Maternal smoking is often associated with allergic disease and increased skin test reactivity, serum IgE, and prevalence of eosinophilia in children. (5–9) However, other studies have failed to see such an association (10) and experimental data supporting these claims is scant. Although the role of pollutants in allergic inflammation has been extensively studied in many animal models, few studies have used these models to study the association between environmental tobacco smoke (ETS)<sup>3</sup> (3) (commonly referred to as “secondhand smoke”) and allergy/asthma. Seymour et al. (11) have shown that ETS can exacerbate allergic responses in mice previously sensitized i.p. to OVA before airway OVA exposure; i.e., that ETS can enhance secondary responses. However, a role of ETS in atopic sensitization is still unproven.

In this study we investigated whether ETS can induce sensitization to OVA (i.e., induce a primary response). In most murine models, repeated aerosolized exposure to protein Ag normally in-

duces tolerance. Although some studies have shown that certain exposure regimes can sensitize mice, in the absence of an adjuvant, to aerosolized OVA (normally an innocuous Ag), no eosinophilia or cytokine changes are observed in these animals. (12) Additionally, increases in Ab responses only occur in IgE high-responder strains of mice (e.g. BALB/c). Sensitization of IgE low-responder strains of mice (e.g. C57BL/6), without prior i.p. immunization, normally requires modulation of the immune system such as over-expression of GM-CSF (13).

We and others (4, 14) have previously shown the potential of particulate pollutants to alter immune function. Thus, the model airborne pollutant, diesel exhaust particles, can induce allergic sensitization in murine models (15–18) and in the human upper airways. (19) In this study we show that ETS can induce sensitization to OVA in both high and low IgE-responder strains of mice via the airway in a manner highly relevant to normal exposures. We demonstrate that not only did ETS induce Ag-specific IgE and IgG1 responses, but also it could promote cytokine changes and airway eosinophilia.

## Materials and Methods

### Animals

Female BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in the UCLA vivarium under specific pathogen-free conditions. The study was approved by the Chancellor’s Animal Research Committee in adherence to guidelines set forth by the National Institutes of Health. Mice were 6–8 wk of age at the onset of each experiment. Additionally, study animals were fed an egg-free chow to preclude them from any environmental exposure to OVA.

### Ag and ETS exposure

OVA (Grade V; Sigma-Aldrich, St. Louis, MO) exposure was used as the Ag in this study. Exposure was to a nebulized 1% (w/v) solution of OVA dissolved in PBS for 20 min following a 15-min chamber equilibration. Nebulization was achieved by the Schuco 2000 (Allied Health Care Products, St. Louis, MO) with a flow rate of 6 L/min at the nebulizer cup yielding particle sizes within 0.5–4.0  $\mu$ m. A control group was exposed to saline alone with no OVA in an identical fashion.

ETS exposure was achieved from the side-stream smoke from 1R4F cigarettes (University of Kentucky Tobacco and Health Research Institute, Lexington, KY). ETS is composed primarily (95%) of side-stream smoke (emitted from the burning zone) and also (4%) smolder stream smoke (emitted from the puffing zone) (20). The reference-filtered cigarette is known to contain 9.2 mg of tar and 0.8 mg of nicotine. (21) It was stored

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<sup>3</sup> Abbreviations used in this paper: ETS, environmental tobacco smoke; BAL, bronchoalveolar lavage; DEP, diesel exhaust particles; PAH, polyaromatic hydrocarbons.

in a sealed plastic bag at 4°C to hold proper moisture levels in the cigarettes. Fifteen minutes before use, they were immediately brought to room temperature and used in an RM G1 Borgwaldt smoking machine (Hamburg, Germany) set at one inhalation/55 s (where an inhalation/exhaust cycle is of a 5-s duration) to conform to Federal Trade Commission guidelines for generation of side-stream smoke. No mainstream smoke entered the system. ETS was mixed with saline administered as before through a nebulizer delivering an airflow of 6 L/min. This acted as a pressurizing agent carrying ETS into and out of the exposure chamber, and it diluted fatally anoxic concentrations of CO. Mice were challenged with the smoke from 5 cigarettes administered over the duration of 1 h.

Both ETS and OVA exposures were performed in a sealed system plexiglas exposure chamber (Ejay International, Glendora, CA) with an internal exposure area of 18 × 9 × 9 in. Chambers were cleaned between exposures to each agent to ensure no accidental contamination.

#### Exposure protocol

Groups of six BALB/c or C57BL/6 mice were used. Mice received exposures as detailed above to either: saline, 1% OVA for 20 min, 1 h ETS, or 1 h ETS followed by 1% OVA for 20 min. This procedure was repeated for 10 consecutive days. It is important to note that mice receiving either ETS alone or ETS plus OVA were placed together in the same chamber and were challenged simultaneously to ensure identical ETS exposure to both groups. Similarly, the second and fourth groups received simultaneous OVA exposure.

Thirty days after the initial challenge (20 days after its completion), mice were re-exposed to 1% OVA for 20 min. Bronchoalveolar lavage (BAL) was performed 24 h later by standard methods as previously described. (22)

#### Collection of BAL and blood

Bleeds were performed from the periorbital sinus of the eye under methoxyflurane anesthesia (Pitman-Moore, Mundelein, IL) before the initial exposure (day 1) and at several times subsequent to that. Sera were stored at -70°C until ready for use. BAL was spun at 300 × g for 20 min, and the supernatant was used for cytokine determination (see below). A total BAL cell count was performed using a hemacytometer. Cells were fixed onto slides following cytocentrifugation, and differential staining was then performed by Wright-Giemsa staining (Dade Behring, Newark, DE). Two hundred cells were counted under a microscope by two different investigators and the absolute numbers of each cell type were calculated.

#### Cytokine and Ig determination

Cytokine levels were determined in BAL fluid. Specifically, IL-2, IL-4, IL-5, IFN- $\gamma$ , and GM-CSF were detected using commercial ELISA kits consisting of paired Abs and standards (BD PharMingen, San Diego, CA) as per the manufacturer's instructions. Cytokine levels were measured from standard curves constructed from serial dilutions of the reference standard provided with each kit. The threshold of detection for IL-2 was 10 pg/ml, IL-4 was 4.1 pg/ml, IL-5 was 15 pg/ml, IFN- $\gamma$  was 5 pg/ml, and GM-CSF was 7 pg/ml.

Both total IgE and IgG1 were assayed by sandwich ELISA in the peripheral blood sera of the mice. Briefly, 96-well ELISA plates (Corning Glass, Corning, NY) were coated overnight with 5  $\mu$ g/ml Fc region, isotype-specific Abs (anti-IgG1 and anti-IgE; BD PharMingen). After washing and blocking with 1% BSA-PBS, sera samples were placed in wells overnight. The sera was removed from the plate and washed. Biotinylated, Fc-specific detecting Abs were added to the corresponding wells in question after being diluted 1/500 in a PBS-Tween buffer containing 0.1% bovine  $\gamma$  globulin and 0.5% BSA plus streptavidin alkaline phosphatase at 1/500. The detection mixture was allowed an incubation of 4 h at room temperature before detection with PNPP (10 mg/ml). The resultant color was read at 405 nm on a microplate reader (DPC Cirrus, Randolph, NJ). For a comparison standard, purified Abs (polyclonal IgG1 and IgE; PharMingen) were serially diluted and plated to establish a reference curve. OD readings were compared with these curves and were given appropriate values.

A similar method was used to quantify allergen-specific Abs. Plates were coated with the specific allergen used in the study; OVA at 50  $\mu$ g/ml in PBS. Reference curves for OVA-IgG1 were constructed using an OVA-specific IgG1 mAb (Sigma-Aldrich). The detection limit was 5  $\mu$ g/ml. Pooled sera from hyperimmunized BALB/c mice was obtained and the amount of OVA-IgE in the pool was given an arbitrary value of 100,000 U/ml. Reference curves for OVA-IgE were then constructed from serial dilutions from this pool, and the concentration of OVA-IgE in the test samples were estimated by comparison to this curve. Similarly, reference curves for OVA-IgG2a were constructed from LPS-immunized mice.

#### Statistical analysis

The Statview II computer package (Abacus Concepts, Berkeley, CA) for the Macintosh was used for all analysis. Comparisons of Ig levels at different times within a group were calculated using a paired *t* test. Comparisons of Ig and cytokine levels between groups were analyzed using the Mann-Whitney *U* test.

## Results

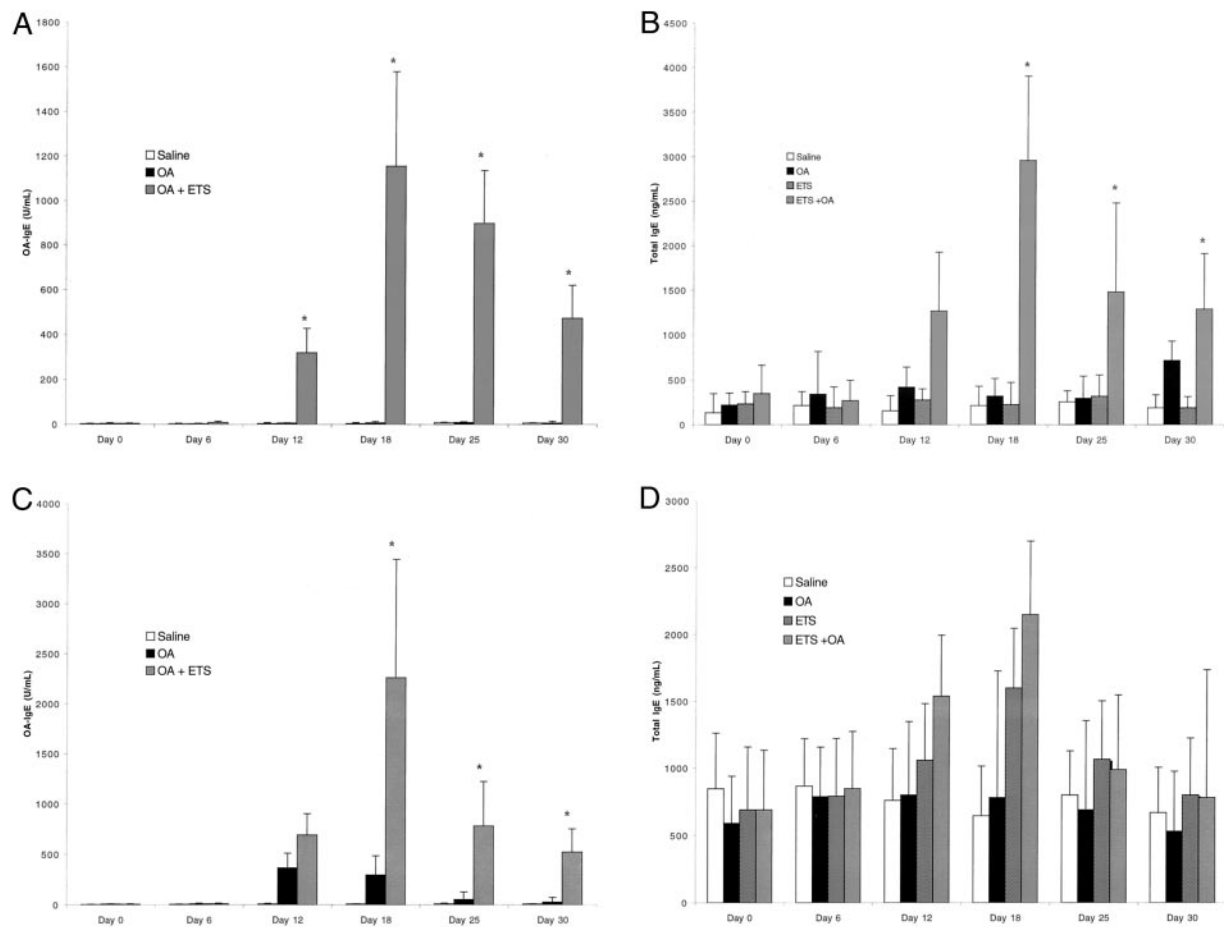
### Allergic Ab induction by OVA plus ETS

ETS induced sensitization to OVA in our murine model. When the low (C57BL/6) IgE-responder mice were exposed to aerosolized OVA alone for 10 days, no OVA-specific IgE was observed at any of the time points studied. In contrast, in the group exposed to ETS plus OVA for 10 days, Ag-specific IgE was apparent 12 days (day 12) after the initial exposure (mean = 318 ± 108 U/ml) and persisted until day 25 (Fig. 1A). Exposure to OVA alone did result in a significant increase on day 12 in the high responder BALB/c strain (Fig. 1B) compared with baseline, but this response was transient and levels were back to baseline by day 25. BALB/c mice exposed to both ETS/OVA made significantly higher OVA-IgE levels than those receiving OVA alone at day 18, and this response was still significantly above baseline levels at day 30. ETS also synergized with OVA to significantly elevate total serum IgE in the low responder C57BL/6 strain (Fig. 1C). Mice exposed to ETS or OVA alone did not exhibit any change in IgE levels, nor did the control group that was exposed to saline. In contrast, total IgE levels in the ETS/OVA groups were significantly elevated from baseline and were higher than in OVA-exposed animals at days 12, 18, and 25. There was no difference in total IgE levels between BALB/c mice exposed to either OVA alone or ETS/OVA (Fig. 1D).

In mice, IgG1 is also an "anaphylactic Ab." Similar to IgE levels, OVA-IgG1 could not be detected at any time in sera from C57BL/6 mice who had received aerosolized OVA alone. However, by day 12, OVA-IgG1 could be detected in mice receiving both ETS and OVA (Fig. 2A), and it remained elevated up to 30 days after initial exposure. OVA alone did induce OVA-IgG1 responses in BALB/c mice, but this response was significantly greater in the OVA/ETS group at all time points after day 12 (Fig. 2C). In contrast, ETS did not enhance production of a Th1-driven isotype, so that in both strains of mice, OVA-IgG2a levels were similar between OVA- and OVA/ETS-challenged animals (Fig. 2, B and D).

### Induction of eosinophilia upon rechallenge of ETS and OVA sensitized mice

The ability of ETS to induce eosinophilia was investigated in high- and low-responder mice. Mice were exposed to OVA, ETS, or both for 10 days as before, and then 20 days later (30 days after initial exposure), the mice were re-exposed to 1% OVA for 20 min. BAL fluid obtained 24 h after re-exposure was assessed for airway eosinophilia (Fig. 3). Confirming previous studies (12, 23), BALB/c mice exposed to OVA alone showed no significant changes in the cellular profile. Total eosinophil, neutrophil, and mononuclear cell counts were similar in mice groups exposed to saline, OVA, or ETS alone. However, exposure to the combination of OVA/ETS resulted in significant eosinophilia following OVA re-exposure (5.98 × 10<sup>5</sup> cells/ml vs 0 cells/ml saline control group, *p* < 0.01). This increase in eosinophil cell numbers was accompanied by an increase in total cell numbers in this OVA/ETS group. Similarly, in the C57BL/6 strain, only in the BAL from those mice previously exposed to both OVA/ETS was an accumulation of eosinophils detected. Unlike the BALB/c strain, this group was also characterized by significant increases in neutrophil



**FIGURE 1.** ETS induced IgE responses to OVA in low and high IgE responders. C57BL/6 (A and C) and BALB/c mice (B and D) were exposed to either saline, 1% OVA, ETS, or both OVA plus ETS for 10 days (see *Materials and Methods*). Bleeds were taken before exposure and 6, 12, 18, 25, and 30 days afterward. OVA-specific (A and B) and total serum (C and D) IgE was measured. \*,  $p < 0.01$  vs OVA-exposed group.

as well as total cell numbers, but this was less marked than the eosinophil response.

#### Cytokine induction by ETS

Airway eosinophilia and allergic Ab responses are normally associated with a Th2 cytokine milieu (24, 25). We therefore studied whether ETS exposure could cause a change in the cytokine profile in BAL obtained from C57BL/6 mice following re-exposure to OVA (Table I). In saline-exposed animals, re-exposure to OVA did not result in changes to any of the cytokines measured (IL-2, IL-4, IL-5, GM-CSF, or IFN- $\gamma$ ). Previous exposure to 10-day aerosolized OVA resulted in elevated IFN- $\gamma$  BAL levels upon OVA re-exposure, but no change was seen in other cytokines. In contrast, increases in IL-5, GM-CSF, and IL-2 were observed in BAL from mice with prior exposure to OVA/ETS. In addition, this group also had significantly reduced levels of IFN- $\gamma$  as compared with the OVA alone exposed group. It is noteworthy that in the ETS alone-exposed animals, compared with the saline control group, significantly increased levels of GM-CSF and IL-2 could be detected.

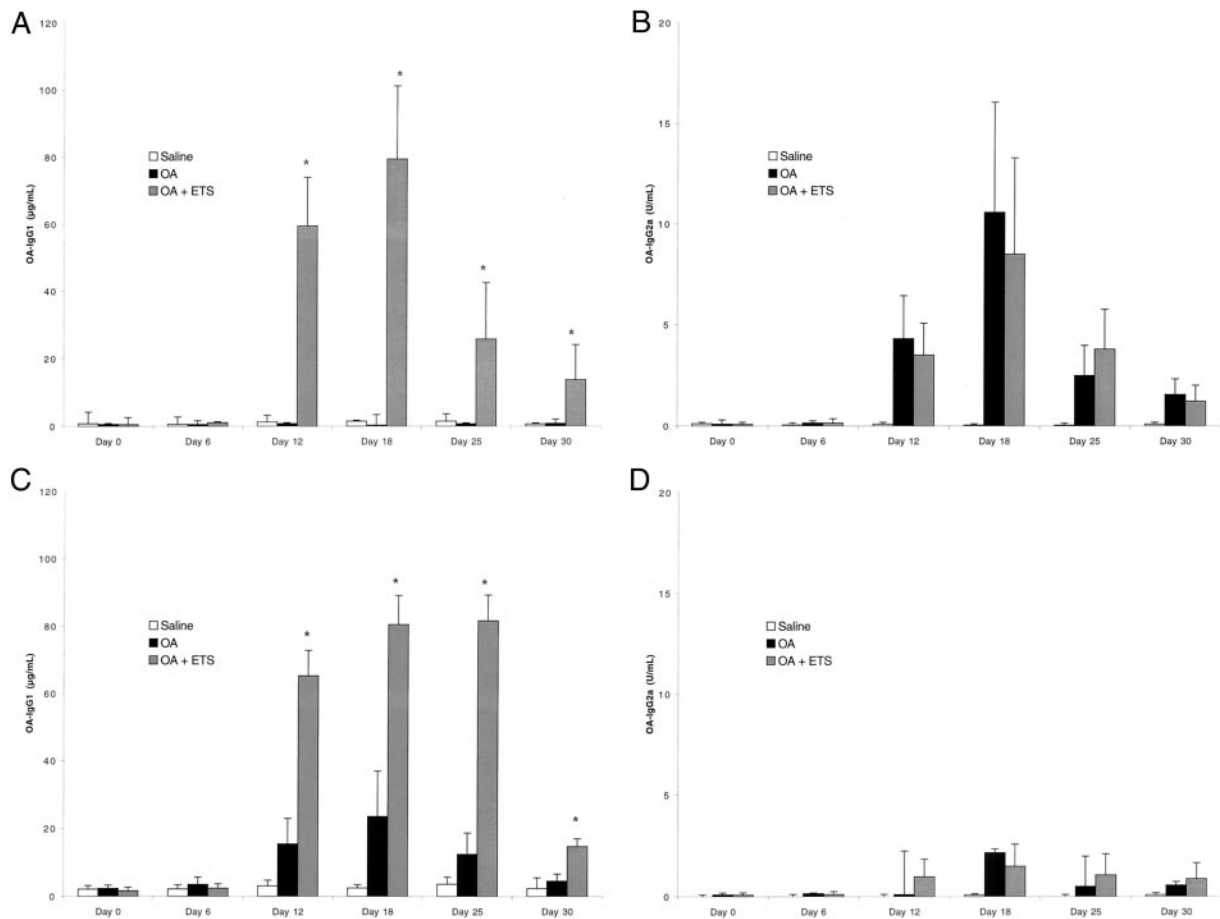
#### Discussion

The ability of ETS, also known as secondhand smoke, to increase the risk of middle ear effusion, bronchitis, and pneumonia in children is widely accepted (20). Several studies on the epidemiology of the allergic response in children have also implicated ETS in the exacerbation of allergic disease (5–9, 26) and asthma (10). ETS is

thought to be especially harmful to children with asthma. The EPA estimates that for between 200,000 and one million asthmatic children, exposure to ETS worsens their condition. (27) ETS can be linked to severity of asthma symptoms, increased prevalence of asthma, increased frequency of medication use, and increased emergency room visits by asthmatic children. (28–31)

The role of ETS in atopic sensitization is more controversial. Our studies aimed to study the association between tobacco smoke and allergic sensitization by direct experimentation. The production of specific allergic Abs against inhaled protein allergens is the hallmark of the sensitization phase of allergic airway disease (allergic rhinitis and asthma); subsequent seasonal exposure leads to a secondary immune response. Many studies have demonstrated elevated IgE levels in active mild smokers (32–35). Studies investigating passive smoke exposure have shown differing results. Among others, Wagner et al. (5) have described a significant increase in serum IgE levels in children of smoking parents. Weiss et al. (6) described a 2.2-fold increased risk of being atopic (as defined by the presence of at least one positive skin test) when maternal smoking occurred. Ronchetti et al. (7) reported both increased skin test reactivity, serum IgE, and increased prevalence of eosinophilia in 9-year-old children of smoking parents. In contrast, other studies have failed to find such a link. Ownby and McCullough (36) found no increase in either total or allergen-specific IgE in children aged 1–19 years exposed to parental smoking. Although Osaka et al. (8) reported an increase in mite-specific IgE levels in Japanese children of smoking parents, Ozasa et al. (9)





**FIGURE 2.** ETS induced IgG1, but not IgG2a, responses to OVA in low and high IgE responders. C57BL/6 (A and B) and BALB/c mice (C and D) were exposed to either saline, 1% OVA, ETS, or both OVA plus ETS for 10 days (see *Materials and Methods*). Bleeds were taken before exposure and 6, 12, 18, 25, and 30 days afterward. OVA-specific IgG1 (A and C) and IgG2a (B and D) was measured. \*,  $p < 0.01$  vs OVA-exposed group.

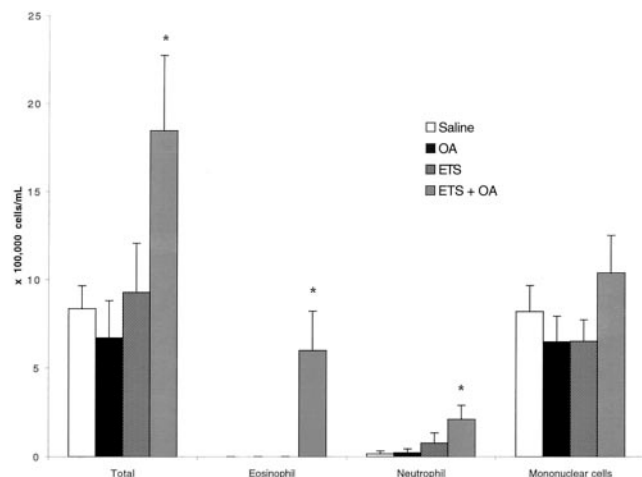
found a negative association between Japanese cedar pollen-specific IgE and passive smoking in the same population.

In this study we show that ETS can augment primary sensitization to an innocuous protein. Our studies demonstrate that ETS can initiate de novo responses. Previous reports have studied the ability

of ETS to augment pre-established Th2 responses. Seymour et al. (11) sensitized BALB/c mice to OVA and then exposed them to ETS or ambient air. Using this murine model of allergy, this showed that ETS had an adjuvant effect on IgE production and eosinophil numbers in the blood. Moreover, IL-4 and IL-10 was significantly higher in aerosolized allergen-sensitized mice exposed to ETS when compared with those exposed to ambient air. Raised serum IgE levels were found in rats exposed to tobacco smoke twice daily 5 days a week for 8 wk, however, it is unclear whether exposure was limited to only side-stream smoke in these experiments.

Aside from the evidence cited above, the effect of ETS on atopy can be inferred from experiments using other substances. Tobacco smoke contains ~6000 known chemical components, as well as nitrogen dioxide and sulfuric acid (20). Some of these components such as lead acetate, mercuric chloride, nickel sulfate, and tungsten have been shown to increase allergenicity in animal models. We have previously shown that, experimentally, diesel exhaust particles (DEP), a model environmental pollutant, can induce allergic sensitization in a human nasal model. DEP shares many characteristics with tobacco smoke including having a particulate phase and the presence of many similar polyaromatic hydrocarbons (PAH). Of note are the prototypical PAHs benzo(a)pyrene (which is particularly high in side-stream smoke) and phenanthrene, which can also induce IgE and Th2 cytokine responses in mice and in vitro.

The mechanism by which ETS induce primary sensitization has yet to be established. One possibility is that ETS may be improving



**FIGURE 3.** Cellular responses in BAL to ETS. C57BL/6 mice were exposed to either saline, 1% OVA, ETS, or both OVA plus ETS for 10 days. At day 30 animals were re-exposed to 1% OVA. BAL was performed 24 h later and total and differential cell counts were performed. \*,  $p < 0.05$  vs OVA-exposed group.

Table I. Cytokine levels in BAL<sup>a</sup>

	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)	IFN- $\gamma$ (pg/ml)
Saline	ND	6.7 $\pm$ 3.2	15.6 $\pm$ 9.7	8.2 $\pm$ 4.8	26.6 $\pm$ 7.4
OVA	ND	9.6 $\pm$ 7.4	18.3 $\pm$ 8.2	7.3 $\pm$ 4.3	77.4 $\pm$ 28.2
ETS	58.9 $\pm$ 15.2**	10.3 $\pm$ 4.3	ND	40.5 $\pm$ 11.2*	33.7 $\pm$ 12.3
OVA + ETS	55.3 $\pm$ 31.1**	14.2 $\pm$ 8.3	73.2 $\pm$ 21.4*	106.7 $\pm$ 32.6*	6.9 $\pm$ 5.2**

<sup>a</sup> C57BL/6 mice were exposed to either saline, 1% OVA, ETS, or OVA plus ETS for 10 days. At day 30 animals were re-exposed to 1% OVA. BAL was performed 24 h later and cytokine levels measured in the fluid. ND, not detectable.

\*,  $p < 0.05$  vs. OVA-exposed group.

\*\*\*,  $p < 0.01$  vs. OVA-exposed group.

Ag presentation either by causing structural modifications to the allergen itself, or by absorption of allergen, thereby causing a more persistent allergen exposure. Allergen adsorption has been observed in vitro after mixing of DEP with the allergen Lol p1. (17) However, it is unlikely that this is the case in these experiments, where ETS and allergen were administered separately. Of course, it is still possible that absorption/modification occurs in the airways themselves. It is more plausible to believe that ETS is altering the environment to one more conducive to allergic sensitization by increasing induction of adhesion molecules, cytokine cascades, or proinflammatory cells. PAHs have been demonstrated to enhance MHC-II gene expression in murine macrophages and to up-regulate CD80 (B7-1) protein (14). In addition, they can affect production of inflammatory cytokines such as GM-CSF, IL-1, and TNF- $\alpha$  (4).

Under normal circumstances, the lungs can be viewed as sites of immunological homeostasis in which repeated aerosolized exposure to protein Ag induces a T cell-mediated immunological tolerance (13, 37). In this study, in the low responder C57BL/6 strain IgE and IgG1, responses were observed following ETS/OVA exposure, but not following exposure to OVA alone. Similar to previous studies, aerosolized OVA alone induced OVA-IgE responses in BALB/c mice; however, this was not accompanied by eosinophilia. In contrast, mice exposed to both OVA and ETS developed eosinophilia had significantly less IFN- $\gamma$  and had an increase in the Th2 cytokine IL-5. Thus, in our model we have shown that ETS can disrupt the initial lung homeostatic mechanism via the airway in a manner highly relevant to normal exposure to permit allergic sensitization characterized by formation of allergic Abs, eosinophilia, and a Th2 cytokine response.

In conclusion, this work demonstrating the potential of ETS to interact with allergen and augment allergic sensitization provides experimental evidence to support studies that suggest that maternal smoking as a risk factor in the development of atopy. Because the prevalence of parental smoking in the U.S. is estimated to be from 40 to 60%, (38, 39), these results may have serious public health implications.

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