Exposure to environmental tobacco smoke (ETS) has been shown to increase allergic sensitization and reactivity and there has been some suggestion that the influence of ETS on the allergic response is dissimilar in males and females. It is to be determined whether gender differences exist in the IgE response to ovalbumin (OVA) sensitization following ETS exposure from the neonatal period through adulthood. To address this thesis, we examined gender differences in OVA sensitization of BALB/c mice housed from birth through adulthood under smoking and nonsmoking conditions. At 6 weeks of age (day 0) all mice were injected i.p. with OVA in aluminum hydroxide adjuvant followed by three 20 min exposures to 1% aerosolized OVA between day 14 and 80. There were significantly more total and OVA specific IgE and IgG1 in the serum of females compared to males. Moreover, these sex responses, along with eosinophilia, were further enhanced in mice exposed to ETS. There were also significantly more IgE positive cells in the lungs of female, but not male, mice exposed to ETS compared with ambient air (p < 0.05). There was also an elevation of Th2 cytokines (IL4, IL5, IL10, and IL13) after re-stimulation of lung homogenates following ETS exposure. These data demonstrate that female animals are significantly more susceptible than males to the influence of ETS on the allergic response.

Keywords: Cytokines; Gender; In vivo animal models; Lung; Tobacco smoke; Th2

Abbreviations: AL, aluminum hydroxide; ETS, environmental tobacco smoke; NIP, nitroiodophenyl; OVA, ovalbumin; T, TWEEN 20

INTRODUCTION

Several reports have shown that gender differences exist in the severity of many diseases involving the immune system due to sex-linked genetic and hormonal factors (Cua et al., 1995; Beebe et al., 1997; Bebo et al., 1999; Ito et al., 2001). This is particularly true for autoimmune diseases (Duquette et al., 1992; Beeson, 1994). Furthermore, the clinical course of diseases such as multiple sclerosis have been shown to be influenced by hormones that can cause a shift in the balance of Th1/Th2 cytokines (reviewed in Ito et al., 2001). In contrast, the role of gender as a factor in the development of allergic asthma and particularly in allergic enhancement by environmental tobacco smoke (ETS) exposure has not been well examined. Although, a genetic predisposition to the development of allergic asthma is recognized (Daniels et al., 1996; Gern et al., 1999) evidence strongly suggests that environmental agents such as ETS increase its phenotypic expression (el-Nawawy et al., 1996; Gilliland et al., 2001). Some studies have shown that males are more likely to be allergic from exposure to ETS or mainstream smoke than females (Ronchetti et al., 1992; Omenaas et al., 1994; Jarvis et al., 1995), while others have reported no sex differences in the prevalence of allergy (Osaka et al., 1985). These studies, while intriguing, are also controversial due to the multiple environmental factors that affect human beings, making it difficult to evaluate the true impact of ETS on the prevalence and severity of allergy.

We have previously shown that exposure of adult BALB/c mice to ETS enhances the allergic response in ovalbumin (OVA) sensitized mice when compared to those in ambient air (Seymour et al., 1997). The ensuing response was characterized by enhancement of blood eosinophils, serum IgE, IgG1 and Th2 cytokines particularly IL4 and...
IL10. In the present study, we have attempted to model the environment of a child born into a home of smokers and to compare this environment with a non-smoking one. To accomplish this, female BALB/c mice gave birth in either ETS or ambient air and their litters were raised in those environments. Mice of both sexes from these environments were sensitized to OVA and gender differences in the allergic response were examined. The allergic response was evaluated by quantification of the cytokines in the lungs, peripheral blood eosinophils, serum antibodies and immunohistochemistry with histological examination of the lungs.

MATERIALS AND METHODS

Animals

Pregnant pathogen-free BALB/c female mice were obtained from Animal Technologies (Fremont, CA). They were housed in either ETS or ambient air and their litters were used in the experiment.

Research Cigarettes

The cigarette used in this study was the IR4F, a filtered cigarette used for research produced by the Tobacco and Health Research Institute (University of Kentucky, Lexington, KY) and stored at 4°C until used. Two days before use, they were placed at 23°C in a chamber containing water and glycerol (at a ratio of 0.76:0.26) to achieve a relative humidity of 60%.

ETS Exposure

The smoke generation system was designed by Teague et al. (1994). Whenever animals were not receiving ETS, filtered air was delivered to the mice. Mice were exposed to ETS for 6 h per day from Monday through Friday through adulthood but daily during the neonatal period between birth and 3 weeks of age, as shown in Fig. 1. At the end of the daily exposure, the smoke generator was turned off, but the animals remained in the exposure chambers. The total suspended particulates concentration in ETS was 1.01 ± 0.05 mg/in³. The nicotine and carbon monoxide concentrations were 146 ± 54 μg/m³ and 6.1 ± 0.7 ppm, respectively. The relative humidity was 52.5 ± 10.0%, while the temperature was 71.3 ± 1.5°F. Measurements of nicotine after the system was turned off revealed a rapid decline to nondetectable levels (<15 μg/m³) during the nonsmoking period.

Antigen Exposure and Immunization

At six weeks of age, the mice were injected i.p. with 10 μg OVA (Sigma, St. Louis, MO) in 2 mg of aluminum hydroxide adjuvant. The animals were later exposed to aerosolized OVA from 1% OVA (wt/vol.) in PBS (Fig. 1). Aerosolization was performed for 20 min using a Passport aerosol compressor (Invacare Corp, Elyria, OH) connected to a box 3 ft³ in size which served as the deposition chamber for the mice.

Immunohistochemical Evaluation of IgE Positive Cells in the Lung Parenchyma

On day 4 after the last aerosol, lungs from all groups were inflated and fixed with 1% paraformaldehyde at 30 cm water pressure for 1 h and placed into 70% ethanol until they were processed into paraffin. A rat anti-mouse antibody, EM95 (DNAX) was used to detect IgE positive cells in 5μm thick paraffin sections. The sections were deparaffinized in xylene, rehydrated through a graded series of ethanol and treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% rabbit serum in 0.01 M PBS, pH 7.4, and sections were incubated in primary antibody diluted at 1:5000 in blocking serum for 1 h at 37°C. A biotinylated rabbit anti-rat, mouse adsorbed, secondary antibody was used, including Vectastain elite ABC immunoperoxidase reagents and a DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Hematoxylin was used as the nuclear counterstain. For a negative reagent control, the primary antibody was substituted with rat serum and used on OVA-challenged tissue sections. The negative control did not show positive staining for IgE positive cells.

To calculate the number of IgE positive cells present in lung parenchyma, 10 random, non-overlapping fields were examined. Fields containing large airways and blood vessels were excluded. Positive cells with abundant

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**FIGURE 1** The time illustrates the exposure and sensitization protocol, beginning with pregnant females that were housed in either ETS or ambient air. Litters were retained in the same environment of the mother throughout the experiment.
amounts of cytoplasm strongly staining for IgE as well as positive cells with thin rims of cytoplasm strongly staining for IgE were observed and counted in the tissue sections. The final results were expressed as number of positive cells/10 fields.

**In-vitro Restimulation of Lung Cells**

Mice were euthanized and their lungs removed aseptically, cut into small pieces and forced against a sterile No. 100 steel mesh (Tylinter Inc., Mentor, OH) with the piston of a 12 ml plastic syringe. The suspended cells were washed three times in Hanks solution and cells were stimulated in 1 ml cultures consisting of RPMI 1640 (J.H.R. Bioscience, Lenexa, KS) with 10% heat-inactivated FCS (Sigma, St. Louis, MO), 0.05 mM 2-ME (Sigma), 2 mM L-glutamine (J.H.R. Bioscience, Lenexa, KS) and penicillin/streptomycin (GIBCO BRL, Life Technologies Inc., Grand Island, NY). Lung cells were stimulated at $5 \times 10^6$ cells/ml in culture medium containing 0.25 mg/ml OVA. The supernatant was harvested at 72 h.

**Cytokine Analysis**

Sandwich ELISAs were performed to measure IL5, IL10 and IFN-γ as described (Abrams, 1995). IL-4 from cultured supernatant was detected by a bioassay using the IL4 dependent CT.4S cell line (kindly donated by Dr William Paul, NIH) as described (Seymour et al., 1997).

**Analysis of Total IgE and OVA Specific Serum IgE**

Total serum IgE was determined using a two-step sandwich ELISA as described (Coffman et al., 1986). OVA-specific serum IgE was determined using a two-step sandwich ELISA (Seymour et al., 1998). Briefly, the coating antibody was a monoclonal anti-IgE antibody called EM95. The serum samples were added and allowed to bind; subsequently the DIG-OVA was added. Final detection was accomplished with anti-digoxigenin-Fab fragments coupled to horseradish peroxidase.

**Analysis of OVA Specific IgG1**

ELISA plates were coated with 5 μg/ml of OVA in PBS. The detecting antibody was a biotinylated rabbit anti-IgG1 (3099D) from DNAK which was used at 0.5 μg/ml. (Seymour et al., 1998). The positive control for OVA specific IgG1 was pooled sera from hyperimmunized IgG1 standard run in parallel on anti-IgG1 coated plates.

**Quantification of OVA in the Lung of Aerosolized Mice**

Immediately after a single OVA exposure, lungs were removed and a cell suspension was prepared for an OVA specific ELISA as previously described (Seymour et al., 1998).

**Quantification of Eosinophils in the Peripheral Blood**

Eosinophils were quantified by dilution of heparinized blood in Discombe's fluid (Colley, 1972).

**Statistics**

Levels of antibodies, eosinophils and cytokines were calculated as mean and SEM. The two-tailed $p$ values were calculated according to the Mann–Whitney Test. Statistics for the morphometric analysis of IgE positive cells were performed using the Unpaired Students $t$-Test (Statview 4.5, Abacus Concepts, Inc. Berkeley, CA).

**RESULTS**

**Gender Differences in the Antibody Levels from the Sera of OVA Sensitized Mice**

By day 28, all mice exposed to ambient air produced increased levels of total and OVA specific IgE (Fig. 2).
However, at all time points, female mice made significantly more total and OVA specific IgE when compared to males ($p < 0.05$). The differences in OVA specific IgE between the sexes widened markedly at all time points assessed for IgE. For example, we observed approximately a four-fold increase in total and OVA specific IgE in female mice when compared to males on day 28. The largest increase in IgE was observed on day 94 (2 weeks after the final aerosolized OVA challenge). Here, there was approximately a two-fold increase in total and OVA specific IgE in female mice compared to males (total IgE in males was 1979 $\pm$ 170 ng/ml and OVA specific IgE was 1586 $\pm$ 240 ng/ml compared to 3980 $\pm$ 724 ng/ml and 3407 $\pm$ 656 ng/ml, respectively).

At the time of the last aerosol exposure (d80 from OVA/AL priming), all mice had elevated levels of OVA specific IgE (Fig. 3A). However, collectively, all mice that were housed in ETS had higher concentrations of OVA specific IgE than those in ambient air. Yet, the differences between ETS and ambient air groups were not statistically significant until the groups were divided by sex. Resulting comparisons then showed that the female mice in ETS made significantly more OVA specific IgE than all other groups including females housed in ambient air (OVA specific IgE concentrations in ETS females were 441 $\pm$ 142 ng/ml compared to 231 $\pm$ 67 ng/ml from females in ambient air, $p = 0.03$). Maximal responses with respect to OVA specific IgE were observed seven days after the last aerosolized OVA exposure (Fig. 3A). Female mice housed in ETS had significantly higher serum concentrations of OVA specific IgE than all male mice. However, at this time point, levels of significance were not attained when females in ETS were compared to females in ambient air. Although males housed in ETS also had higher serum concentrations of OVA specific IgE than males in ambient air, these data did not attain levels of significance (IgE concentrations in ETS exposed males were 1465 $\pm$ 295 ng/ml compared to 852 $\pm$ 191 ng/ml, $p = 0.11$, respectively). Female mice in ambient air had much greater serum concentrations of OVA specific IgE than males in ambient air (1976 $\pm$ 283 ng/ml vs. 852 $\pm$ 191 ng/ml, $p = 0.0008$, respectively). However, when female mice in ambient air were compared with males in ETS at the same time point, there was no longer a significant difference in OVA specific IgE between them (1976 $\pm$ 283 ng/ml vs. 1465 $\pm$ 295 ng/ml, respectively, $p = 0.22$) due to the enhancing effect of ETS on the IgE response. Moreover, females in ETS had significantly higher serum concentrations of OVA specific IgE that males housed in the same environment (3296 $\pm$ 555 vs. 1465 $\pm$ 295, respectively, $p = 0.019$).

Significant gender differences in OVA specific IgG1 were observed only on day 7 after the last exposure to aerosolized OVA (Fig. 3B). At this time, females housed in ETS showed the highest response when compared to all other groups. The OVA specific serum IgG1 concentrations in females were significantly higher than those in males regardless of ETS or ambient air exposure ($p < 0.005$). However, among mice of the same sex, significant differences relevant to ETS or ambient air exposure were seen only between the males. Thus, male mice housed in ETS had a mean serum concentration of 427 $\pm$ 80 $\mu$g/ml OVA specific IgG1, while males in ambient air had a mean serum concentration of 197 $\pm$ 37 $\mu$g/ml OVA specific IgG1 ($p = 0.002$).

**IgE Positive Cells in the Lung Parenchyma of OVA Sensitized Mice Exposed to ETS or Ambient Air**

Mononuclear cells that stained positive for IgE were found throughout the lung parenchyma, in the perivascular influx of inflammatory cells and occasionally in the airway epithelium and lamina propria of OVA sensitized mice. Morphometric analysis of the lung parenchyma demonstrated that exposure to ETS significantly increased ($p < 0.05$) the number of IgE positive cells in females sensitized with OVA compared to females that were housed in ambient air (Fig. 4). There was no statistically significant
difference in the number of IgE positive cells between OVA sensitized males exposed to ETS or ambient air. Mice not sensitized with OVA had few IgE positive cells (not shown).

**Eosinophils in the Peripheral Blood**

Quantitative differences in eosinophilia were not observed between the genders at the early time points. Thus, results from both sexes were pooled and mice in ETS were compared with those in ambient air (Fig. 5). Mice (naïve with respect to OVA) exposed to ETS, from birth to adulthood at 6 weeks of age, showed a significant enhancement of eosinophils in the blood when compared to similar naïve mice in ambient air (53 ± 5 × 10^{-4}/ml vs. 27 ± 4 × 10^{-4}/ml, respectively, p = 0.0002). Exposure of mice to OVA led to additional enhancement of blood eosinophils from ETS housed mice as seen at day 21 and again at day 36. At day 36, extremely significant differences in eosinophils were observed when ETS exposed mice were compared to mice housed in ambient air (184 ± 14 × 10^{-4}/ml vs. 107 ± 13 × 10^{-4}/ml, p = 0.0006). By this time, significant differences were apparent in blood eosinophil levels between genders; this was particularly evident in the data obtained from the males and females housed in ambient air. Female mice had

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**FIGURE 4** IgE positive cells in the lung parenchyma of OVA sensitized mice exposed to ETS/ambient air. Four days after the last aerosol OVA, lungs were removed and tested for IgE positive cells. Asterisks indicate p < 0.05.

**FIGURE 5** Eosinophil levels in the blood of OVA/AL sensitized mice housed in either ETS or ambient air since birth after exposure to aerosolized OVA. Asterisks indicate p < 0.05.
a mean eosinophil level of $157 \pm 13 \times 10^{-4}$/ml, while male mice had a mean level of $60 \pm 8 \times 10^{-4}$/ml eosinophils ($p < 0.0001$).

Eosinophils were found throughout the lung parenchyma of the OVA sensitized mice. The groups that were not sensitized with OVA did not show evidence of lung inflammation and eosinophils were rarely seen (not shown).

**Gender Differences in Lung Levels of Cytokines**

On day 87, lungs were removed from all mice and cell suspensions of the whole lungs and associated lymph nodes were restimulated in vitro for 72 h with OVA; supernatants of these cultures were assayed for cytokines (Table I). A polarized Th2 response with no detectable IFN-$\gamma$ was apparent in all mice. Greater concentrations of cytokines were detected from cells of female than from those of males from the same environment. Although the IL-4 concentration produced by cells from ambient air housed females was greater than that from similarly housed males, the difference was not significant. However, when concentrations of IL13 were measured extremely significant ($p < 0.0001$) differences were observed. Moreover, extremely significant differences were also observed between males and females housed in ambient air for IL5 ($p < 0.0001$) and IL10 ($p = 0.0008$).

The enhancing effect of ETS on the cytokine concentrations was most apparent for males. All cytokines examined, except for IFN-$\gamma$, were significantly enhanced in ETS exposed males when compared to males in ambient air ($p < 0.05$). However, when the effect of ETS on cytokine production in females was examined, the greatest enhancements were seen in IL4 and IL10, when ETS housed females were compared to females in ambient air (Table I).

**Deposition of OVA in Lung**

The quantity of OVA that was deposited in the lung of mice from both sexes was determined after 20 min of exposure to a 1% aerosolized OVA (Fig. 6A). Six week old male mice received $2588 \pm 300$ ng of OVA, while female mice received $2120 \pm 200$ ng OVA. A greater quantity of OVA was deposited in the lung of 12 weeks old mice of both sexes when compared to 6 weeks old mice. Although male mice consistently inhaled greater quantities of OVA than females, these differences were not significant. The clearance of OVA from the lung of 6 weeks old females was rapid. At 48 h after the mice inhaled 2000 ng of OVA, 99.5% of the antigen was cleared from the lung (Fig. 6B). The quantity of OVA remaining in the lung at 72 h was $6.3 \pm 0.9$ ng. Results from males exposed to a 1% aerosolized OVA for 20 min revealed the same clearance.

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**TABLE I** OVA specific cytokines in the lung of OVA sensitized mice exposed to ETS/ambient air

<table>
<thead>
<tr>
<th>Gender</th>
<th>Exposure</th>
<th>IL4 (pg/ml)</th>
<th>IL13 (pg/ml)</th>
<th>IL10 (U/ml)</th>
<th>IL5 (pg/ml)</th>
<th>IFN-$\gamma$ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (9)</td>
<td>Air</td>
<td>140 ± 66</td>
<td>855 ± 23</td>
<td>0.943 ± 0.21</td>
<td>404 ± 13</td>
<td>&lt;156</td>
</tr>
<tr>
<td>Male (12)</td>
<td>ETS</td>
<td>252 ± 43*</td>
<td>1499 ± 263*</td>
<td>1.420 ± 0.13*</td>
<td>1003 ± 100*</td>
<td>&lt;156</td>
</tr>
<tr>
<td>Female (9)</td>
<td>Air</td>
<td>187 ± 37</td>
<td>2891 ± 324</td>
<td>2.470 ± 0.26</td>
<td>3099 ± 309</td>
<td>&lt;156</td>
</tr>
<tr>
<td>Female (9)</td>
<td>ETS</td>
<td>404 ± 63**</td>
<td>3079 ± 252</td>
<td>3.594 ± 0.65</td>
<td>3328 ± 487</td>
<td>&lt;156</td>
</tr>
</tbody>
</table>

In vitro stimulation to detect lung cytokines was done on day 7 after the last aerosolized OVA exposure.

* $p < 0.05$ compared to newborn males in air.

** $p < 0.05$ when compared to newborn females in air.

Data are presented as means ± standard error.

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**FIGURE 6** Measurements of OVA in the lung of BALB/c mice after exposure to a 1% aerosolized OVA. (A) Clearance of OVA from the lung of BALB/c mice after receiving 2000 ng of OVA. (B) Mice were exposed to OVA aerosol and lungs were removed immediately, minced, and then suspended in 10 ml of RPMI. Suspension was centrifuged and supernatant assayed by ELISA for OVA. Ten mice of each gender were used in part A and an average of two mice/time point were sacrificed in part B, except for the 74 h time point in which five mice/group were used.
rate as seen in females; 72h after exposure, there were 4.4 ± 1.0 ng of OVA in the lungs.

**DISCUSSION**

Mice born into and raised in ETS are a unique model for the human infant and child born into and raised in a home of smokers. Using this model we tested our earlier hypothesis that male and female children will respond differently to the Th2 adjuvant effect of ETS (Seymour et al., 1998). The data demonstrate a significant difference in OVA sensitization of females when compared to males in ambient air. This difference is based on Th2 cytokines and IgE and IgG1 responses to OVA sensitization. This enhancement effect in females was seen with multiple parameters of the allergic response including: IgE, IgG1, Th2 cytokines, peripheral blood eosinophil counts, and cells staining for IgE within the lung. The combination of female gender and ETS exposure resulted in the highest IgE concentrations and the greatest number of IgE containing cells in the lung. However, the effect of ETS exposure on the males was significant for all of the Th2 cytokines, indicating that even though the levels of cytokines produced were lower in males than in females, the ETS exposure effectively increased those levels.

These observations raise questions concerning the mechanisms involved in this gender difference. One potential mechanism we considered was that differences in lung size, development, or breathing pattern could cause males and females to inhale different amounts of aerosolized OVA resulting in different amounts being deposited in the lung. Data obtained to test this hypothesis was not supportive of this mechanism. Hormones may also be partly responsible for the modulation of the allergic response and several hormones have been implicated in enhancing the activity of Th cells (discussed in Romagnani, 1994). In vitro, progesterone has been shown to cause human Th cells to produce Th2 cytokines (Hamano et al., 1998). Other investigators have speculated that the maternal immune response is biased towards humoral immunity and away from cell mediated immune responses; cytokines such as IFN-γ have been shown to be deleterious in the microenvironment during pregnancy (Chauvat et al., 1990). Indeed, investigators have also shown that Th2 cytokines are up regulated in female mice during pregnancy (Wegmann et al., 1993).

Patients with multiple sclerosis and other cell mediated autoimmune diseases involving Th1 lymphocytes (Steinman, 1996) go into remission during pregnancy probably due to increases in the production of sex hormones which down regulate Th1 responses (Ito et al., 2001). In a murine model of experimental autoimmune encephalomyelitis, induced by myelin basic protein, female mice are more susceptible than males. Furthermore, a reduction in the severity of disease occurs in females implanted with dihydrotestosterone pellets (Dalal et al., 1997). Others have shown that this protection is lost in castrated males, thereby demonstrating gender-related differences due to hormonal influences in disease processes (Bebo et al., 1999). Herein, we demonstrate that even in the absence of ETS, the Th2 cytokine response and the allergic phenotype appears to be greater in the female when compared to males. In particular, IL5 and IL13 concentrations were approximately eight and three-fold higher in females than males, respectively. Blood eosinophilia has been shown to be a risk factor in acute asthma (Janson and Heralu, 1992); IL13 is a key cytokine in the pathogenesis of allergic asthma (Grunig et al., 1998).

In a report by the American Thoracic Society, deaths from asthma between 1979 and 1992 were significantly greater among females than males (Society, 1995). Therefore, it is possible that the Th2 bias of females, in conjunction with environmental factors such as ETS and diesel exhaust particles that can enhance the Th2 response (Diaz-Sanchez et al., 1997), may play a role in the increased mortality from allergic asthma as seen in females. An epidemiological study on the relationship of active and passive exposure to tobacco smoke on total IgE and asthma in human subjects further supports the validity of this murine model. Specifically, it was found that while both men and women, who were first-degree relatives of asthmatics had increased total serum IgE as a result of passive smoke exposure, the relationship was only statistically significant in the women (Oryszczyn et al., 2000). In summary, the murine data presented herein provides support for the hypothesis that early and sustained exposure to ETS stimulates development of an allergic phenotype and that there is a gender bias for this effect.

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