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ORIGINAL ARTICLE

Tobacco Smoke is an Adjuvant for Maintained Airway Sensitization in Guinea Pigs

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Tobacco smoke (TS) exposure exacerbates asthma and may induce airway hyperresponsiveness in asymptomatic individuals. We hypothesized that TS exposure is an adjuvant to airway responsiveness. Ovalbumin (OA) sensitized guinea pigs were TS or air exposed. At 30 exposure days OA airway responsiveness was demonstrable in OA-treated animals exposed to either TS or air. After 130 exposure days only TS-exposed guinea pigs demonstrated OA airway responsiveness. Capsaicin airway responsiveness developed in non-sensitized and OA-sensitized guinea pigs exposed to TS. Therefore TS-exposure acts as an adjuvant to antigenic and neurogenic airway responsiveness. Combined antigen and adjuvant avoidance may attenuate or reverse airway responsiveness.

Keywords tobacco smoke, adjuvant, asthma, guinea pigs, airway responsiveness

INTRODUCTION

Growing evidence supports a causal relationship between tobacco smoke (TS) exposure and increased asthma severity (1, 2). In humans (3) and animal models of asthma (4–6), TS exposure increases airway responsiveness to certain airway mediators. Recently TS exposure has also been shown to induce antigenic sensitization in mice (7, 8). The mechanism of the relationship is unknown. However, second-hand smoke is an adjuvant for Th-2 helper cell responses in a murine model of allergy (9).

A salient feature of asthma and animals models of asthma is eosinophilia. In humans maternal smoking is associated with eosinophilia in children (10). In OA-sensitized guinea pigs eosinophils become a prominent cell type in BALF (6). Eosinophil activation releases cationic proteins, tachykinins and leukotrienes that induce airway inflammation and hyperresponsiveness (11).

On the other hand several studies in mice have failed to establish a relationship between environmental TS exposure and enhanced airway responsiveness. Bowles et al. (12) report that environmental TS exposure failed to enhance OA-specific antibody production, airway inflammation or hyperresponsiveness. Robbins et al. (13) report that TS exposure attenuated airway inflammation in sensitized mice. The effect of TS exposure on airway responsiveness remains equivocal.

Therefore, we tested the hypothesis that chronic TS exposure maintains airway responsiveness to antigenic challenge. In addition we tested the hypothesis that TS acts as an adjuvant to neurogenic airway responsiveness in both non-sensitized and antigen sensitized individuals.

METHODS

Experimental Groups

Hartley guinea pigs weighing from 300 to 350 grams were randomly divided into 4 experimental groups with 5 animals per group. The groups were non-sensitized, air exposure (NS-A); ovalbumin-sensitized, air exposure (OA-A), non-sensitized, tobacco smoke exposure (NS-TS) and sensitized and tobacco smoke exposure (OA-TS).

Airway Sensitization

OA sensitization was induced as per Andersson (14). Guinea pigs received intraperitoneal injections of 10 μ g of OA and 100 mg of aluminum hydroxide in 0.5 cc distilled water. After 12 days a booster injection of 10 μ g of OA in 0.5 cc distilled water was intraperitoneally administered. NS-A and NS-TS animals received vehicle injection (distilled water).

Tobacco Smoke (TS) Exposure

NS-TS and OA-TS groups were exposed 5 days per week to TS from 5 standard 2R4F reference cigarettes with the filters detached (Tobacco and Health Research Institute, Lexington, KY). NS-A and OA-A groups were exposed to air in the exposure chambers of similar airflow and duration of the TS exposures. TS was drawn from a mixing chamber into an environmental chamber (60 $\times$ 40 $\times$ 15 cm) housing a maximum of 10 guinea pigs. Each cigarette burned for approximately 7 minutes. Therefore the daily duration of the exposure was 35–40 minutes. During the TS exposure the environmental chamber was filled with approximately 5 mg total particulate matter/L air. Variables of the exposure to TS are based upon studies that resulted in airway hyperresponsiveness to capsaicin challenge in OA-sensitized guinea pigs exposed to TS (6).

Assessing Airway Responsiveness

Airway responsiveness to OA and capsaicin aerosol challenges was determined by monitoring Penh units obtained

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from plethysmographs that measure several respiratory variables from non-restrained animals (Buxo Electronics Inc.). The Penh unit is a dimensionless product of respiratory pause and peak expiratory pressure divided by peak inspiratory pressure, where respiratory pause is expiratory time (T_e) minus expiratory relaxation time ($T_r = 36\%$ of T_e) divided by T_r (15). In guinea pigs Penh units and specific airway resistance are correlated measurements of airway responsiveness (6). The variables of the OA and capsaicin aerosol challenges used in this study were chosen from a study that induced significant but not life-threatening airway responsiveness (6). Aerosols ($1-5\ \mu\text{M}$ in diameter) of the agents were generated and delivered by an ultrasonic nebulizer (DeVilbiss, Model 65, Somerset, PA) directly into the plethysmographs. All agents were purchased from Sigma Chemical Co. (St. Louis, MO). OA aerosol challenges were conducted at 30 and 130 exposure days. Capsaicin aerosol challenges were conducted at 32, 90 and 132 exposure days.

Total and Differential Cell Counts of Lung Lavage

At the end of the study the animals received intraperitoneal injections of 75 mg/kg pentobarbital to induce respiratory arrest. After cardiac arrest, determined by auscultation, the lungs were lavaged with $5\ \text{ml} \times 4$ of Hank's solution (pH 7.4, 37°C). The lavage was then centrifuged at $671 \times g$ (4°C) for 15 minutes. The supernatant was separated from the cell pellet. The cell pellet was reconstituted in 3 mls of Hank's solution and vortexed. Aliquots of the solution were placed in a hemocytometer to determine total cell counts and placed in a cytocentrifuge (Shandon) to obtain slides for differential cell counts. The slides were stained with Leukostat (Fisher) and 300 cells were counted at $400\times$ magnification as macrophages/monocytes, eosinophils or neutrophils.

Data Analysis

Data are presented as means $\pm$ standard error of the mean. Base level Penh units are 15-second averages just prior to aerosol challenge. Peak Penh units were determined from a 15-second segment after aerosol challenge irrespective of time. A two-factor analysis of variance (ANOVA) determined if statistical separation occurred among treatment groups with time and treatment being the 2 factors. BALF analysis of total and differential cell counts among treatment groups was performed using a one-way ANOVA. When the ANOVA indicated statistical separation ($p < 0.05$), the Newman Keul's test was used to determine where statistical separation occurred.

RESULTS

Weight gained was similar in all groups indicating that the guinea pigs remained in good health. The average weight in grams of each group at the end of the study is shown in Figure 1.

Airway Hyperresponsiveness to Ovalbumin (OA) Aerosol Challenge

Airway responsiveness to OA aerosol challenge was determined at 30 and again at 130 days of exposure to TS or air. Base level Penh units were monitored one minute immediately prior to OA aerosol challenges (0.04%, 60 seconds). Base level Penh units were similar in all four exper-

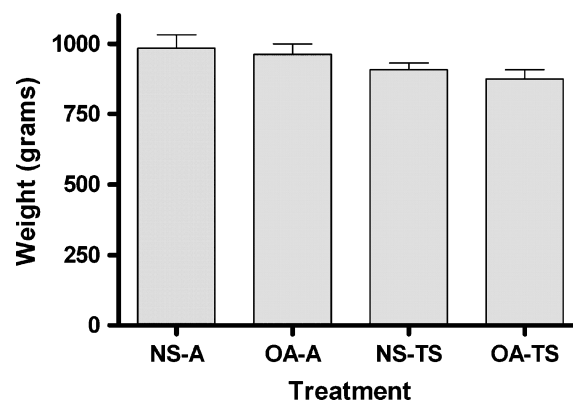


FIGURE 1.—Mean weight (grams) of 4 experimental guinea pig groups determined at the study's end. No statistical separation occurred among the means. The abbreviations are non-sensitized, air exposed (NS-A), ovalbumin-sensitized, tobacco smoke exposed (OA-A), non-sensitized, tobacco smoke exposed (NS-TS) and ovalbumin-sensitized, tobacco smoke exposed (OA-TS).

imental groups throughout the study. Penh units were then monitored for 8 minutes after OA aerosol challenges. OA airway responsiveness as a function of time is presented in Figure 2A. The averaged peak Penh units for each of the 4 treatment groups after OA aerosol challenge irrespective of time is presented in Figure 2B. In OA-treated guinea pigs OA aerosol challenges increased Penh units. Peak Penh units were higher in the OA-TS versus the OA-A group. OA aerosol challenge did not increase Penh units in NS-A or NS-TS groups.

After 130 days of TS or air exposure, OA challenges were repeated in the two OA-sensitized guinea pig groups. Penh units are presented as a function of time in Figure 3A. Airway responsiveness to OA aerosol challenge was enhanced in OA-TS treated guinea pigs versus OA-A treated guinea pigs as demonstrated by comparison of peak Penh units after aerosol challenge (Figure 3B). The BALF of one animal of the OA-A group was then analyzed. It was determined that the percentage of macrophages/monocytes and eosinophils of that animal was similar to that of the non-sensitized groups either exposed to air or TS, being 66.7% macrophages/monocytes, 26.0% eosinophils and 7.3% neutrophils. The remaining 4 animals then once again underwent OA sensitization protocol in order to determine if airway responsiveness to OA aerosol challenge as well as airway eosinophilia could be reestablished. Airway responsiveness to OA aerosol challenge was reestablished in this group (Figure 3A & B) and eosinophilia was demonstrable (Figure 7B).

Airway Hyperresponsiveness to Capsaicin Aerosol Challenge

Airway responsiveness to capsaicin aerosol challenge (0.001%, 30 seconds) was determined after 32, 90 and 132 days of exposures. Base level Penh units were monitored one minute immediately prior to capsaicin aerosol challenge (0.001%, 30 seconds). Base level Penh units were similar in all experimental groups throughout the study. Penh units were then monitored for 5 minutes after the aerosol challenge. TS exposure induced airway hyperresponsiveness to capsaicin with time. At 32 exposure days there was no statistical separation among TS or air exposed groups for peak Penh units

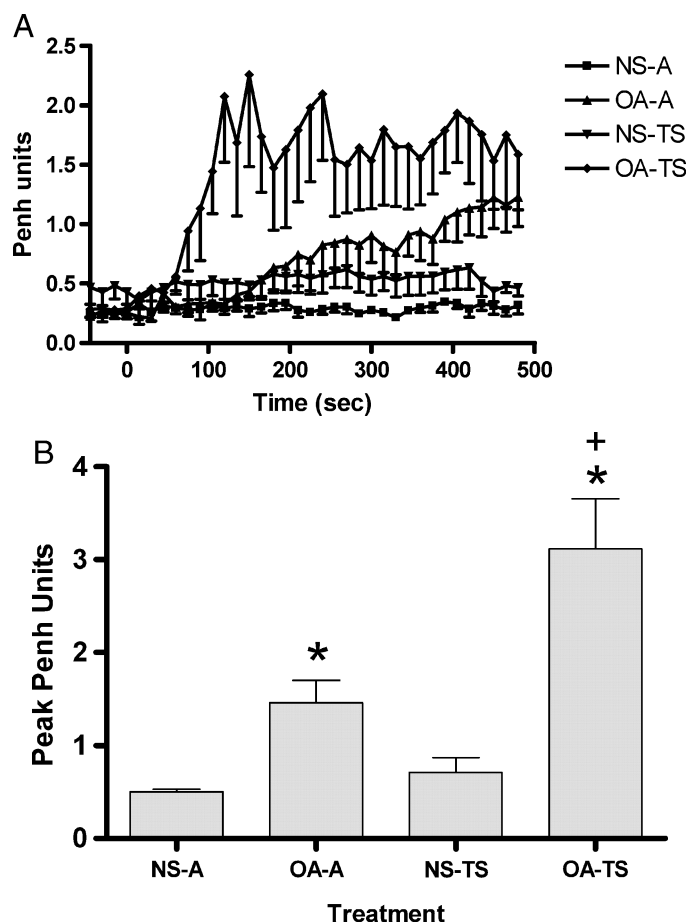


FIGURE 2.—(A) Airway responsiveness (Penh units) to OA aerosol challenge (0.04%, 60 s) in guinea pigs housed in whole body plethysmographs ($n = 5/\text{group}$). Challenges were conducted beginning 30 exposure days with either TS or air. Abbreviations are as stated in Figure 1. (B) Peak Penh units irrespective of time after OA aerosol challenge (0.04%, 60 sec). * = $p < 0.001$ versus NS-A, + = $p < 0.01$ versus OA-A.

(Figure 4A & B). At 90 days exposure airway responsiveness increased in both TS exposed groups versus air-exposed groups (Figure 5A & B). Similar results were observed after 132 days of exposure although peak Penh units were numerical less than those of 90 days of exposure (Figure 6A & B). Therefore airway hyperresponsiveness to capsaicin aerosol challenge was enhanced in guinea pigs daily exposed to TS compared to both NS-A and OA-A groups.

Total and Differential BALF Cell Counts

Total and differential cell counts of the BALF in the 4 groups are shown in Figure 7. No statistical separation occurred among total cells counts (Figure 7A). OA sensitization increased the percentage of eosinophils and decreased the percentage of macrophages/monocytes in the BALF in the OA-A group compared to the NS-A group (Figure 7B). With the combination of OA sensitization and TS exposure, the percentage of macrophages/monocytes and eosinophils in the OA-TS group fell between that of the NS-A and OA-A groups.

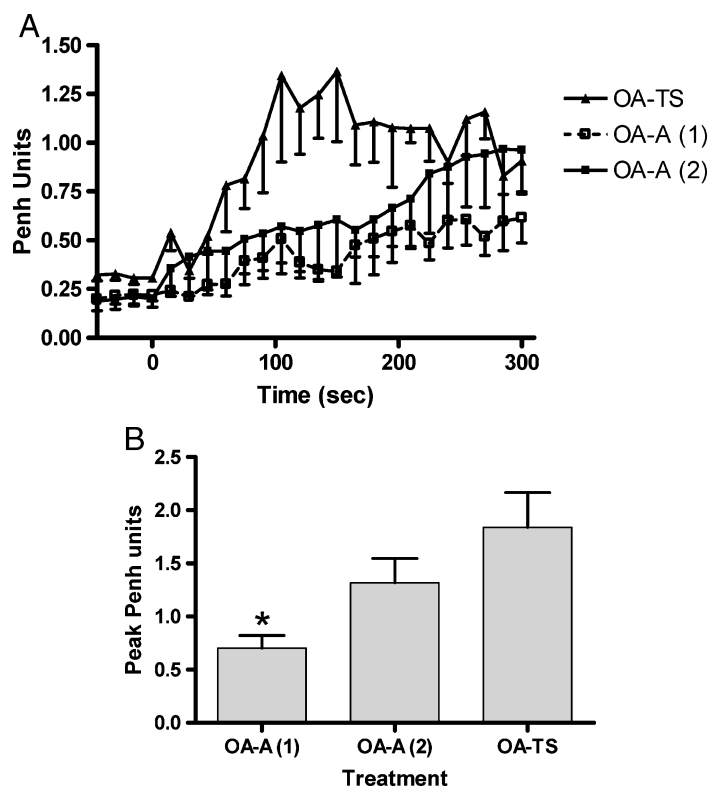


FIGURE 3.—(A) Airway responsiveness (Penh units) to OA aerosol challenge (0.04%, 60 s) in sensitized guinea pigs housed in whole body plethysmographs ($n = 5/\text{group}$). Challenges were conducted at 130 exposure days. Abbreviations are as stated in Figure 1. OA-A (1, $n = 5$) and OA-A (2, $n = 4$) is OA challenge before and after resensitization respectively. (B) Peak Penh units irrespective of time after OA aerosol challenge (0.04%, 60 s) in OA-A and OA-TS groups. OA-A (1) is the first aerosol challenge of the OA-A group ($n = 5$). This challenge resulted in attenuated airway responsiveness compared to the OA-TS group (* $P < 0.05$ versus OA-TS). Therefore a booster injection of OA (10 $\mu\text{g}/\text{i.p.}$) was administered and the OA challenge repeated OA-A (2) after 14 days ($n = 4$). The booster injection reestablished airway hyperresponsiveness to the OA-A group of guinea pigs.

DISCUSSION

TS exposure can exacerbate airway responsiveness in both animal models (4–6) and humans (2, 3). In addition TS exposure, through ill defined mechanisms, may act as an adjuvant to allergic sensitization (7, 8). In the present study we present evidence to support these previous studies. Furthermore this airway sensitization is at least partially reversible with antigen and adjuvant avoidance. In addition neurogenic airway hyperresponsiveness was enhanced with chronic TS exposure independent of OA sensitization. However there was indication of developing tolerance regarding neurogenic inflammation with prolonged TS exposure.

Few studies have investigated the effect extended TS exposure on airway responsiveness in animal models. In the present study 132–146 exposures was conducted 5 days/week. With antigen avoidance airway responsiveness to OA challenge decreased in the OA-A group. Resensitization restored airway OA airway responsiveness, but as was the case at 30 exposure days, airway responsiveness to either OA or capsaicin was not as great as with the combination of OA sensitization and TS exposure. Therefore

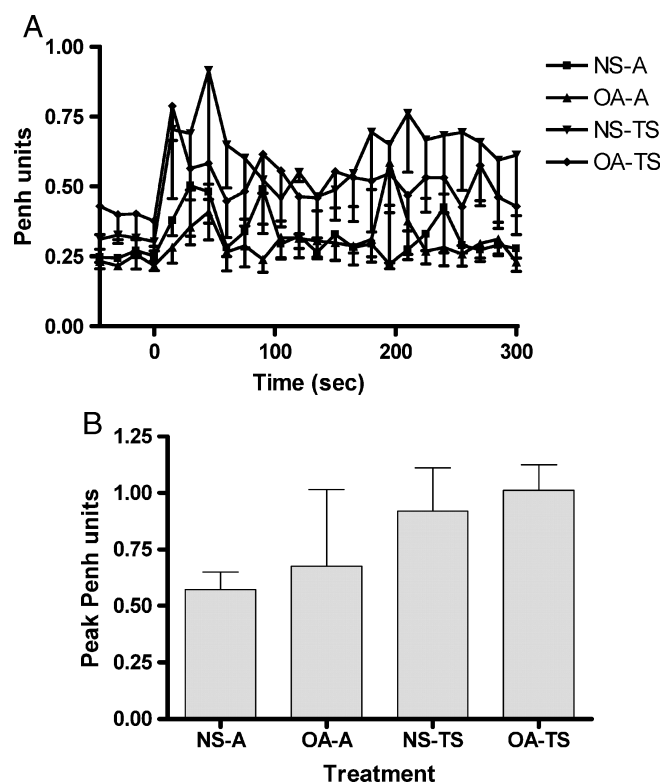


FIGURE 4.—(A) Airway responsiveness (Penh units) to capsaicin aerosol challenge (0.001%, 30 s) in the 4 groups housed in whole body plethysmographs ($n = 5/\text{group}$). Challenges were conducted 32 days after the initial sensitization and treatment with either TS or air. Abbreviations are as stated in Figure 1. (B) Averaged peak Penh units irrespective of time after airway challenge with capsaicin (0.001%, 30 seconds) in the 4 groups. No statistical separation occurred among the groups.

clearly TS exposure acts as an adjuvant for OA airway responsiveness.

Not all studies support that TS exposure enhances airway inflammation. Robbins et al. (13) and Melgert et al. (16) both report suppressive effects of TS exposure on airway responsiveness and inflammation in mice. Possible explanations of these conflicting data could include species variation and contributing factors of variation in experimental protocol. Robbins et al. (13) report reduced airway eosinophilia and CD4 T lymphocytes in BALB/c mice despite increased serum levels of each. Melgert et al. (16) exposed C57Bl/6j mice to mainstream TS over a 3 week period. These investigators report high levels of carboxyhemoglobin. Carbon monoxide decreases airway responsiveness to methacholine challenge in 2 strains of OA-sensitized mice (17).

Eosinophils

Airway eosinophilia is a hallmark of atopy although the role in airway inflammation is poorly understood. Eosinophil metabolism includes leukotriene, platelet activating factor and substance P production (18, 19). In addition cationic proteins cause mast cell and basophil degranulation releasing additional inflammatory mediators such as histamine (20). Eosinophil peroxidase enhances oxidation forming reactive oxygen species (21). Therefore these agents would induce airway inflammation, airway smooth muscle contraction and

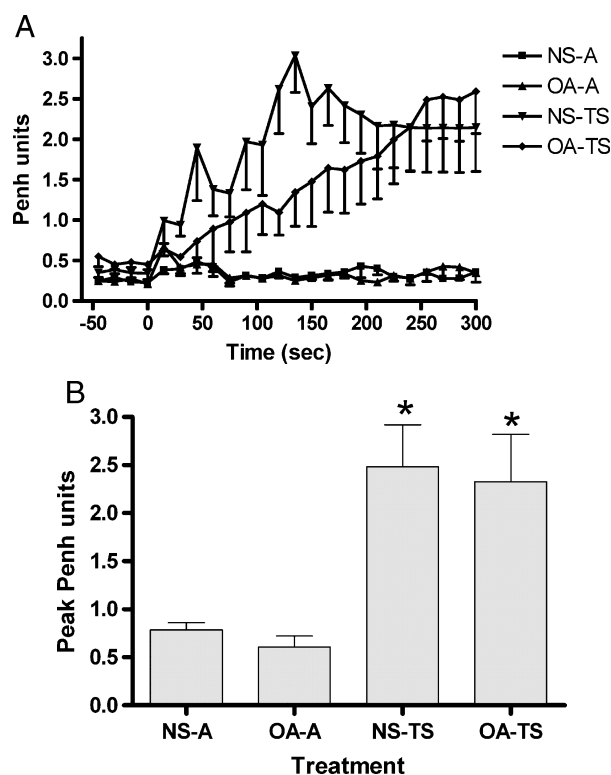


FIGURE 5.—(A) Airway responsiveness (Penh units) to capsaicin aerosol challenge (0.001%, 30 s) in the 4 groups housed in whole body plethysmographs ($n = 5/\text{group}$). Challenges were conducted at 92 exposure days. Abbreviations are as stated in Figure 1. (B) Peak Penh units irrespective of time in the 4 guinea pig treatment groups before and after capsaicin aerosol challenge (0.001%, 30 seconds). $p < 0.01$ versus NS-A and OA-A.

mucus production. Eosinophils activation releases cationic proteins that are toxic to airway epithelium (22). Denudation of the protective epithelium may thereby enhance neurogenic inflammation through C-fiber activation.

In addition eosinophils may act as antigen presenting cells amplifying Th2 cell responses (23, 24). A mechanism through which TS may act as an adjuvant to airway responsiveness to antigen challenge may be that the combination of antigen sensitization and TS exposure increases antigen specific IgE which likely contribute to eosinophil migration into mouse airways (9). The processes of antigenic desensitization may include reducing airway eosinophilia. In both animal models and human studies attempts to reduce antigenic airway responsiveness through reducing airway eosinophilia has been both successful (25–28) and unsuccessful (29–33).

Capsaicin

Capsaicin induces airway responsiveness in guinea pigs largely by activating airway C-fibers (34). Airway hyper-responsiveness to capsaicin occurs in asthma (35) and animal models of asthma (6, 36). C-fibers activation induces bronchoconstriction through the central reflex arc resulting in enhanced parasympathetic tone to the airways and through local reflex mechanisms (37, 38). During the local reflex C-fiber activation releases tachykinins such as substance P (6, 37, 38), which are potent chemotactic factors and

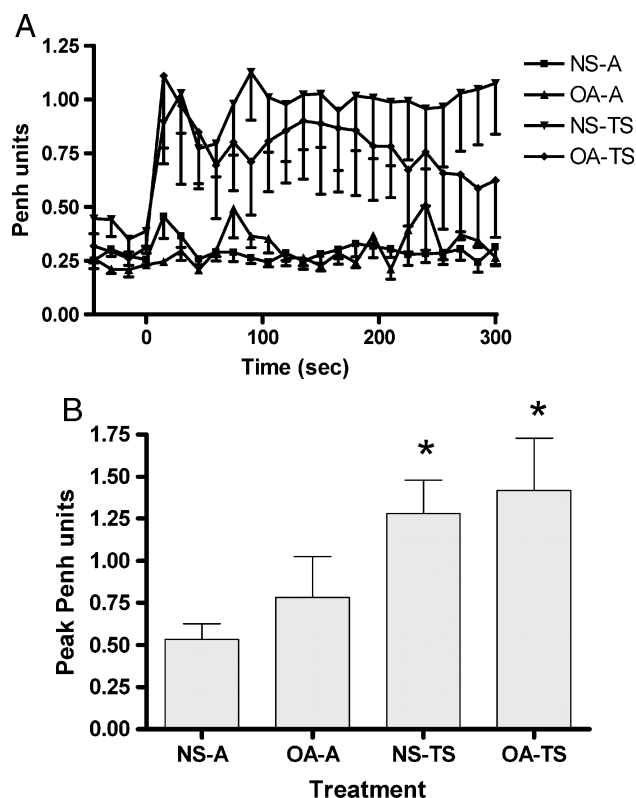


FIGURE 6.—(A) Airway responsiveness (Penh units) to capsaicin aerosol challenge (0.001%, 30 s) in the 4 groups housed in whole body plethysmographs ($n = 5/\text{group}$). Challenges were conducted at 132 exposure days after the initial sensitization and treatment with either TS or air. Abbreviations are as stated in Figure 1. (B) Averaged peak Penh units irrespective of time after airway challenge of capsaicin (0.001%, 30 seconds) in the 4 groups. Statistical separation occurred among the groups exposed to tobacco smoke and those exposed to air. $p < 0.05$ versus NS-A.

degranulation agents of eosinophils (39). Airway responsiveness to capsaicin is further enhanced in OA-sensitized guinea pigs chronically exposed to TS (6). Chronic TS exposure induces enhanced C-fibers responsiveness to capsaicin and this effect is also further enhanced in OA-sensitized guinea pigs (36). It is not known if TS exposure enhances eosinophil responsiveness. The relationship between airway C-fiber responsiveness and eosinophilia is poorly understood. However airway hyperresponsiveness to capsaicin in the present study was more apparent with TS exposure rather than OA sensitization.

Summary and Conclusions

Maintained airway responsiveness to OA aerosol challenge in sensitized guinea pigs exposed to TS and the attenuation of airway responsiveness to OA challenge in guinea pigs exposed to air demonstrate that TS acts as an adjuvant to OA airway sensitization over an extended time period. TS exposure independently enhanced airway responsiveness to capsaicin aerosol challenge. This study supports the hypothesis that TS exposure is an adjuvant to atopy and an agent that independently enhances neurogenic inflammation.

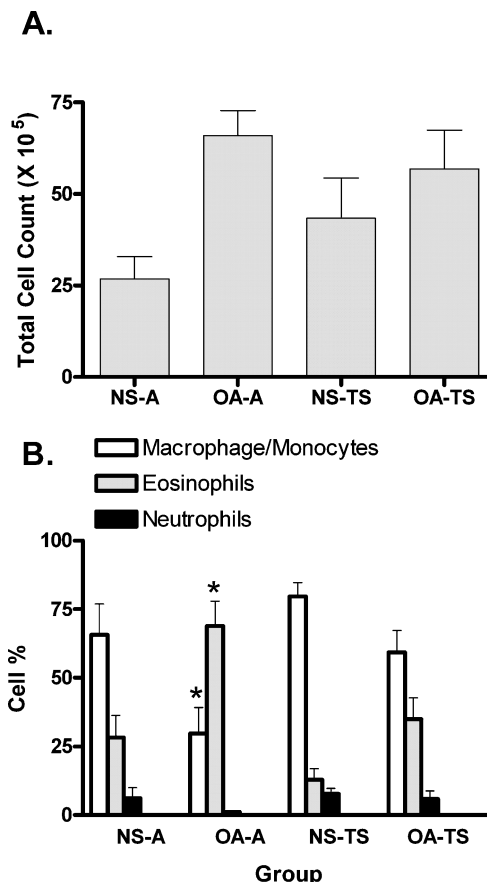


FIGURE 7.—Total (A) and differential (B) cell counts of bronchial alveolar lavage fluid (BALF) from the 4 groups. The abbreviations are described in Figure 1. No statistical separation occurred among the means in Figure 7A. In Figure 7B $p < 0.05$ versus all other groups.

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