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ORIGINAL ARTICLE

Effect of TRFK-5 on Airway Responsiveness in Ovalbumin-Treated Guinea Pigs Exposed to Tobacco Smoke

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Tobacco smoke (TS) exposure can induce airway hyperresponsiveness, especially in asthma. A feature of asthma is eosinophilia. We hypothesized that tobacco smoke exposure enhances eosinophil responsiveness in sensitized guinea pigs. Tobacco smoke-exposed, ovalbumin (OA)-sensitized guinea pigs were treated with TRFK-5 (1.0 mg/kg, intraperitoneal), an anti-interleukin (IL)-5 agent, or its vehicle. Guinea pigs were challenged with aerosols of OA, capsaicin, histamine, and methacholine. TRFK-5 attenuated airway responsiveness to OA but not to capsaicin, histamine, or methacholine. Bronchial alveolar lavage fluid analysis confirmed TRFK-5 attenuated airway eosinophilia in OA-treated guinea pigs. Therefore, airway responsiveness to OA is enhanced by eosinophils or IL-5 itself.

Keywords TRFK-5, eosinophils, antigen, capsaicin, plethysmography

INTRODUCTION

Tobacco smoke exposure can induce airway responsiveness in certain individuals (1), particularly those with asthma (2–4) and in ovalbumin (OA)-sensitized guinea pigs (5). Eosinophil infiltration into the lungs correlates with airway responsiveness in asthma (6–8), as well as in guinea pigs (9). The actions of eosinophils include release of cationic proteins, substance P, and leukotrienes (10). Substance P and leukotrienes are potent bronchoconstrictors and activators of mucus secretion. Cationic proteins induce mast cell degranulation (11) and attenuate muscarinic M2 receptor activity causing enhanced acetylcholine release and airway constriction (12, 13). Eosinophils are also antigen presenting cells to T-lymphocytes (14, 15). Therefore, eosinophils are believed to contribute to airway inflammation and hyperresponsiveness.

Migration of eosinophils from the capillaries into the airways depends greatly on interleukin-5 (IL-5) (16). Administration of anti-IL-5 agents in animal models of allergic asthma attenuates both antigen-induced airway eosinophilia, as well as bronchoconstriction (17–20). However, the action of anti-IL-5 agents in asthmatics has been disappointing despite efficacious inhibition of eosinophil migration into the lungs (21, 22).

In guinea pigs, chronic tobacco smoke exposure causes airway hyperresponsiveness to certain mediators of bronchoconstriction including antigenic and non-antigenic stimuli (23–26). Chronic tobacco smoke exposure enhances airway responsiveness to capsaicin (5, 27), which is further enhanced in OA-sensitized guinea pigs (5, 28). Capsaicin is an agent that selectively activates C-fibers and thereby induces neurogenic inflammation.

Therefore, we tested the hypothesis that eosinophil recruitment into the airways contributes to airway hyperresponsiveness to antigenic and non-antigenic mediators in ovalbumin-sensitized guinea pigs chronically exposed to tobacco smoke. OA-sensitized guinea pigs were treated with either the monoclonal anti-IL-5 antibody TRFK-5 or its vehicle and then chronically exposed to tobacco smoke. Two additional groups of guinea pigs were treated with the vehicles for OA and TRFK-5 with one group chronically exposed to tobacco smoke and the other to air. Subsequently the airways were challenged with aerosols of OA, capsaicin, histamine, or methacholine and airway responsiveness was monitored. An airways bronchial-alveolar lavage analysis was conducted to determine TRFK-5 effectiveness to attenuate eosinophil migration into the lungs.

METHODS AND MATERIALS

Animals

The Creighton University Animal Use Committee approved the study. Male Hartley guinea pigs (Harlan, Minneapolis, MN) weighing approximately 300 grams at the time of purchase were housed in the Creighton University School of Medicine Animal Resource Facility with food and water *ad libitum*. Guinea pig weights were determined on arrival and at day 30. There was no difference in mean weight gain among treatment groups. Guinea pigs were randomly selected for treatment with either TRFK-5 or its vehicle and also with either ovalbumin and its adjuvant or the vehicle. The four experimental guinea pig groups were non-sensitized air exposed (NS-A) that received the vehicle for OA and TRFK-5; non-sensitized tobacco smoke exposed (NS-TS) that received the vehicle OA and TRFK-5; OA-sensitized tobacco smoke exposed (OA-TS) that received the vehicle for TRFK-5 and OA-sensitized tobacco smoke exposed treated with TRFK-5 (OA-TS + TRFK-5).

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TRFK-5 or Vehicle Treatment

Five hundred μL containing either 1.0 mg/kg TRFK-5 or an equal volume of its vehicle (10 mM phosphate buffer plus 150 mM NaCl, pH 7.2) was intraperitoneally injected. The dose of TRFK-5 was based on previous studies where TRFK-5 blocked eosinophil accumulation into the guinea pig lungs (17).

Ovalbumin Administration

On the same day after injection of TRFK-5 or its vehicle guinea pigs were injected intraperitoneally with OA (10 μg) and aluminum hydroxide (100 mg) in distilled water (0.5 ml) or distilled water only (vehicle). A booster injection of OA (10 μg /0.5 ml-distilled water) or distilled water only was administered 12 days later (29). Airway reactivity to OA aerosol challenge (0.04%, 60s) was determined on day 30 of the study.

Chronic Tobacco Smoke Exposure

One day after the OA or vehicle injection, guinea pigs were exposed daily to mainstream tobacco smoke from four standard 2R4F reference cigarettes with the filters detached (Tobacco and Health Research Institute, Lexington, KY). The tobacco smoke was drawn by vacuum into an exposure chamber (36 liters in volume). Air exposed groups were placed in an identical chamber with a similar airflow. Bias flow in the chamber was 25 L/min, which was well above the estimated combined minute volume of 3 to 5 liters for a maximum of 8 guinea pigs housed in the chamber at one time. This prevented development of either a hypoxic or hypercapnic environment within the chambers. CO_2 content of the exhaust air from the chamber was monitored (LB-2 CO_2 monitor, Beckman, Schiller Park, IL) and it remained less than 1% of the exhaust air.

Exposures were approximately 30 min/day and 7 days/week for 36 days. The concentration of tobacco smoke inside the chamber was 5.3 ± 0.1 mg/L during the exposure ($n = 10$ determinations). The variables of the tobacco smoke exposure were based on our previous experiments that induced airway hyperresponsiveness to capsaicin challenge (5).

Measurement of Airway Responsiveness

Beginning after 30 consecutive days of tobacco smoke or air exposure, airway responsiveness was determined by monitoring Penh units obtained from plethysmographs that measure a number of respiratory variables in unrestrained animals (Buxco Electronics Inc., Wilmington, NC). The Penh unit is a dimensionless product of respiratory pause and peak expiratory pressure divided by the peak inspiratory pressure. Respiratory pause is the expiratory time (T_e) minus expiratory relaxation time ($T_r = 36\%$ of T_e) divided by T_r (30). In guinea pigs Penh units and specific airway resistance are correlated measurements of airway responsiveness (5).

Mediator Challenge

Mediator challenges by aerosol inhalation included OA, capsaicin, histamine, and methacholine. Doses of agents used in this study were chosen from a previous study that induced significant but not life-threatening airway responsiveness (5). Agent aerosols (1–5 μm in diameter) were generated and

delivered by an ultrasonic nebulizer (model 65; DeVilbiss, Somerset, PA) directly into the plethysmographs.

Total and Differential Cell Counts of Lung Lavage

At the end of the study the animals received a peritoneal injection of 75 mg/kg pentobarbital to induce respiratory arrest. After cardiac arrest the lungs were lavaged with 5 mL $\times$ 4 of Hank's solution (pH 7.4, 37 $^\circ\text{C}$). The lavage was then centrifuged at $671 \times g$ (4 $^\circ\text{C}$) for 15 minutes. The supernatant was separated from the cell pellet. The cell pellet was reconstituted in 3 mL 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, neutrophils, or lymphocytes.

Agents

All agents were purchased from Sigma Chemical Co. (St. Louis, MO) except TRFK-5 that was purchased from PharmMingen (San Diego, CA).

Statistical Analysis

Data are presented as means $\pm$ standard error of the mean. Base level Penh units are 15-second averages just before aerosol challenge. Peak Penh units were determined from a 15-second segment after aerosol challenge irrespective of time. Penh unit area under the curve (AUC) above base level was determined as the sum of all the averaged 15-second segments after mediator challenge divided by the averaged base level multiplied by the number of segments after mediator challenge. Analysis of variance (ANOVA) determined whether statistical separation occurred among treatment groups. When the ANOVA indicated statistical separation ($p < 0.05$), the Newman Keul's test was used to determine where statistical separation occurred.

RESULTS

The time course of Penh units before and after OA aerosol challenge (0.04%, 60s) of the three guinea pig groups is presented in Figure 1A. OA increased both peak Penh units and the AUC above the NS-A and NS-TS treated group versus the OA-TS treated group but not for the OA-TS + TRFK-5 treated group (Figure 1B and C). No statistical separation occurred between peak Penh units and AUC of the NS-A or NS-TS treated group versus the OA-TS + TRFK treated group. In addition, airway responsiveness to OA aerosol challenge was also attenuated in the OA-TS + TRFK-5 treated guinea pigs versus that of the OA-TS treated guinea pigs for both peak Penh units and the AUC.

The time course of Penh units before and after capsaicin aerosol challenge (0.001%, 60s) is presented in Figure 2A. Capsaicin challenge increased both Penh units (Figure 2B) and AUC above base level (Figure 2C) in all four groups. No statistical separation occurred among groups for either variable.

Methacholine aerosol challenge (0.01%, 30 s) increased Penh units in all guinea pig groups above base level (Figure 3). Peak Penh units were elevated in the OA-TS group versus the

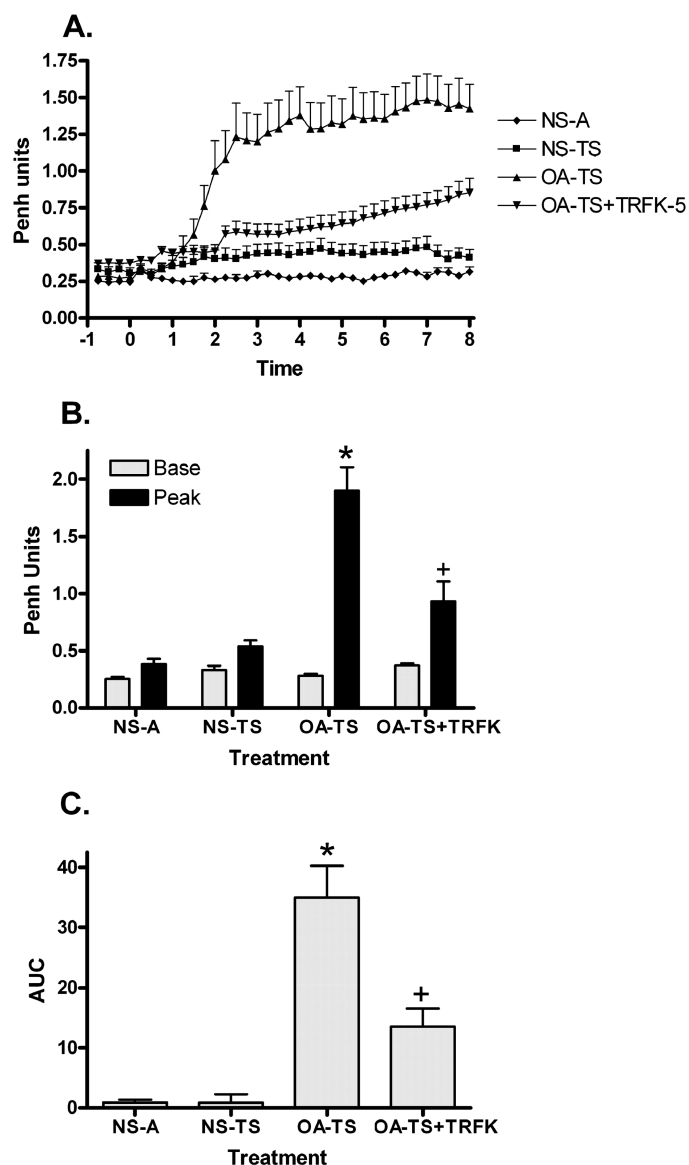


FIGURE 1.—Ovalbumin (OA) aerosol challenge (0.04%, 60 s) of non-sensitized air exposed (NS-A); non-sensitized tobacco smoke exposed (NS-TS); ovalbumin-sensitized tobacco smoke exposed (OA-TS); and OA-sensitized tobacco smoke exposed and TRFK-5 treated (OA-TS + TRFK-5) guinea pigs ($n = 12, 13, 16$, and 16 , respectively). Aerosol challenges were conducted beginning after 30 consecutive days of exposure. (A) Time course of Penh units before and after OA challenge. (B) Peak Penh units after OA challenge irrespective of time. (C) The area under the curve (AUC) above base level after OA challenge for each group. * $p < 0.001$ for peak OA-TS versus peak NS-A and NS-TS Penh units in B and C. + $p < 0.01$ for peak OA-TS + TRFK versus peak OA-TS Penh units.

NS-A group. No other statistical separation was demonstrable among the groups. Histamine aerosol challenge (0.01%, 30 s) also increased Penh units in all guinea pig groups above base level (Figure 4), and peak Penh units were similar among the guinea pig groups.

Analysis of the bronchial alveolar lavage fluid (BALF) is presented in Table 1. Total cell counts were elevated in the OA-TS above the NS-A treated group. Regarding the

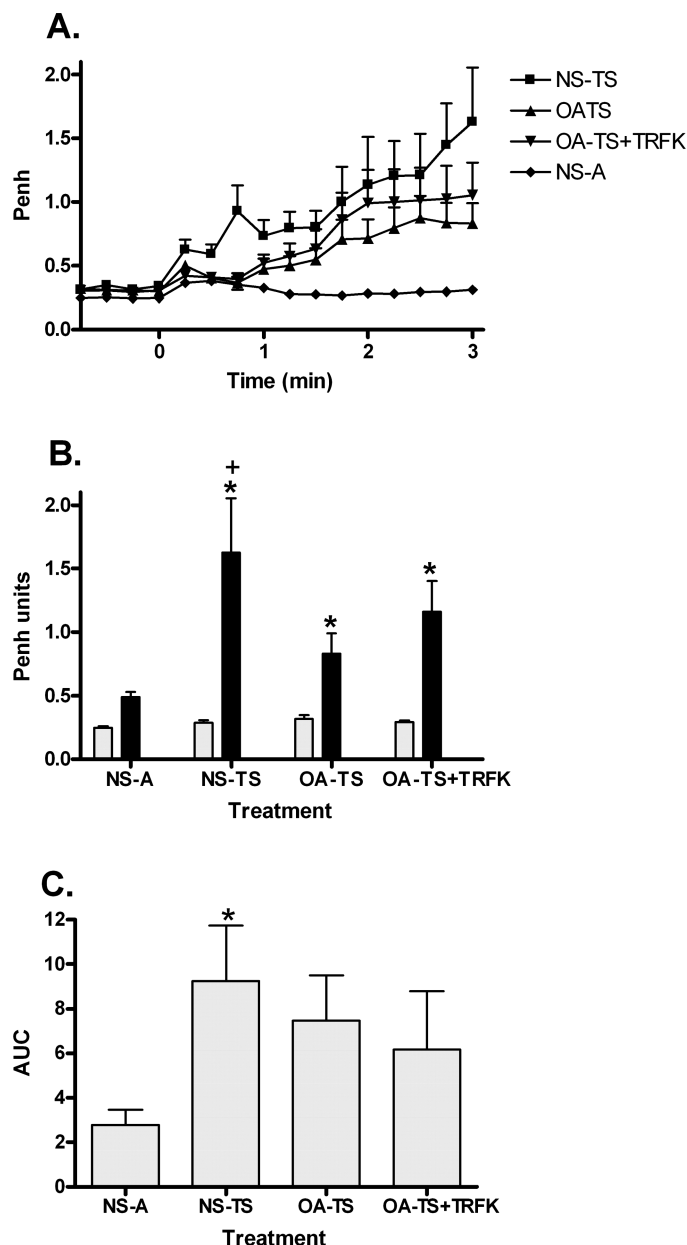


FIGURE 2.—Capsaicin aerosol challenge (0.001%, 30s) of NS-A, NS-TS, OA-TS, and OA-TS + TRFK-5 treated guinea pigs. Abbreviations and number of animals are defined in Figure 1. (B) Capsaicin increased peak Penh units above base level in all groups except NS-A treated group. * $p < 0.001$ and + $p < 0.01$ for peak NS-TS versus peak NS-A Penh units. (C) The AUC above base level. * $p < 0.01$ for peak NS-TS versus peak NS-A Penh units.

differential cell counts the percentage of macrophages was lower in the OA-TS group versus all other groups. The eosinophil percentage was higher in the OA-TS treated group versus all other groups. No statistical separation occurred among the groups for either the percentage of neutrophils or lymphocytes.

DISCUSSION

The present study supports the hypothesis that anti-IL-5 administration attenuates airway responsiveness to OA aerosol

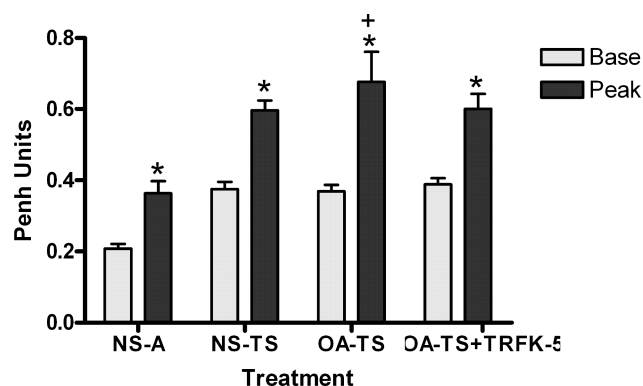


FIGURE 3.—Methacholine aerosol challenge (0.01%, 30 sec) of NS-A, NS-TS, OA-TS, and OA-TS + TRFK-5 treated guinea pigs. Abbreviations and number of animals are defined in Figure 1. Methacholine increased Penh units above base level in all groups (* $p < 0.001$) and peak OA-TS Penh units increased versus peak NS-A Penh units (+ $p < 0.05$).

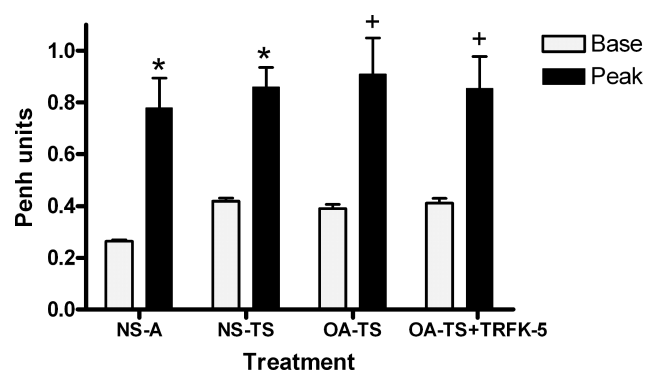


FIGURE 4.—Histamine aerosol challenge (0.01%, 30 s) of NS-A, NS-TS, OA-TS, and OA-TS + TRFK-5 treated guinea pigs. Abbreviations and number of animals are defined in Figure 1. Histamine increased Penh units above base level in all groups (* $p < 0.01$ and + $p < 0.001$). No statistical separation occurred among groups.

challenge in sensitized, non-anesthetized guinea pigs exposed daily to tobacco smoke. Analysis of airway lavage confirmed TRFK-5 attenuated eosinophil migration into the lungs. These data support the hypothesis that airway eosinophils or IL-5 itself contributes to airway responsiveness to OA challenge in sensitized guinea pigs exposed daily to tobacco smoke. However, TRFK-5 treatment did not attenuate airway responsiveness to the non-antigenic mediators capsaicin, histamine, or methacholine.

TRFK-5, an interleukin-5 receptor antibody, antagonizes eosinophil migration from the circulation into the lungs (17, 19, 20). Interleukin-5 (IL-5) is a chemotactic factor for eosinophils (17). IL-5 also prolongs eosinophil viability (32) and enhances eosinophil degranulation (32). Therefore TRFK-5 administration would be expected to decrease accumulation of eosinophils into the lungs after antigen challenge, decrease eosinophil life span, and thereby reduce airway inflammation and responsiveness to mediator challenge.

TRFK-5's antagonism of eosinophil infiltration into the lungs has been documented to last at least 12 weeks after a single administration in guinea pigs (33), mice (34), and monkeys (20). The effective dosage of TRFK-5 in guinea pigs for complete blockade of eosinophil migration into the lungs is 0.1 to 1 mg/kg and 0.01 mg/kg for 50% blockade of eosinophil infiltration into the lungs (17). The TRFK-5 dosage of 1.0 mg/kg used in the present study is within the range effective in preventing eosinophil infiltration into the lungs.

The role of eosinophils in inflammation of the airways is complex and incompletely understood. Eosinophils may

act as antigen-presenting cells (15) and amplify Th2 cell responses (14). In the airways, eosinophils activation causes release of cationic proteins that are toxic to airway epithelium (35). In addition, these cationic proteins can induce mast cell and basophil degranulation (11). Eosinophil peroxidase enhances oxidation by forming reactive oxygen species (36). Eosinophil metabolism includes leukotriene, platelet-activating factor, and substance P production (37, 38) that would enhance airway smooth muscle contraction and mucus production. Activation of eosinophils in the airways would therefore be expected to enhance airway inflammation and responsiveness.

Additional effects of TRFK-5 on inflammatory mediators have been largely unexplored. In guinea pigs TRFK-5 inhibited LTD4-induced airway eosinophilia but had no effect on LTD4-induced bronchoconstriction (39). In mice TRFK-5 prevented antigenic but not lipopolysaccharide (LPS) eosinophil mobilization into the lungs (40). Additionally, TRFK-5 attenuated enhanced acetylcholine contractility of isolated human bronchial smooth muscle independent of eosinophils (41). Therefore IL-5 ligand-receptor interaction may induce airway hyperresponsiveness independently of eosinophil migration.

Capsaicin induces bronchoconstriction in guinea pigs by activating airway C-fibers (42). C-fibers activation induces bronchoconstriction through both central and local reflex mechanisms (43–45). C-fiber activation releases tachykinins such as substance P (5, 45), which are potent chemotactic factors and degranulation agents of eosinophils (46). The effect of capsaicin is further enhanced in OA-sensitized guinea

TABLE 1.—Total and differential (%) cell counts from bronchial alveolar lavage fluid (BALF) from four experimental guinea pig groups.

Group	Total cell Count $\times 10^4$	Macrophages %	Eosinophils %	Neutrophils %	Lymphocyte %
NS-A	21.1 $\pm$ 1.8	65.7 $\pm$ 5.1	25.8 $\pm$ 4.4	5.0 $\pm$ 1.2	3.5 $\pm$ 0.9
NS-TS	31.3 $\pm$ 4.2	59.0 $\pm$ 3.4	21.7 $\pm$ 2.5	12.0 $\pm$ 2.6	7.3 $\pm$ 1.2
OA-TS	41.6 $\pm$ 5.8*	44.6 $\pm$ 4.6†	40.8 $\pm$ 3.3†	10.5 $\pm$ 2.7	4.2 $\pm$ 0.9
OA-TS + TRFK	41.2 $\pm$ 9.3	58.6 $\pm$ 6.5	19.8 $\pm$ 5.3	14.2 $\pm$ 5.8	7.1 $\pm$ 1.9

NS-A = non-sensitized air exposed; NS-TS = non-sensitized tobacco smoke exposed; OA-TS = ovalbumin-sensitized tobacco smoke exposed; OA-TS + TRFK-5 = OA-sensitized, tobacco smoke exposed and TRFK-5 treated guinea pigs.

* $p < 0.01$ versus NS-A and OA-TS + TRFK; † $p < 0.01$ versus all other groups.

pigs chronically exposed to tobacco smoke (5, 28). Chronic tobacco smoke exposure induces enhanced C-fiber responsiveness to capsaicin (47). This effect is further enhanced in OA-sensitized guinea pigs (48). It is not known whether tobacco smoke exposure enhances eosinophil responsiveness and therefore enhanced airway responsiveness. Unlike the OA challenge, airway responsiveness to capsaicin was not attenuated by TRFK-5 despite a reduced eosinophil airway population.

The effect of reducing the eosinophil airway population on methacholine and histamine airway responsiveness was also determined in the present study. The interaction of eosinophils with the parasympathetic nervous system includes inactivation of the M2 receptors by the release of cationic proteins such as major basic protein (12, 13). Therefore parasympathetic tone to airway smooth muscle as well as glandular secretion due to enhanced acetylcholine release would be increased after eosinophil degranulation. In guinea pigs IL-5 administration increased histamine airway responsiveness and increased eosinophils in lung lavage fluid (18, 49). However, in the present study reducing eosinophil airway population had no effect on either methacholine- or histamine-induced airway responsiveness.

The role of the eosinophil in airway responsiveness and asthma has become controversial. In mouse models of asthma, IL-5 inhibition depresses lung eosinophilia but not airway responsiveness in all cases. The discrepancy may be due to strain variations. In IL-5 deficient C57BL/6 mice both airway eosinophilia and responsiveness are attenuated (50). Conversely, in IL-5 deficient BALB/c mice only eosinophil migration is attenuated (51). A high resident population of airway eosinophils in BALB/c mice may contribute to the existing airway responsiveness. In another study, TRFK-5 attenuated both murine airway eosinophilia and tracheal smooth muscle responsiveness to electric field stimulation (52).

Studies of antagonism of IL-5 in human asthma have been disappointing. In asthmatic individuals anti-IL-5 treatment with mepolizumab reduced the numbers of circulating and sputum eosinophils but did not improve the late asthmatic response, airway hyper-responsiveness, or the forced expiratory volume in 1 second (FEV₁) (21, 22). One explanation of the equivocal results in human studies is that this anti-IL-5 agent only partially depletes eosinophils in asthmatic airways (21). Conversely, SCH55700, a humanized anti-human IL-5 antibody, produced a short-lived reduction in blood eosinophils and increased baseline FEV₁ (53). The potential for anti-IL-5 therapy in asthma remains controversial.

In conclusion, TRFK-5 administration to OA-sensitized guinea pigs exposed to tobacco smoke attenuates airway responsiveness to OA antigen challenge. Therefore, either eosinophils or IL-5 itself contributes to airway responsiveness. However, TRFK-5 attenuation of airway responsiveness does not include capsaicin, histamine, or methacholine aerosol challenges. Eosinophils or IL-5 appears not to significantly contribute to airway responsiveness to non-antigenic mediators in OA-treated guinea pigs chronically exposed to tobacco smoke.

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