

Toxocara-induced Eosinophilic Inflammation

Airway Function and Effect of Anti-IL-5

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The immunoinflammatory response to parasitic nematode infections and allergic diseases have some similarities, the most profound being the increases in eosinophils and serum total IgE concentration. Whether parasitic infections stimulate or inhibit allergic asthma is a matter of debate. We investigated the effect of *Toxocara canis* (*T. canis*) infection on airway function in BALB/c mice at various days post-infection. Within 24 h after infection, the trachea responded hyperreactive to carbachol stimulation. Eosinophils, and to a lesser degree lymphocytes, infiltrated the airways causing interstitial and alveolar inflammation (7 d post-infection). Concurrently with cell infiltration, the trachea became hyporesponsive to carbachol whereas the pulmonary resistance was increased and the dynamic compliance decreased. The hyporeactive response could be simulated *in vitro* by incubating normal tracheae with eosinophil-enriched bronchoalveolar lavage cells obtained from infected mice. The response depended on the number of cells added to the medium, a lower number causing a hyper- and a higher number a hyporeactive response. Anti-interleukin-5 (anti-IL-5) producing hybridoma cells given simultaneously with *T. canis* infection inhibited eosinophil infiltration in the airways but not that of lymphocytes. Anti-IL-5 treatment prevented tracheal hyporeactivity but not perivascular and peribronchial edema, increased pulmonary resistance, or decreased dynamic compliance. Treatment with isotype control antibody did not affect eosinophil number nor the observed changes in airway functions. It was concluded that *T. canis*-induced airway inflammation coincided with increased pulmonary resistance, decreased dynamic compliance, and perivascular/peribronchial edema. These phenomena were independent on the presence of eosinophils, whereas tracheal hyporeactivity was clearly associated with airway eosinophilia. **Buijs J, Egbers MWE, Lokhorst WH, Svelkoul HFJ, Nijkamp FP. *Toxocara*-induced eosinophilic inflammation: airway function and effect of anti-IL-5. *Am J Respir Crit Care Med* 1995;151:873-8.**

The tissue-migrating stage (larva) of the parasitic worms species *Toxocara* causes pulmonary inflammation in all kinds of mammals including man and small rodents. The inflammatory reaction is characterized by infiltrating eosinophils (1, 2) and, to less extent, lymphocytes (3). Furthermore, toxocarosis goes together with increased IgE levels in serum (2, 3) and bronchoalveolar lavage (BAL) fluid (3, 4). Toxocarosis has these phenomena in common with allergic asthma (2, 5, 6). Whether parasitic infections predispose to or protect against allergic asthma has been a matter of debate for several years (7, 8).

Eosinophil-derived products may affect the airways in several ways as has been shown in *in vitro* experiments by various investigators. Major basic (MBP) and eosinophil cationic proteins (ECP)

and eosinophil peroxidase (EPO) cause damage to the tracheal epithelium (9). Eosinophil-derived leukotriene C₄ (LTC₄) and platelet activating factor (PAF) induce bronchoconstriction and airway hyperreactivity (10, 11). Tissue-invading parasites stimulate IgE production through polyclonal B cell activation and only part of the newly produced IgE is parasite-specific (12, 13). Eosinophils can be stimulated via their low affinity IgE receptors (Fc_εR).

Proliferation and maturation of eosinophils and IgE production are regulated by the cytokines interleukin-5 (IL-5) and IL-4, respectively. Both cytokines are in turn released by the T helper cells of subset 2 (T_H2; 14, 15) and by mast cells (16). Furthermore, interleukin-4 was shown to be produced by Fc_εR⁺, non-T, non-B cells (17). T cell clones producing IL-5 and IL-4 were cultured from peripheral blood lymphocytes of both *Toxocara*-infected (18) and atopic (19) individuals. We showed before in guinea pigs that IL-5 induced bronchial hyperreactivity, whereas antibodies against IL-5 prevented allergen-induced bronchial hyperresponsiveness (20). The present study reports about the effect of a primary infection with *T. canis* on airway functions in BALB/c mice and about the role of eosinophils and IL-5 in the observed changes.

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METHODS

Animals

Male, inbred BALB/c mice, specified pathogen free, 6 to 8 wk old, were obtained from the animal breeding department of the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands.

Toxocara Strain and Infection

T. canis, donated in 1986 by Janssen Pharmaceutica, Parasitology Division, Beerse, Belgium, was maintained in Beagles. Eggs, collected from female worms, were transferred to petri dishes containing 0.1 M H₂SO₄ for embryonation occurring at ambient temperature in 4 to 6 wk. The infective eggs were stored in 0.1 M H₂SO₄ at 4° C until used. Before use, the eggs were washed (3×) with phosphate-buffered saline (PBS), and doses of 1,000 eggs/0.3 ml PBS were prepared. Administration was done by oral intubation on Day 0 of the experiment. Control mice were treated similarly with 0.3 ml PBS. Estimation of tracheal reactivity and cell collection by bronchoalveolar lavage were done at 14 d post-infection as changes in cell composition and tracheal reactivity were marginal from that day until at least 5 wk post-infection (21). Estimation of RL and C_{dyn} were measured at 28 d post-infection as the mice tended to support anaesthesia less well at earlier days post-infection.

Cholinergic Receptor Activity of Isolated Tracheae

Mice received intraperitoneally a lethal dose of pentobarbital sodium (0.3 ml of a solution containing 60 mg/ml). The tracheae were removed and dissected free of attaching tissue. Lengths of nine trachea rings were mounted in a continuously gassed (carbogen O₂/CO₂: 95%/5%) organ bath containing Krebs bicarbonate buffer.

The isometric method as described by Garssen and colleagues (22) was applied to measure isolated tracheal smooth muscle tone. Cumulative concentration-response curves were performed using carbachol (carbamylcholine chloride OPG Farma, Utrecht, the Netherlands) as cholinergic receptor agonist. Carbachol concentrations ranged from 10⁻⁹ M to 3 × 10⁻⁴ M. Results were recorded in g contraction. No differences were measured in basic muscle tone between infected and noninfected animals, as assessed by the induction of tracheal relaxation using aminophylline and ethylene-diamine-tetra-acetic acid (Na₂ EDTA) as relaxants.

Trachea Incubations with Pulmonary Eosinophils

Mice were inoculated intraperitoneally with 0.3 ml (60 mg/ml) pentobarbital sodium. The lungs were flushed with 5 ml of Ca²⁺-Mg²⁺ free Hanks' Balanced Salt Solution (HBSS) via the right heart ventricle and were then removed with the connected trachea. Bronchoalveolar lavages (BAL) were performed using 3 to 4 ml of HBSS per mouse. The BAL fluid (kept on ice) was pooled and centrifuged at 400 × g (4° C) for 5 min. The supernate was discarded. The pellet resuspended in Tyrode buffer (NaCl 0.137 M, KCl 2.7 mM, NaH₂PO₄ 0.4 mM, NaHCO₃ 12 mM, glucose 5.6 mM, with 1.0 g gelatin, 30 mg DNase per 1,000 ml H₂O destillata), was layered on a discontinuous metrizamide gradient (20%/21%/22%) (Pharmachemie, Haarlem, the Netherlands) and centrifuged at 1,200 × g for 20 min. Cells sedimented at the 22% interphase were collected, washed with PBS (3×), and suspended in Gibco's RPMI-1640 containing L-glutamine and antibiotics. The density of the eosinophils was close to that of the lymphocytes, resulting in co-sedimentation. About 70% of the cells were eosinophils, 24% lymphocytes, and 6% macrophages as estimated from May Grünwald-Giemsa stained smears. Tracheae isolated from normal mice (six to eight/group) were incubated in the presence of either 1 × 10⁵, 5 × 10⁵, 1 × 10⁶, or 5 × 10⁶ cells. Serum (0.1 ml of a 1:5 dilution) from the mice serving as cell donors, added to the incubation medium for enrichment, did not affect tracheal reactivity. The final volume was adjusted to 0.3 ml with RPMI-1640. The tubes were incubated overnight at 37° C, the tracheae were then removed, and cumulative carbachol concentration response curves were measured as described above. Controls consisted of tracheae incubated without cells.

Lung Function Tests

Lung function measurements, i.e., pulmonary resistance (RL) and dynamic compliance (C_{dyn}) were performed according to a technique developed

for guinea pigs (23) and adapted to the mouse by Garssen and associates (24). Measurements were performed in sublethally anaesthetized (1.25 mg of pentobarbital sodium/mouse), spontaneously breathing mice, placed in a heated chamber (37° C). A canule filled with saline was inserted into the esophagus and another into the trachea. Airflow (V) and tidal volume (V_t) were determined (Gould Godart Pneumotachograph, Bilthoven, the Netherlands). Transpulmonary pressure (P_L) was measured (Validyne MP-45-2 pressure transducer, Northridge, CA). RL and C_{dyn} were determined breath-by-breath according to a modified method of Ambudur and Mead (25) using a computerized respiratory analyzer (24). RL is expressed in cm H₂O ml⁻¹ s⁻¹ and C_{dyn} in ml cm⁻¹ H₂O.

Hybridoma Cell Lines and Encapsulation of Hybridoma Cells in Alginate

Hybridoma cell lines producing rat monoclonal antibody to mouse IL-5 (TRFK-5) and the isotype-matched control anti-*Escherichia coli* β-galactosidase (GL-113) were kindly donated by Dr R. L. Coffman, DNAX Research Institute, Palo Alto, CA. The cells were maintained in Gibco's RPMI-1640 supplemented with 25 mM HEPES, 10% FCS, L-glutamine, and antibiotics. A method of encapsulation (26) modified by Savelkoul and co-workers (27) was used. Hybridoma cells, collected from the growth medium by centrifugation, were washed thoroughly with saline and adjusted to 6 × 10⁶/ml. The cell suspension was mixed with 1.2% alginate (FMC Corp., Rockland, ME) at a ratio of 1:2, and transferred to a syringe fitted with a 26-gauge needle. Enclosure of the cells in capsules was obtained by injecting the suspension in 80 mM CaCl₂ solution in a petri dish mounted on a rotator. The capsules were washed immediately with saline (3×). 1 ml of the final suspension (1 × 10⁶ cells) was inoculated intraperitoneally on Day 0 of the experiment. This procedure was repeated using freshly prepared capsules on Day 7 post-infection. Control groups included a) noninfected/nontreated mice; b) noninfected and anti-IL-5 treated mice; c) noninfected and isotype-treated mice. Infection and treatments, when not applied, were replaced by saline and administered similarly as to the test groups. A control group given empty alginate capsules showed a negligible effect on tracheal reactivity. Each group consisted of eight animals.

Histology

Mice (five animals/group) were anaesthetized intraperitoneally with 0.5 ml KRA/250 g body weight (KRA containing 7 vol of 50 mg/ml Ketalar, Park and Davis, Barcelona, Spain; 3 vol of 2% Rompun, Bayer, Leverkusen, Germany; 1 vol of 1 mg/ml Atropin, Brocacef, Maarssen, the Netherlands). Lungs, removed as described for bronchoalveolar lavage, were fixed by intratracheal instillation of buffered formalin 4% followed by immersion in the fixative. The tissues were processed using a Tissue Tek vacuum infiltration processor (Bayer/Miles). Five-micrometer sections were prepared and conventional staining techniques were performed, i.e., Haemalum Eosine (HE, general impression), May Grünwald-Giemsa (MG, eosinophils).

Statistics

Levels of significance of the concentration response curves between experimental and control groups were calculated by analysis of variance (ANOVA). Significance of differences in E_{max} (maximal contraction) was calculated using the unpaired Student's *t* test. *p* Values ≤ 0.05 were considered significant. Results are presented as the mean ± standard error of the mean (SEM).

RESULTS

Reactivity of Isolated Tracheae to the Cholinergic Receptor Agonist Carbachol

Cumulative carbachol concentration-response curves were measured on tracheae from control (saline-treated) and infected mice at various days post-infection (Figure 1). The overall mean concentration-response curve and E_{max} obtained from infected mice at 14 d post-infection were significantly decreased as compared with the saline treated control group (*p* ≤ 0.01 and *p* ≤ 0.02, respec-

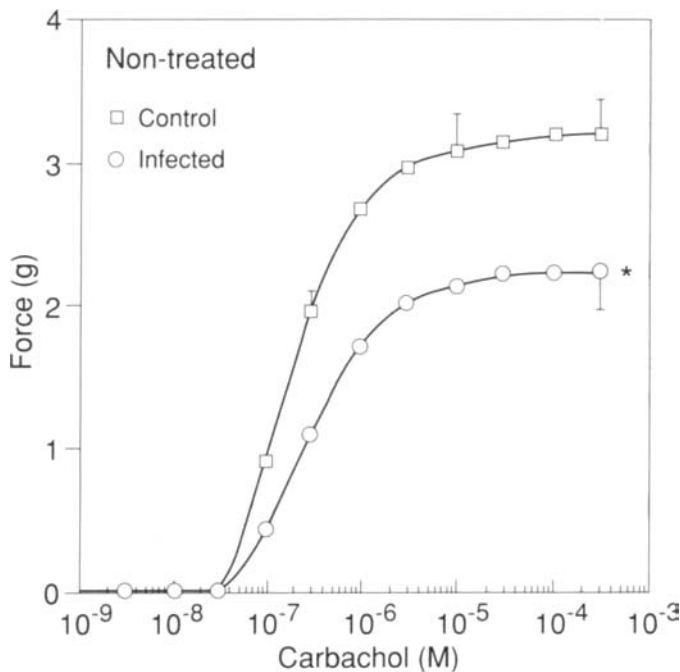


Figure 1. Mean carbachol concentration-response curves of isolated tracheae of noninfected and infected mice at 14 d post-infection. The mean reactivity of the infected group is significantly decreased ($p \leq 0.01$). The $E_{\max}$ is significantly lower in the infected group (* $p \leq 0.02$) ($n = 6$).

tively). *In vitro* incubation of tracheae from normal mice with 1×10^5 and 5×10^5 BAL cells from infected mice (70% eosinophils) shifted the mean carbachol concentration response curve significantly upwards ($p \leq 0.01$; Figure 2), whereas incubation in the presence of 5×10^6 BAL cells significantly decreased the curve ($p \leq 0.01$). Tracheae incubated in the presence of 1×10^6 cells reacted to carbachol stimulation similarly to those of the control group (Figure 2).

Tracheal Reactivity of Infected Mice Treated *In Vivo* with Anti-IL-5 Producing Hybridoma Cells

The tracheae isolated from infected mice treated with isotype control antibody-producing hybridoma cells were hyporeactive to cholinergic receptor stimulation compared with the tracheae from the treated and noninfected control subjects. This difference in responses was similar to that of corresponding groups of mice that were not treated with control antibody. The mean carbachol concentration-response curves of tracheae isolated from noninfected and infected mice treated with anti-IL-5 producing hybridoma cells were similar. Comparing tracheal reactivity of noninfected, nontreated mice with that of noninfected, anti-IL-5 treated mice, a downward shift in the concentration response curve of the latter was observed. The $E_{\max}$ dropped by 35% (Figure 3).

Pulmonary Resistance and Dynamic Compliance and the Effect of Anti-IL-5 Treatment

The effect of airway inflammation on parameters of pulmonary function was examined 28 d post-infection. The pulmonary resistance (RL) was significantly increased ($p \leq 0.001$; Figure 4A) and the lung dynamic compliance (C_{dyn}) was significantly decreased ($p \leq 0.05$; Figure 4B) in the infected mice as compared to the noninfected control subject.

Treatment with anti-IL-5 and isotype control antibody had no

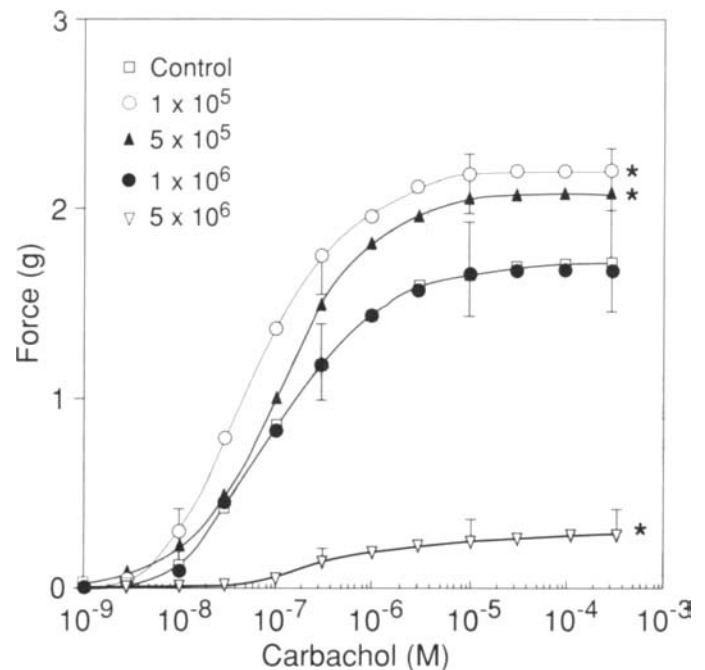


Figure 2. Mean carbachol concentration-response curves of isolated tracheae from normal mice after incubation (16 h) with various numbers of BALF cells (70% eosinophils). Control, 1×10^5 and 5×10^5 cells, 1×10^6 , and 5×10^6 cells. The decrease in reactivity is dependent on the number of cells added to the incubation sample ($n = 6$) (* $p \leq 0.05$).

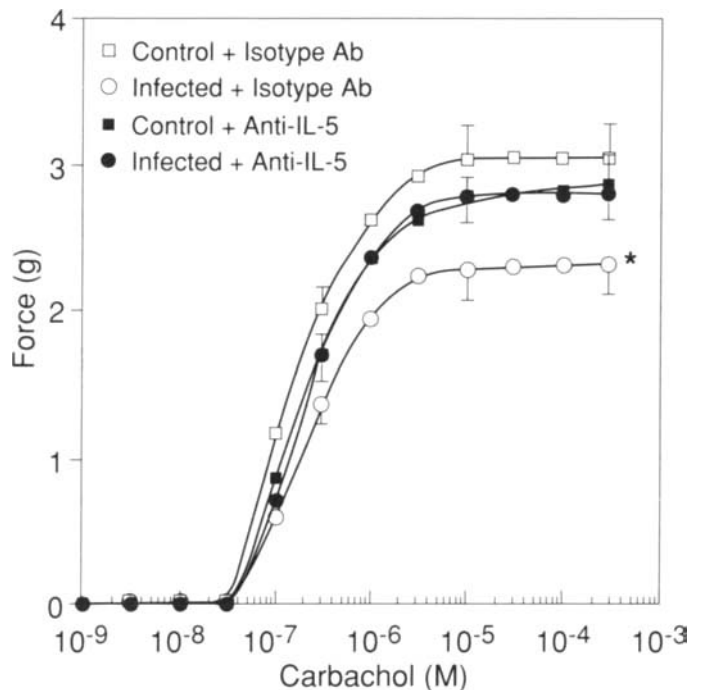


Figure 3. Mean carbachol concentration-response curves of isolated tracheae from noninfected and infected mice, treated with either isotype control antibody or anti-interleukin-5. Isotype control antibody did not affect the tracheal response. Anti-IL-5 prevented tracheal hyporeactivity. (* $p \leq 0.05$).

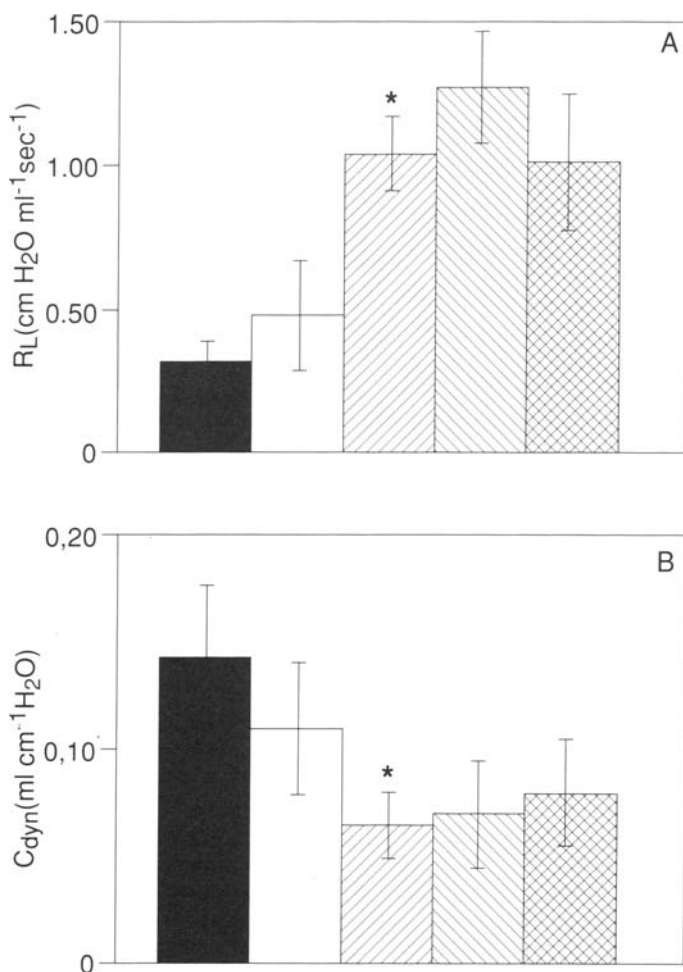


Figure 4. Pulmonary resistance (RL) (panel A) and lung dynamic compliance (C_{dyn}) (panel B) of noninfected (black bars) and infected mice measured at 28 d post-infection. The RL of the infected mice is significantly increased ($p \leq 0.01$) and the C_{dyn} is significantly decreased ($p \leq 0.05$) (hatched bars) as compared with the noninfected control subjects (black bars). RL and C_{dyn} of infected mice treated with anti-IL-5 (cross-hatched bars) were not different from the infected isotype-treated (horizontally striped bars) and from the infected nontreated mice (hatched bars). Noninfected anti-IL-5 treated mice (white bars) were not different from the nontreated controls (black bar).

significant effect on RL (Figure 4A) and C_{dyn} (Figure 4B) of the noninfected or the infected mice.

Effect of Treatment with Anti-IL-5 on the Eosinophil Response in Lung Tissue

Lung sections of infected mice showed mixed cellular infiltrates in which eosinophils were dominating; lymphocytes and macrophages were observed to a lesser extent. Perivascular and peribronchial edema and activated airway epithelium was observed. Anti-IL-5 treatment prevented the infiltration of eosinophils in the airways, but not that of lymphocytes and macrophages at both days examined (Figure 5A, 28 d post-infection). Treatment with isotype control antibody did not prevent eosinophil infiltration at either day examined (Figure 5B, 28 d post-infection). Histologically, except for the absence of eosinophils in the anti-IL-5-treated mice, the lung sections of infected mice nontreated or treated with either anti-IL-5 or isotype control antibody were similar. All infected mice showed inflammatory cell infiltration, swollen airway epithelium, and perivascular/peribronchial edema at both days examined (Figure 6A). A section of the lungs of noninfected, nontreated mice showing normal histology is presented in Figure 6B.

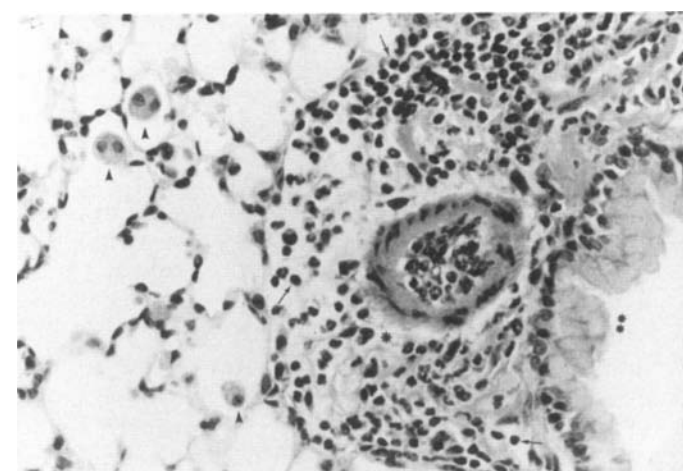
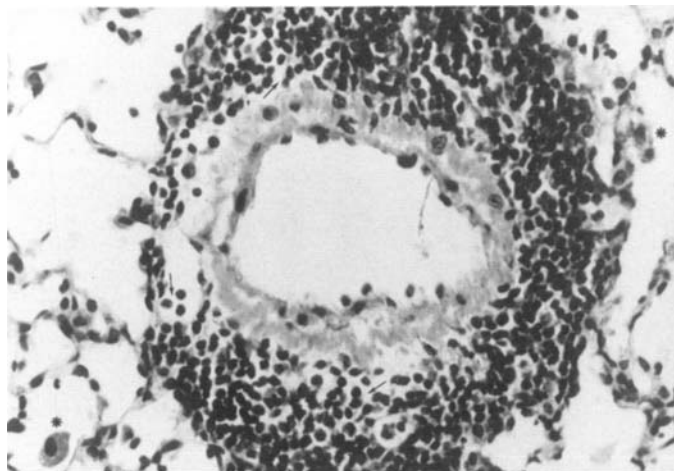


Figure 5. Cross-section of the lungs of *Toxocara canis*-infected mice treated with anti-interleukin-5 (A) showing mononuclear cell infiltration (arrows—lymphocytes; asterisk—macrophages); or (B) treated with isotype control antibody showing mixed cellular infiltration and activated airway epithelium (asterisk—eosinophils; arrows—lymphocytes; arrowheads—macrophages; two asterisks—activated airway epithelium). (HE stained; objective 40 $\times$; ocular 10 $\times$).

lium, and perivascular/peribronchial edema at both days examined (Figure 6A). A section of the lungs of noninfected, nontreated mice showing normal histology is presented in Figure 6B.

DISCUSSION

Infection of BALB/c mice with *T. canis* caused chronic airway inflammation of mixed cellular character in which eosinophils were the dominating cell type. The number of eosinophils in the bronchoalveolar lavage fluid (BALF) increased from $\leq 10^4$ in noninfected animals, to about 1.5×10^6 /BALF in infected animals (3). A decrease in cholinergic receptor responsiveness of the trachea, an increase in pulmonary resistance (RL), and a decrease in dynamic compliance (C_{dyn}) occurred concurrently with eosinophil infiltration in the upper and lower airways. The present study aimed at elucidating the role of eosinophils in the observed changes in airway function parameters. Cholinergic receptor responsiveness of normal tracheae was shifted upward after incubation with a low number of BAL cells (70% eosinophils), and downward after incubation with a larger number of cells. *In vivo* treatment with anti-

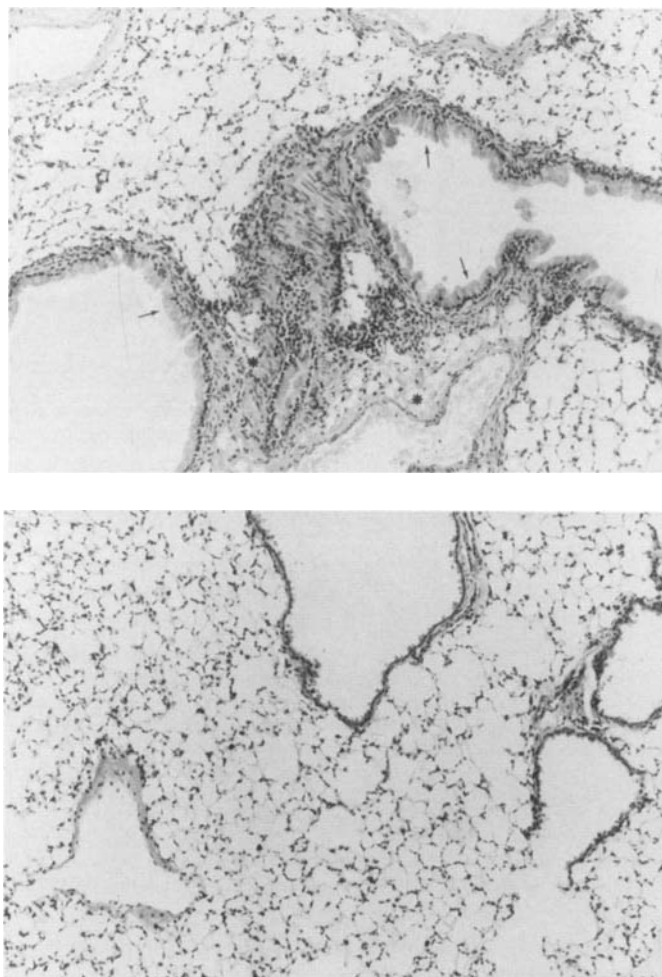


Figure 6. Cross-section of the lungs of *Toxocara canis*-infected mice treated with anti-IL-5 (A) showing edema (asterisk) and activated airway epithelium (arrows). B: cross-section of the lungs of a noninfected mouse showing normal histology. (HE stained; objective 40 $\times$; ocular 10 $\times$).

IL-5 abolished eosinophil response and tracheal hyporesponsiveness but not the increase in pulmonary resistance, the decrease in dynamic lung compliance, or edema formation. This study also demonstrated the dependency of eosinophil proliferation on IL-5 in *T. canis*-infected mice, as was shown for other tissue nematodes before (28, 29).

Perhaps the downward shift of the incubated tracheae (5×10^6 cells) was a consequence of inflicting serious damage to the trachea. Eosinophils may release various mediators injurious to tissues. Eosinophil cationic (ECP) and major basic (MBP) granule proteins and eosinophil peroxidase have been shown to damage guinea pig tracheal epithelium *in vitro* (9). Uchida and associates (30) showed that airway instillation of MBP caused a short-lived increase in airway responsiveness, for which epithelial shedding was not a prerequisite. It can be argued that contractile responses of the airways initiated by inflammatory mediators is concentration-dependent. Involvement of lymphocytes in the observed decrease in cholinergic receptor responsiveness could not be excluded in the incubation experiments. However, the finding that tracheal hyporeactivity did not occur after *in vivo* administration of anti-IL-5, thus eliminating eosinophil proliferation, suggested that directly or indirectly, primarily eosinophils were

responsible. Eosinophils were present in small numbers in the BALF of noninfected and nontreated mice (3). Anti-IL-5-treated, noninfected mice showed a decrease in tracheal reactivity reaching a level similar to the upward shifted curve of anti-IL-5-treated and -infected mice. This finding may indicate that eosinophils codetermine airway tone. Various substances, released in consequence of the inflammatory process, may have caused the tracheal hyporesponsiveness. Frossard and colleagues (31) showed that sensory neuropeptides, in particular substance P, induced the release of relaxant factors from airway epithelium, including cyclooxygenase-derived products and epithelium-derived inhibitory factor (EpDIF). Nijkamp and coworkers (32) investigated normal and epithelium-denuded guinea pig tracheae for nitric oxide (NO) synthesis. They suggested that trachea epithelium contributed to NO synthesis and played an important role in modulating airway tone. Furthermore, Rettori and associates (33) suggested that NO stimulated the release of prostaglandin E_2 (PGE $_2$) by activating cyclooxygenase. Eosinophils also have the potential to release PGE $_2$ (34). The possibility of epithelium- or/and eosinophil-derived PGE $_2$ playing a role in the downward shift of tracheal reactivity needs further investigation. We found that the PGE $_2$ concentration in BAL fluid was significantly increased at 2 and 4 wk after infection (preliminary results).

Contrary to the reactivity of the trachea, the observed increase in airflow resistance and decrease in dynamic compliance were not affected by eosinophil ablation and seemed to be dissociated from eosinophil infiltration. This finding was supported histologically by demonstrating that the perivascular and peribronchial edema and activated airway epithelium were not affected by anti-IL-5 treatment. Submucosal edema is often seen in airway inflammation and is a feature of asthma. Edema may cause a decrease in airway caliber and thus affect RL (35).

In conclusion, the present investigation showed that *Toxocara*-induced eosinophilic airway inflammation caused opposite effects on tracheal cholinergic receptor responsiveness and RL and C_{dyn} . The *in vitro* incubation experiments suggested that induction of tracheal hyporesponsiveness depended on the height of tissue eosinophilia. Effects observed at the tracheal level could be inhibited by the elimination of eosinophils, which was not the case for the effects observed in the lungs. The increased airway resistance and decreased lung dynamic compliance seemed to be dissociated from eosinophil infiltration. The mechanisms regulating airway functions in the trachea appear to differ from those in the lungs. We compared airway functions in the presence and absence of the intact eosinophilic cells and not that of isolated products. A role for eosinophils in edema formation and lung function parameters *in vivo* could not be substantiated in the present study.

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