

Infection of BALB/c mice with *Angiostrongylus costaricensis* decreases pulmonary inflammatory response to ovalbumin

L. A. PINTO, P. M. C. PITREZ, G. R. FONTOURA, D. C. MACHADO, M. H. JONES, C. GRAEFF-TEIXEIRA & R. T. STEIN

Instituto de Pesquisas Biomédicas/Pontifícia Universidade Católica do Rio Grande do Sul

SUMMARY

The prevalence of asthma in developing countries is lower than in developed countries. Viral, bacterial and parasitic infections may be associated with this discrepancy. The relationship between parasitic infection and asthma prevalence is not clear. Previous controversial data have demonstrated that parasitic infection may either predispose or protect against the development of asthma. The aim of this study is to determine whether infection with Angiostrongylus costaricensis (A. costaricensis) decreases inflammatory lung response to ovalbumin (OVA) in mice. Seven BALB/c mice were infected with A. costaricensis by orogastric gavage (10 larvaelmouse) on day (D) 0. The mice were immunized against OVA by intraperitoneal injection on D 5 and D 12 and received an intranasal OVA challenge (40 µL) on D 15 and D 17. On D 19 bronchoalveolar lavage (BAL) was performed. Six BALB/c mice (control group) were immunized with OVA using the same protocol, but were not infected with A. costaricensis. Interleukin (IL)-1β and IL-6 levels were measured in the BAL fluid by using commercial ELISA assays. Total cell counts and differential cell counts were performed in the BAL fluid samples. The group infected with A. costaricensis had lower total cell count in the BAL fluid when compared with the control group (0.11×10^6 cells/mL and 0.3×10^6 cells/mL, respectively; $P = 0.013$). BAL fluid IL-1β levels in the infected group were significantly lower than in the control group ($P = 0.008$). IL-6 levels in BAL fluid were not different between the groups studied. We conclude that Angiostrongylus costaricensis infection in mice decreases pulmonary inflammatory response to OVA.

INTRODUCTION

Epidemiological studies have demonstrated an elevated and increasing prevalence of asthma in developed countries. However, asthma and allergies have shown a lower prevalence in the developing world (1). Von Mutius and colleagues also demonstrated that the prevalence of asthma in an area of East Germany was lower when compared to cities in West Germany, suggesting that environmental factors may play a significant role in the pathogenesis of asthma and atopy (2).

Viral, bacterial and parasitic infections may be associated with this discrepancy (3). The relationship between parasitic infection and allergic disorders is not clear. Epidemiological studies have shown that different parasitic infections are associated with reduced risk of allergic skin sensitization and allergen-specific IgE levels in children (4,5). In relation to asthma, however, the data are less clear. Controversial data have demonstrated that parasitic infection may either predispose or protect against the development of asthma (6,7).

Murine models have been useful in understanding the mechanisms of the development of asthma. Wang and colleagues demonstrated that a pre-existing helminth infection with *Strongyloides stercoralis* may suppress allergic response in the lungs of mice sensitised with OVA (8).

Angiostrongylus costaricensis (*A. costaricensis*) is a nematode of wild rodents with widespread occurrence in Central and South America (9). Transmission to humans occurs by ingesting infected slugs (intermediate host) or contaminated food or water. The infection affects adults and children. In Brazil, the incidence of infection by *A. costaricensis* is increasing (10). Geiger *et al.* have demonstrated a predominantly Th1 cytokine profile in *A. costaricensis* infected BALB/c mice, with the gut as its primary site of infection (11). Therefore, this nematode could be useful as an experimental model for the study of the association between asthma and intestinal parasitic infection. To the authors' knowledge, experimental studies with this parasite have not been published.

The aim of this study was to determine whether infection with *A. costaricensis* decreases inflammatory lung response to ovalbumin (OVA) in mice.

Correspondence: Paulo Pitrez, Instituto de Pesquisas Biomédicas/Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6690, Porto Alegre – RS, CEP 90.610-000, Brazil (e-mail: pmpitrez@puers.br).

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MATERIALS AND METHODS

Parasites

Angiostrongylus costaricensis infective third-stage larvae (Santa Rosa-Brazil strain) were isolated from previously infected *Biomphalaria glabrata* as intermediate host. Infective third-stage larvae were obtained from infected molluscan fibromuscular tissues after the snails had been minced and artificially digested with a 2 h incubation, at 37°C, in 0.03 g percentage pepsin solution. Larvae were isolated according to the Baermann technique.

Mouse infection

Thirteen BALB/c mice were used for the study. Seven BALB/c mice were infected with *A. costaricensis* by orogastric gavage (10 larvae/mouse) on the first day. Six non-infected mice were used as the control group.

Ovalbumin immunization

Both groups (infected and non-infected), received the first dose (100 µg) of ovalbumin (OVA, Grade V, Sigma, USA), plus 100 µg of alum (aluminium hydroxide hydrate, Sigma, USA), diluted in phosphate-buffered saline (PBS, total volume: 200 µL), by intraperitoneal (i.p.) injection on day 5. After 7 days (day 12), a second dose was injected, as previously described.

Ovalbumin challenge

OVA solution (100 µg of OVA, in 50 µL of PBS) was instilled by the nasal route on days 17 and 18. To facilitate pulmonary aspiration, mice were lightly anaesthetized with ketamine (2 mg) and xylazine (0.2 mg), by i.p. injection, before intranasal OVA administration.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed on day 19. Mice were anaesthetized with ketamine (5 mg) and xylazine (2 mg) solution by i.p. injection before the procedure. Tracheostomy and BAL were performed. BAL was obtained with a single 1 mL aliquot used three times. After BAL procedure, mice were killed with a lethal dose of ketamine/xylazine solution (0.3 mL).

Total cell counts

The samples were centrifuged at 400 g for 2 min and the supernatant removed and frozen (at -80°C) for cytokine

analysis. The cell pellet was resuspended in 1 mL of PBS and the total cell count (TCC) assessed by use of a haemocytometer (Boeco, Germany) and viability assessed with trypan blue.

Differential cell counts

For preparation of slides for differential cytology, 40 µL of the sample was centrifuged (FANEM, São Paulo, model. 218) at 100 g for 5 min. Slides were fixed with methanol and stained with May-Grunwald Giemsa. Cells were analysed according to their morphology. The cell types at light microscopy were expressed in percentage after counting 200 cells.

Cytokine analysis

Levels of IL-1β and IL-6 were measured in the BAL supernatant using the manufacturer's recommended ELISA protocol (Pharmingen, USA).

Statistical analysis

Values are described as mean and median, and the statistical difference was calculated by the Mann-Whitney test. Differences were considered significant with $P < 0.05$.

Ethics

The study was approved by the Ethics Committee for Animal Research of our Institution and was based on animal models research guidelines.

RESULTS

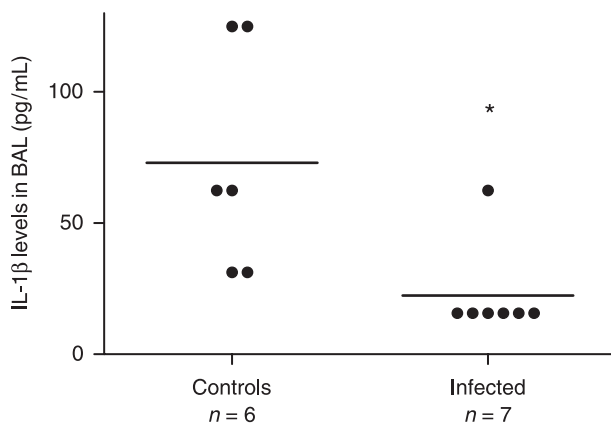
BAL was performed successfully in all studied mice. The mean of returned volume of BAL was 72.3% (SD: 11.7) and median cell viability was 100%. The mean TCC in all mice studied was 0.2×10^6 cells/mL.

The group infected with *A. costaricensis* had significantly lower TCC in the BAL fluid when compared with the control group (0.11×10^6 cells/mL and 0.3×10^6 cells/mL, respectively; $P = 0.013$, Table 1). Differential cell counts have shown a predominance of macrophages and neutrophils (> 70%), in both infected and non-infected mice. However, in the infected group there were significantly less neutrophils and macrophages than in the control group (Table 1). On the other hand, mean eosinophil percentage was higher in mice with pre-existing helminth infection (18% and 8%, respectively), but the difference was not statistically significant.

IL-1β levels from BAL fluid in the infected group were significantly lower than in the control group ($P = 0.008$, Figure 1). IL-6 levels in the infected group were not significantly different from the control group (Figure 2).

Table 1 Total cell counts and differential cytology between the groups studied

	Infected $\times 10^6$ cells/mL (%)	Control $\times 10^6$ cells/mL (%)	P-value
Total cell count	0.11	0.3	0.01
Macrophages	0.04 (46.5)	0.08 (33.5)	0.06
Neutrophils	0.04 (25)	0.15 (47)	0.03
Lymphocytes	0.02 (6.6)	0.03 (10.3)	0.24
Eosinophils	0.01 (18)	0.03 (8)	0.18

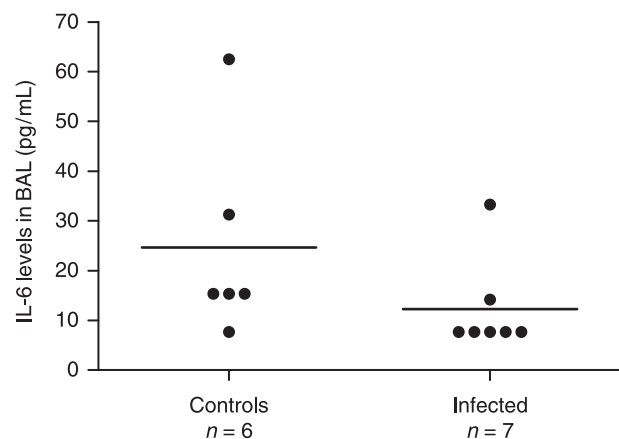
**Figure 1** Comparison of IL-1 β levels in BAL between the groups studied ($P = 0.008$).

The infected mice presented macroscopic changes compatible with *A. costaricensis* disease: 6/7 (85%) of infected mice had inflammatory changes on liver and/or spleen. We have also found *A. costaricensis* larvae in mesenteric arteries in two mice. There were no larvae found in BAL of infected mice.

DISCUSSION

The results of this study suggest that *A. costaricensis* infection in mice decreases pulmonary inflammatory response to OVA. We have shown a suppressive effect in TCC and neutrophils in BAL fluid of infected mice. We have also shown that IL-1 β levels are significantly decreased in the lungs of the infected group. To the authors' knowledge, there are no previous reports studying the impact of the allergic pulmonary response caused by this nematode.

In Gabonese children, *Schistosoma* infection was associated with a lower prevalence of a positive skin reaction to house-dust mite (4). Studies from Venezuela have demonstrated that parasite eradication increases allergic skin sensitization and allergen-specific IgE levels in children and appears to have caused improvement in clinical asthma (4,12). Asthma has also been reported to be less common in Taiwanese children infected with pinworm (13). In Brazil, Medeiros *et al.* have demonstrated that wheezing may be less severe in asthmatic children infected with *Schistosoma*

**Figure 2** Comparison of IL-6 levels in BAL between the groups studied ($P = 0.07$).

(6). On the other hand, in a Gambian study helminthic infection was not associated with wheeze (14). Palmer *et al.* also reported an increased risk of childhood asthma and allergic sensitization in association with *Ascaris* infection in China (7).

Taking all these data together, these controversial results may be explained by the different populations and types of parasites under study. Moreover, a species-specific effect may exist and confusion in the literature could have arisen in part because of infection with more than one parasite by each host (15). Therefore, studies with murine models are useful for this purpose.

Different hypotheses are proposed to explain how certain infections may prevent atopic diseases. Firstly, exposure to an intracellular pathogen, inducing a Th1 immune response, would down-regulate atopic Th2 response later in life. Secondly, a Th2 response to a specific parasite could inhibit Th2 response to aeroallergens, and lastly, a chronic strong anti-inflammatory response triggered by parasitic infection could help to prevent the events leading to allergic diseases (16).

Previous studies in animal models have suggested that nematodes have the capacity to inhibit allergic pulmonary response. *Strongyloides stercoralis* infection in mice inhibits eosinophilic pulmonary inflammation (8). Lima and colleagues has also demonstrated that adult worm extract from

Ascaris suum had a profound inhibitory effect on pulmonary allergic response (17). In contrast to these results, we have shown that infection with *A. costaricensis* did not significantly inhibit eosinophilic airway inflammation. However, IL-1 β levels, total cell counts and neutrophil counts were significantly decreased in BAL fluid of infected mice. These findings support the hypothesis that different parasites may cause different immune responses in the host.

Pro-inflammatory cytokines are strongly associated with airway inflammation in asthma. Levels of IL-1 β in BAL fluid of patients with asthma were found to be increased compared with those of non-asthmatic volunteers (18). IL-1 β induces airway neutrophilia and increases airway responsiveness to bradykinin in rats (19). IL-6 is increased in alveolar macrophages from asthmatic patients after allergen challenge compared with non-asthmatic subjects (20). Moreover, previous reports have demonstrated that neutrophils may play a role in the inflammatory response in asthma, including the acute phase. Jatakanon and colleagues have shown that neutrophil counts are increased in the sputum of subjects with severe asthma when compared with patients with mild asthma and normal control subjects (21). Other studies also demonstrated a relationship between neutrophilic inflammation and asthma exacerbation (22,23). Our study suggests that *A. costaricensis* could inhibit specifically this type of neutrophilic inflammation and IL-1 β production in a murine model.

In regard to the decreased airway inflammatory response in infected mice, one may not exclude that the pulmonary immune response was attenuated because of the severe inflammatory response in the intestinal wall, which is the main site of *A. costaricensis* infection (24). Therefore, one may speculate that infections that affect mostly the gut could induce a protective environment in the airways for either atopic or non-atopic types of asthma.

In summary, *A. costaricensis* infection suppresses the inflammatory response in the airways and inhibits neutrophil influx to the lungs. However, eosinophil counts are not decreased, suggesting that this parasite may trigger a different immune response in OVA-challenged airways, compared to other helminths. Our findings support the species-specific hypotheses, with different parasites inducing different immune responses in the host, and consequently in subjects with asthma. Further investigations in murine models with different parasites analysing other cytokines, such as IL-4, IL-5 and IL-10, are required to better understand the relationship between asthma and parasitic infections.

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