ARTIGO

INGESTION OF INACTIVATED THIRD STAGE LARVAE OF *Angiostrongylus costaricensis* DOES NOT RESULT IN DETECTABLE HUMORAL IgG RESPONSE IN MICE

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ABSTRACT

Angiostrongylus costaricensis is a nematode parasite of wild rodents. In a previous experiment, it was demonstrated that bleach water (1.5%) eliminates the infective third stage larvae that may be present on vegetables and fruits. In that experiment, sera from mice inoculated with inactivated larvae had humoral reactivity against crude antigen of *A. costaricensis* detected by Western-blot. In order to confirm and analyse this finding, we used 3 groups of Swiss females, 8 weeks-old mice: a negative control group (NC), a positive control group (PC) and an experimental group (EG). Mice of EG group were inoculated *per os* with inactivated larvae. After 28 days, the mice of PC and EG groups were inoculated with 15 non-treated infective third stage larvae. No significant differences favoring a protective response were detected in the variables: survival, number of worms, morbidity and humoral reactivity. In conclusion, there were neither evidences of a strong humoral response nor protection after oral inoculation of inactivated *A. costaricensis* larvae.

KEYWORDS: *Angiostrongylus costaricensis*. Zoonosis. Protective immunity. IgG.

INTRODUCTION

Abdominal angiostrongyliasis is an helminthic infection caused by *Angiostrongylus costaricensis*, an intra-arterial rodent parasite (Morera 1973). The source of the infective third stage larvae (L3) are the intermediate hosts, molluscs from the Veronicellidae family. Vertebrates become infected through ingestion of L3, either present in inadvertedly ingested molluscs, foods containing infected mucous of these invertebrates, or contaminated water (Morera 1988, Ubelaker1988). Zanini et al. (2001) have demonstrated the larvicial efficacy of 1.5% bleach water

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solution and reported (unpublished data) the detection of IgG humoral response after oral infection with inactivated larvae, in a murine experimental model. Therefore, the use of natural or induced infections and mucosal immunization with specific vaccine antigens remains an attractive possibility for immunization against infections, especially those acquired through mucosal surfaces (Ogra 2001).The objective of this experiment was to confirm and evaluate the IgG response against inactivated *A. costaricensis* L3.

MATERIAL AND METHODS

Parasite

Infective larvae were obtained from the Santa Rosa strain of *A. costaricensis*, that is maintained in our laboratory through passages in *Oligoryzomis sp.* and *Biomphalaria glabrata* Esteio strain. Female worms were recovered through microdissection of the mesenteric arteries at 40-50 days post-infection and stored at -20° C. First stage larvae and L3 were obtained from feces and from artificial digestion of invertebrate hosts, respectively.

Immunological methods

Female worms were used for antigen preparation and a IgG enzyme linked immunosorbent assay (ELISA) was performed as described elsewhere (Geiger et al. 2001), except for the dilution of sera (1:100) and incubation conditions (1h at 37°C). Cut off was defined as the average + 2 sd (standard deviation) of the optical density from duplicate testing of a pool with serum from 6 uninfected animals. Each experimental serum test was performed in duplicate and the results are expressed as a ration (average/cut off).

Western-blot was performed after separation of the antigenic components by SDS-PAGE and tranference to nitrocellulose membranes. Incubation with serum samples at 1:100, 37°C was followed by incubation with (1:1000) peroxidase-conjugated secondary antibodies and colour developement with diaminobenzidine (DAB).

Parasitological method

Feces were individually collected and weighted, larvae were isolated by a modification of Baermann method, as described in Willcox and Coura (1989), and counted under a stereomicroscope.

The experiment

Three groups of Swiss females, 8 weeks-old mice were employed: a negative control group (NC), a positive control group (PC) and an experimental group (EG).

Third stage larvae (L3) were inactivated with bleach water solution (Clorosul S.A., Porto Alegre) at room temperature, 30 minutes (treated larvae) (Zanini et al. 2001).

Ten mice of the experimental group (EG) were inoculated *per os* with 15 L3 per animal with inactivated larvae. After 28 days, the positive control group (PC) and the EG were further inoculated with 15 non-treated L3. Six animals from the negative control group (NC) were kept in the same experimental conditions. Every 7 days animals were weighted and blood was collected through a retro-bulbar puncture, and the serum samples were stored at -20°C until use. Thus, blood was collected before the challenge and after the challenge every 7 days. Dead or sacrificed animals (at 56 days post-inoculation or 28 days post-inoculation with live L3) were examined with annotation of macroscopical lesions, selection of tissue samples for histopathology and counting and gender identification of intra-mesenteric worms.

Bioethics

Animal handling and experimentation was done in accordance with Brazilian legislation (Lei 6.638, 8 May 1979) and the recomendations from Colégio Brasileiro de Experimentação Animal (COBEA).

Statistical analysis

Whenever appropriate, variance analysis (ANOVA), Tukey's tests and significance of differences in proportions were made using SPSS software.

RESULTS

Two animals died, one in the positive control group and the other in the experimental group, while all animals in the negative control group survived. There was no significant difference in the total body weight and relative spleen weight (0.7 and 1.07) among PC and EG. Worms were recovered in PC and EG, 4.12 and 4.37, respectively (Table 1). Antibodies were never detected before the challenge with infective larvae. Only three weeks after the inoculation with live untreated larvae antibodies could be detected in animals from both PC and EG (Figure 1). Likewise, bands were detected by Western-blot only when a pool of serum samples from PC were tested or after the challenge with untreated larvae in EG (Figure 2).

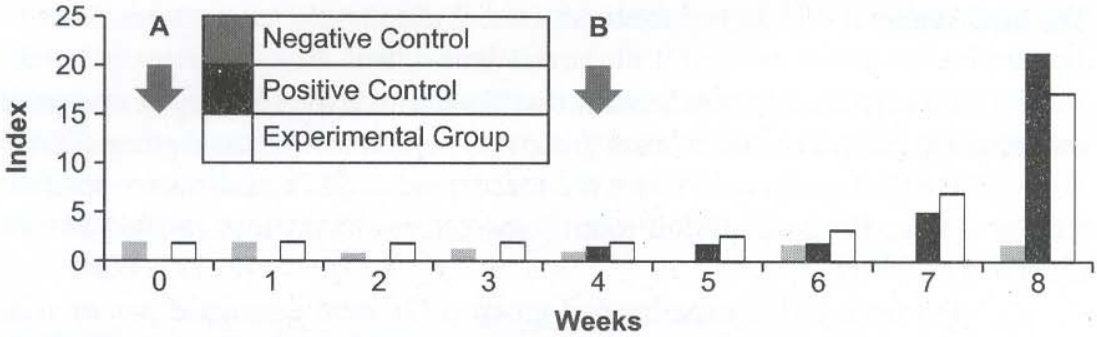

Figure 1. Graph showing results of ELISA tests (IgG), in the different groups: A = inoculation with inactivated larvae, B = inoculation with activated *A. costaricensis* larvae, Index = duplicate averages of the ELISA tests divided by the cut off

Table 1. Recovered worms and larvae and average weights of the spleens in the three groups with experimental infection with *Angiostrongylus costaricensis*

Groups/worms	Females	Males	Total	Average/ animal	% of L3 recovered as worms	Average spleen weights
Positive Control	21	12	33	4.125	27.78	1.07
Experimental	23	12	35	4.37	27.34	0.70
Negative Control	0	0	0	0	0	0.24

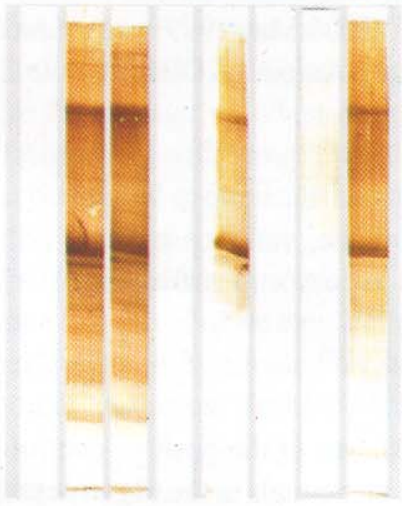

Figure 2. Western-blot analysis of sera (pool) from negative control group (A), external positive control – infected animals from the cycle in the laboratory (B, C), internal positive control: mice inoculated with viable *A. costaricensis* larvae (D = time zero; E = 28 dpi) and experimental group inoculated with inactivated larvae (F = time zero; G = 28 dpi; H = 28 days after challenge with viable larvae)

Anatomopathological examination did not reveal clearcut differences in the distribution and intensity of the lesions, some of them shown in Figures 3 to 6.

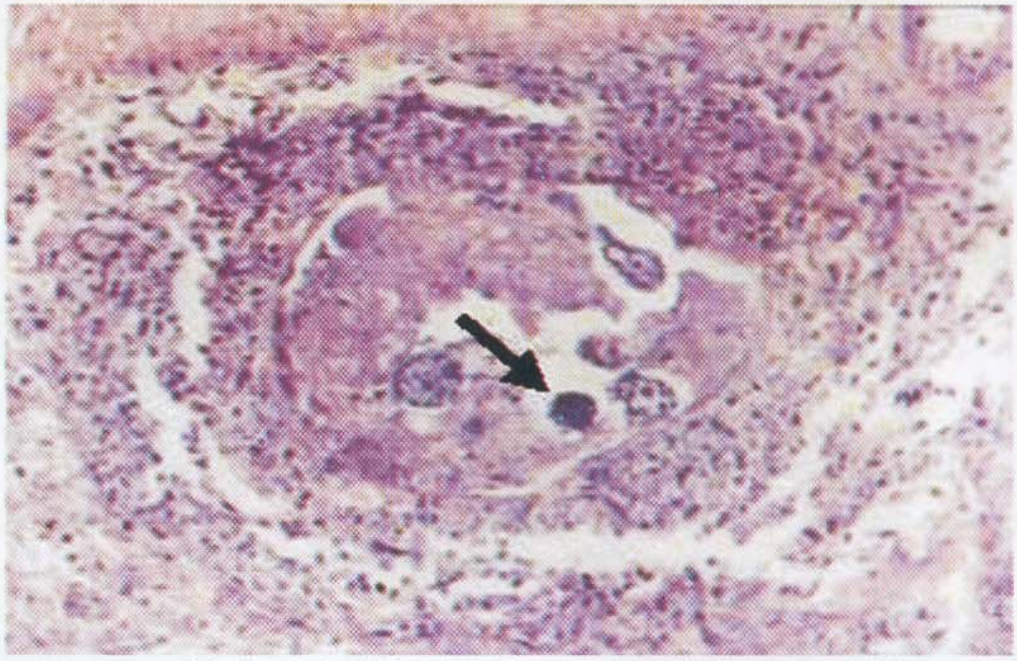

Figure 3. Arterial thrombosis and peri-arterial inflammatory infiltrate in the mesentery of a mice from the experimental group. Degenerated eggs (arrows) at different development stages can be seen amid the intra-arterial thrombi. HE, 400 x

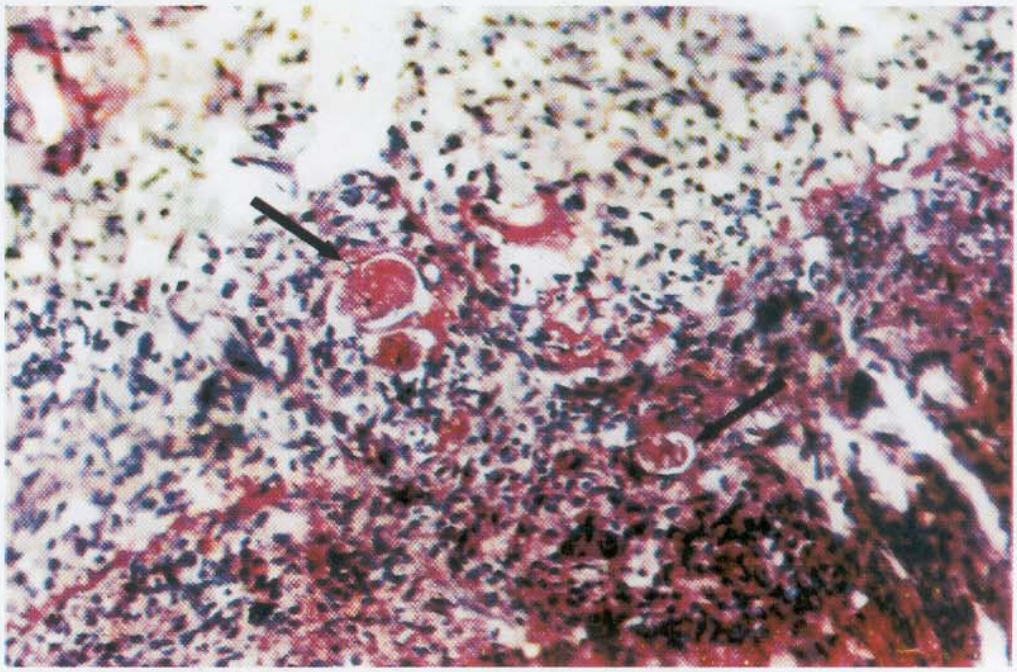

Figure 4. Several degenerated *A. costaricensis* eggs (arrows) are seen in the mesentery next to the serosal surface. Gomori, 400 x

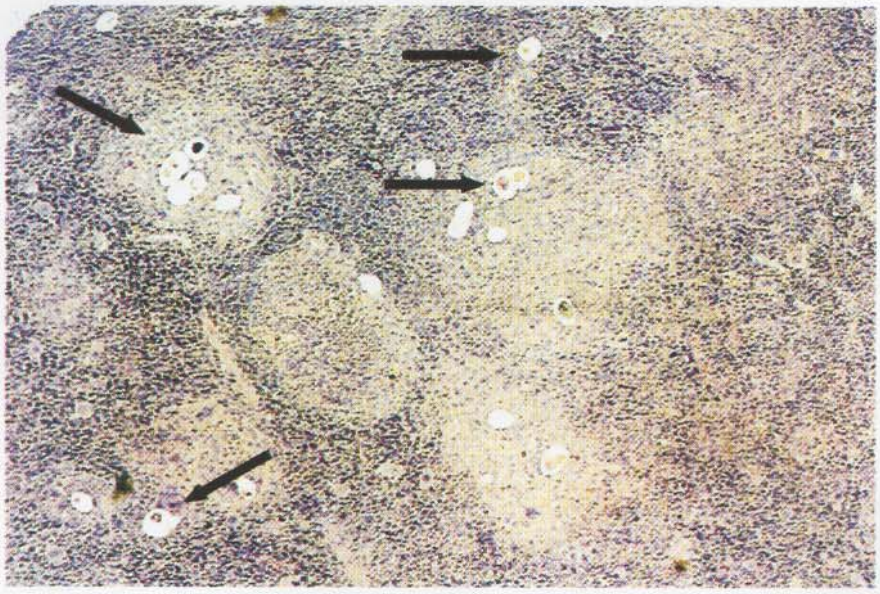

Figure 5. Several *A. costaricensis* eggs (some are indicated by arrows) in the spleen of mice from the experimental group. HE, 100 x

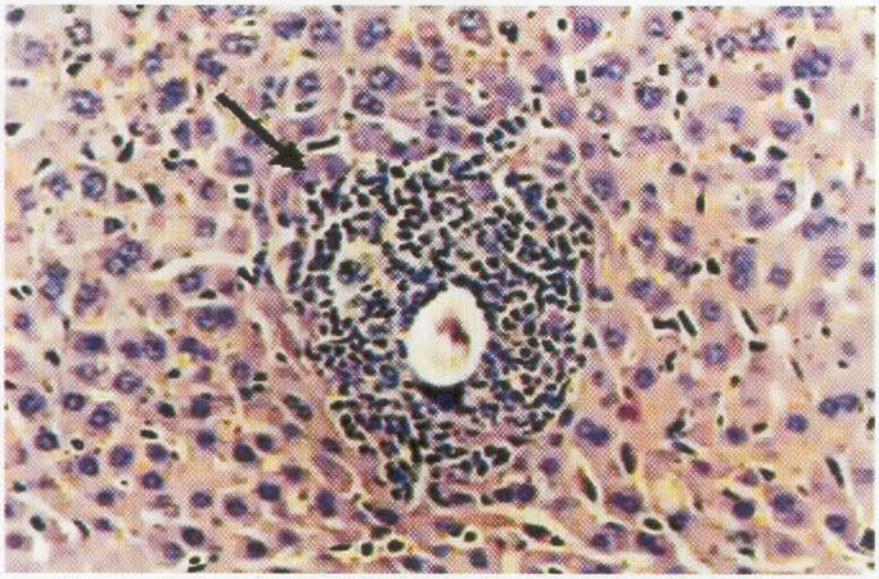

Figure 6. *A. costaricensis* eggs surrounded by granuloma at an exsudative stage in a liver section from a mouse of the experimental group. HE, 400 x

DISCUSSION

To find protective immune responses in experimental models of helminthic infections is a difficult task (Hagan et al. 2004). Therefore the absence of such a response either after exposure with inactivated larvae or after a reinfection with live larvae is not an unexpected result. These experiments were done to

investigate a previous intriguing finding of reactivity shown in immunoblotting with sera from animals inoculated orally with inactivated larvae (GM Zanini, personal communication). Considering the challenge with live larvae in EG, the lack of significant differences in the total body weight, relative spleen weight, survival and morbidity in post-mortem examination, among the EG and PC, clearly demonstrates the lack of any protective response by a previous oral exposure to inactivated L3 larvae. Otherwise this exposure apparently does not elicit any other protective response other than antibody production.

In helminthic infections eventually a large amount of antigenic material is presented to the host's immune system, what may result in downregulation or deviation of the immune response. Another aspect of the experimental model involving an enteroparasite is the developement of immune tolerance after the initial stimulus being presented at the mucosal surface. All these factors may have played a role and may partially explain the observed outcome in the experiments now reported. One strategy to overcome the lack of response after oral antigenic stimulation may be the intra-serosal inoculation with the appropriate adjuvant, leading to antigenic processing in the Payer patches and IgA production by the mucosa. Success will depend on antigens capable of inducing serositis and an increased permeability of serosal surface, paving their way to the Payer lymph patches (Husband et al. 1996).

Adult worms and especially male worms may be missed during the examination of the arterial system and this may explain the "unexpected" reactivity found by Zanini (personal communication). In unisexual infections with male worms, pathogenicity apparently is lower (Santos et al. 1995) and the worms themselves are smaller and not clearly distinguishable inside the mesenteric arterial branches. Other uncontrolled variables could be present in Zanini's experiments, such as cross-reactivity in outbread animals with poor sanitary conditions.

In conclusion, there were neither evidences of a strong humoral response nor protection after oral inoculation of inactivated *A. costaricensis* larvae.

ACKNOWLEDGMENTS

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RESUMO

Ausência de resposta detectável com anticorpos IgG no camundongo após a ingestão de larvas de terceiro estágio de *Angiostrongylus costaricensis*

Angiostrongylus costaricensis é um nematódeo parasito de roedores silvestres cujas larvas infectantes de terceiro estágio podem estar presentes em vegetais e frutas. Essas larvas podem ser mortas com solução de água sanitária a 1,5%,

conforme demonstrado em uma publicação prévia. Nessa publicação, inoculando-se camundongos com larvas inativadas e empregando-se o teste de Western-blot, observou-se que os soros desses animais apresentaram resposta humoral ao antígeno bruto de *A. costaricensis*. Para confirmar e analisar esse achado prévio, foram utilizados, neste trabalho, três grupos de camundongos fêmeas da linhagem Swiss, com oito semanas de idade: um grupo-controle negativo, um grupo-controle positivo e um grupo experimental. Cada animal do grupo experimental foi inoculado, pela via oral, com quinze larvas tratadas. Vinte e oito dias depois, tanto os animais do grupo experimental como os do grupo-controle positivo receberam quinze larvas não tratadas. Usando-se os testes de ELISA e Western-blot, nenhuma diferença significativa a favor de indícios de proteção foi detectada nas diversas variáveis testadas: sobrevida, número de vermes, morbidade e reatividade humoral. Em conclusão, não há evidências nem de forte resposta humoral nem de proteção após a inoculação oral com larvas inativadas de *A. costaricensis*.

DESCRITORES: *Angiostrongylus costaricensis*. Zoonoses. Imunidade protetora. IgG.

REFERENCES

1. Geiger SM, Laitano AC, Sievers-Tostes C, Agostini AA, Schulz-Key H, Graeff-Teixeira C. Detection of the acute phase of abdominal angiostrongyliasis with a parasite-specific IgG enzyme linked immunosorbent assay. *Mem Inst Oswaldo Cruz 96*: 515-518, 2001.
2. Hagan P, Appleton CC, Coles GC, Kusel JR, Tchuem-Tchuenté LA. Schistosomiasis control: keep taking the tablets. *Trends in Parasitology 20*: 92-97, 2004.
3. Husband JÁ, Bao S, Mcclure SJ, Emery DL, Ramsay AJ. Antigen delivery strategies for mucosal vaccines. *Internat J Parasitol 26*: 825-834, 1996.
4. Morera P. Life history and redescription of *Angiostrongylus costaricensis* Morera & Cespedes, 1971. *Am J Trop Med Hyg 22*: 613-621, 1973.
5. Morera P, Andrews KL, Rueda A. Intermediate host of *Angiostrongylus costaricensis* in Honduras. *Rev Biol Trop 36*: 575-576, 1988.
6. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. *Rev Clin Microbiol 3*: 641, 2001.
7. Santos FT, Pinto VM, Graeff-Teixeira C. Evidences against a significant role of *Mus musculus* as natural host for *Angiostrongylus costaricensis*. *Rev Inst Med trop São Paulo 38*: 171-175, 1996.
8. Ubelaker JE, Bullick GR, Caruso J. Emergence of third stage larvae of *Angiostrongylus costaricensis* Morera & Céspedes, 1971 from *Biomphalaria glabrata* (Say). *J Parasitol 66*: 856-857, 1980.
9. Zanini GM, Graeff-Teixeira C. Inactivation of infective larvae of *Angiostrongylus costaricensis* with short time incubations in 1.5% bleach solution, vinegar or saturated cooking salt solution. *Acta Tropica 78*: 17-21, 2001.