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Long-term observations on mouse strains experimentally infected with *Angiostrongylus costaricensis*

Abstract BALB/c and C57BL/6 mice were experimentally infected with *Angiostrongylus costaricensis* and the parasitic parameters and antibody response during the acute and chronic phases of infection were analyzed. Following administration of six third-stage larvae (L3), there was no significant difference in the mean worm recovery or mean larval output. Coinciding with the maturation of worms in infected animals and with the egg output in mesenteric arteries, a strong increase in the humoral immune response was observed in both mouse strains. This response was characterized by a hypergammaglobulinemia, with a predominance of IgA and IgG1 during the acute phase of infection, and IgG1 and total IgE during the patent and post-patent periods. Significantly higher levels of IgM, IgG and IgG1 were found in BALB/c mice compared with C57BL/6 mice. On the other hand, a significantly higher concentration of IgA was detected at 6 and 7 weeks post-infection in C57BL/6 mice compared with BALB/c mice. Specific IgE could not be detected in any of the mouse strains. Our results suggest that immunoglobulins, mainly IgG1, contribute to the outcome of a primary *A. costaricensis* infection with respect to the period of patency and to mortality during the chronic phase.

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Introduction

Angiostrongylus costaricensis is a parasitic nematode with a widespread occurrence throughout Central and South America (Morera 1985; World Health Organisation 1987; Graeff-Teixeira et al. 1991). The natural life cycle of *A. costaricensis*, which may accidentally cause abdominal angiostrongyliasis in humans, involves mainly the rodents *Sigmodon hispidus* and *Oryzomys* spp. as the definitive vertebrate hosts. At present, experimental studies on small rodents offer the sole possibility of characterizing this parasitosis in terms of immune response and parasitological outcome during the course of an infection. However, such experimental investigations in natural hosts, as well as in several inbred mouse strains, have addressed the question of differences in mortality and in humoral and cellular immune responses only during the acute phase, e.g. during the first 4–5 weeks of infection (Ishii and Sano 1989; Ishih and Nishimura 1997; Geiger et al. 1999, 2001) . Longitudinal studies on larval output, worm recovery and host survival rates are scarce. In the present study, we aimed at investigating the parasitological outcome and the humoral immune response after a low dose *A. costaricensis* infection in two different mouse strains throughout the entire patency period.

Materials and methods

Animals

BALB/c and C57BL/6 mice were kept under standard laboratory conditions as recently described (Geiger et al. 2001). At the time of infection, the BALB/c ($n=17$) and C57BL/6 ($n=11$) mice were aged between 2 and

4 months. All animal experiments were conducted in accordance with German law.

Parasites and infection

A. costaricensis infective third-stage larvae (L3) were isolated as previously described (Geiger et al. 1999), and each mouse was infected with six L3 via a stomach tube.

Parasitological examinations

Following the administration of the L3, the survival time of the infected animals was monitored daily. Beginning with 25 days post-infection (p.i.), the excretion of first-stage larvae (L1) in the feces was determined weekly until the end of patency according to Geiger et al. (2003). The recovery of adult worms from the different organs was determined after host death, and in surviving animals at the end of the patency period. Heart, aorta dorsalis, liver, and mesenteric arteries were examined under a stereomicroscope, and parasites were extracted with a fine forceps. *A. costaricensis* adult worms were washed with phosphate buffered saline (PBS) three times and stored at −20°C until used for antigen preparation.

Antigen preparation

A PBS-soluble antigen extract was prepared from fertile male and female *A. costaricensis* as previously described (Geiger et al. 1999). Antigen aliquots were stored at −20°C until further use.

Determination of parasite-specific immunoglobulins by enzyme-linked immunosorbent assay in the sera of infected mice

Blood samples from infected mice were obtained by punctation of the retro-orbital venous plexus under ether anesthesia. Individual serum samples were stored at −20°C until further use. *A. costaricensis*-specific antibodies in the sera of infected mice were determined by indirect ELISAs as described previously (Geiger et al. 1999). The class and subclass specific antibodies were used in the following dilutions: IgM, IgA, IgG 1:1000 (Sigma, Munich, Germany); IgG1 1:2000 and IgG2a 1:500 (Rockland, Gilbertsville, USA). Only serum samples from the animals that survived throughout the experiment were used. Parasite-specific IgE was determined with some modifications. Briefly, plates were coated with antigen and blocked with PBS/10% fetal calf serum (FCS, Sigma). Sera from infected mice were diluted 1:100 in dilution buffer (PBS, 10% FCS, and 4% Tween-20, pH 7.4). A volume of 50 µl of the diluted serum samples was added to each well and the plates were incubated for 90 min at 37°C. Thereafter, the plates were washed three times with PBS/1% Tween-20 and 50 µl of biotinylated anti-IgE monoclonal antibody (ϵ-chain-specific; Serotec, Oxford, UK) at a 1:1000 dilution was added to each well. After incubation for 2 h at 37°C, the plates were washed again (see above) and 50 µl of alkaline phosphatase-conjugated egg white-avidin (Jackson ImmunoResearch Laboratories, USA) was added at a concentration of 2 µg/ml. After another incubation step for 1 h at room temperature, the plates were washed six times and the substrate was finally added as described by Geiger et al. (1999). The plates were read at 405 nm in a microplate autoreader (BioTek, Winooski, Belgium) and the results were expressed as mean optical densities for duplicate determinations.

Determination of total IgE

The determination of total IgE in the sera of infected mice was performed as described by Geiger et al. (1999) and the concentrations were determined according to a mouse immunoglobulin reference serum (ICN, Ohio, USA). Total IgE serum concentrations were expressed in micrograms per milliliter for duplicate assays.

Statistics

For the comparison of mortality in both mouse strains, the Kaplan-Meier survival analysis and χ^2- test were performed. The numbers of L1 excreted in the feces of infected mice of both strains are given as geometric means, and both groups were compared for differences using the Mann-Whitney U-test ($P < 0.01$). For the antibody ELISAs, values are indicated as arithmetic means $\pm$ SD. Again, the Mann-Whitney U-test was used to determine significant differences with values of $P < 0.05$.

Results

Mortality of infected BALB/c and C57BL/6 mice and recovery of adult worms

The mortality of BALB/c and C57BL/6 mice was monitored over 11 weeks (p.i. In both mouse strains, an increased mortality was observed between 14 and 35 days p.i. (Fig. 1). During this acute phase of infection more BALB/c mice died before 28 days than C57B/6 mice. An increasing mortality was observed in the C57BL/6 mice throughout the 11 weeks of infection (81.8%) (Fig. 1), whereas the mortality in BALB/c mice remained constant (41.8%) after 7 weeks p.i. The mean recovery of adult worms in all infected BALB/c mice was 35%, which is equivalent to nearly two adult worms per infected animal. The recovery of adult worms in C57BL/6 mice was higher, 48.5%, however, without reaching significance when compared with BALB/c mice.
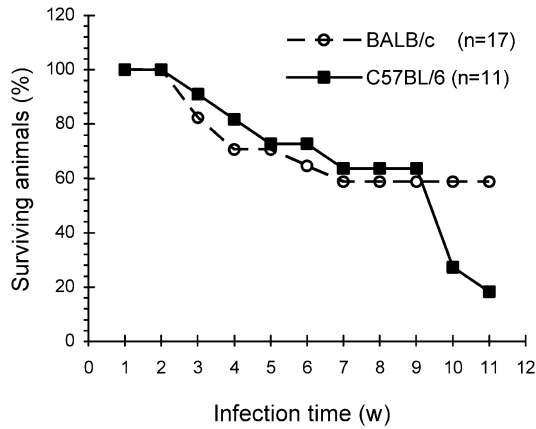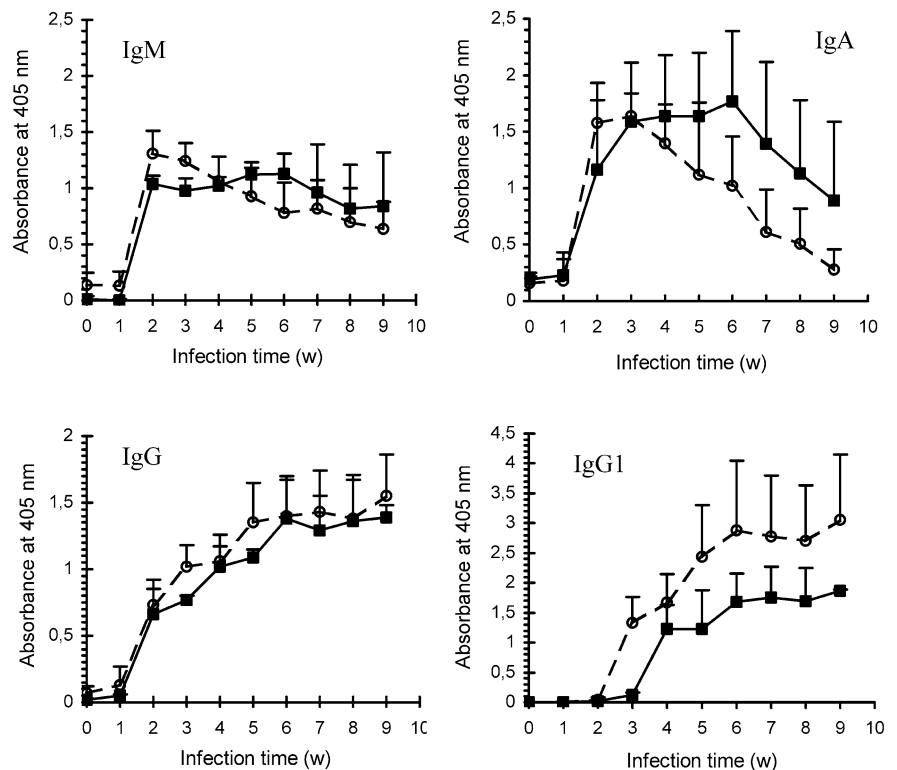
Fig. 1 Survival of mice infected with *Angiostrongylus costaricensis*. All mice were inoculated with six L3; *n* is the number of mice in each group

Excretion of L1 in the feces

Fecal L1 excretion by infected mice showed a high variation among the different animals and within the same animal at different times. There were no significant differences in the mean excretion of first-stage larvae in infected BALB/c and C57BL/6 mice throughout the infection (data not shown). In both mouse strains, L1 were detected from 25 days p.i. onwards and both strains showed a maximum L1 excretion at 39 days p.i. The mean patency period in BALB/c and C57BL/6 mice was 8 and 5 weeks, respectively.

Production of parasite-specific antibodies

Levels of parasite-specific IgM antibodies began to rise 1 week p.i. in both mouse strains (Fig. 2). In BALB/c mice, maximum parasite-specific IgM values were measured at 14 and 21 days p.i. At these early times, the values were significantly higher in BALB/c when compared to C57BL/6 mice ($P < 0.05$). The time course of parasite-specific IgA production in BALB/c mice was similar to that of IgM, with highly elevated values at 2 and 3 weeks p.i., and a subsequent decrease until the end of the experiment. In C57BL/6 mice, parasite-specific IgA values only reached maximum levels after 4 weeks p.i., with significantly higher values at 6 and 7 weeks p.i. ($P < 0.05$) when compared to BALB/c mice (Fig. 2). For parasite-specific IgG, a prominent increase was detected at 2 weeks p.i. (Fig. 2), with gradually increasing values in both mouse strains until the end of the experiment. In general, mean parasite-specific IgG was higher in the sera from infected BALB/c mice when compared to C57BL/6 mice. At 3 and 5 weeks p.i. these differences were significant ($P < 0.05$) (Fig. 2). For parasite-specific IgG2a antibodies, only low optical densities could be detected throughout the experiment (data not shown), whereas for parasite-specific IgG1 an increase was measured after 3 weeks p.i. in both mouse strains. However, IgG1 values in C57BL/6 mice were lower in comparison to BALB/c mice and this difference was significant at most times during the patency period ($P < 0.05$).

Fig. 2 *A. costaricensis*-specific antibodies in BALB/c (*open circles*) and C57BL/6 (*filled squares*) mice infected with six third-stage larvae. In order to compare the humoral response in both mice strains only serum samples from the animals that survived throughout the experiment were used, BALB/c (*n* = 10) and C57BL/6 (*n* = 7). The results are expressed as the mean ELISA values ± SD

Determination of parasite-specific and total IgE

An increase in total IgE was detected at 2 weeks p.i., with gradually increasing values in both mouse strains until 8 weeks p.i. (data not shown). Values for parasite-specific IgE, determined by indirect ELISA, reached only low levels in both mouse strains without any significant differences (data not shown).

Discussion

In order to study the parasitological and immunological aspects of an *A. costaricensis* infection, several mouse strains have already been used as experimental models (Sano et al. 1986; Terada et al. 1987). In the present study, C57BL/6 and BALB/c mice were infected with a low dose of six L3 per animal and the immune response and mortality were registered during the following 9 weeks p.i. The efficacy of achieving a patent infection by the application of low parasite doses has been demonstrated for *A. cantonensis* in Wistar rats (Sano et al. 1982). In our study, in spite of the low dose of inoculated L3, adult worms were found in all animals.

Taking into consideration the excretion of L1 in the feces, the average period of patency in both mouse strains was shorter than the patency period reported for the natural hosts (25 weeks) (Garrido and C. Graeff-Teixeira, pers comm). Similar results with an enormous variation in the excretion of L1 and a high mortality have been reported by Canali et al. (1999) for Swiss mice. These authors considered their observations to be an indication of how little adaptation between the parasite and the murine host was present, and they concluded that *Mus musculus* does not play an important role in the natural parasite life cycle and epidemiology of the disease. Moreover, in our study, the mean patency period differs between BALB/c (8 weeks) and C57BL/6 (5 weeks). The reason for this is not yet clear. In a previous study (Geiger et al 1999), the parasite-specific or unspecific antibody production in experimentally *A. costaricensis* infected BALB/c and C57BL/10 mice did not correlate with the excretion of L1. More recently, it has been proposed that the elimination of first-stage larvae could be regulated by a MHC-II and a CD4+ T-cell-dependent mechanism (Geiger et al. 2003). However, these experiments using inbred mouse strains addressed the question of difference in larval output only during the acute phase, and therefore a role of antibodies during the chronic phase of the infection cannot be ruled out.

An elevated mortality in BALB/c and C57BL/6 mice occurred between the 2nd and 4th weeks p.i. (30% and 20%, respectively). This coincides with the period of maturation of the worms, oviposition, hatching of L1 and penetration of larvae through the intestinal wall in the life cycle of the parasite, events that have been correlated with an increase in mortality during the acute phase of an *A. costaricensis* infection (Geiger 1998). During the first 9 weeks p.i., we did not observe any significant differences in mortality between mouse strains. However, an increasing mortality was observed in the C57BL/6 mice throughout the 11 weeks of infection, whereas the mortality in BALB/c mice remained constant after 7 weeks p.i. These results differ from those published by Geiger et al. (1999), who reported significant differences in the mortality between BALB/c and C57BL/10 mice during the acute phase of infection and after the inoculation of six to eight L3. They correlated the mortality and morbidity in BALB/c mice with the increased production of total IgE and parasite-specific IgG1. Significantly higher levels for both immunoglobulins were determined in the sera of BALB/c mice during the first 4 weeks of infection. Recently, in a study on the cellular immune responses in *A. costaricensis*-infected BALB/c and C57BL/6 mice, Geiger et al. (2001) reported a polarization of the immune response towards cellular hyporesponsiveness, and a predominantly Th1 cytokine profile in BALB/c mice, which, according to the authors, may contribute to pathogenesis and increase morbidity during the acute phase of the infection. In the present study, an increase in the levels of IgE and parasite-specific IgG1 was also measured, starting from the 2nd and the 3rd week p.i., with significantly higher levels for IgG1 in BALB/c mice throughout the whole experiment. These results suggest that antibody levels could play an important role in the outcome of infection, at least during the chronic phase. In the course of helminth infections, strong increases in the IgG_{total} levels and parasite-specific IgG, and specially IgG1, as well as in the IgE_{total} levels and parasite-specific IgE are frequently reported (Finkelman et al. 1991). However, there have been no reports indicating that these immunoglobulins are involved in protection during a primary helminth infection. In contrast, it has been shown that they play an important role during a challenge infection with *Schistosoma* spp. or *Strongyloides ratti* in immunized mice (Delgado and McLaren 1990; Abraham et al. 1995). In experimental *A. cantonensis* and *A. costaricensis* infections, an increase in the serum-specific IgE serum levels was observed. For *A. cantonensis* an increase in parasite-specific IgE was correlated with susceptibility to infection (Pérez et al. 1989; Yoshimura et al. 1994). On the other hand, IgE does not play an important role in the mechanisms of defense against infection with *A. cantonensis* (Watanabe et al. 1993).

Further studies on the role of parasite-specific IgG1 antibodies on mortality and larval output, and on the cellular immune response during the chronic phase of an *A. costaricensis* infection, are necessary.

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