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DEVELOPMENT AND PATHOLOGY OF *ECHINOSTOMA CAPRONI* IN EXPERIMENTALLY INFECTED MICE

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ABSTRACT: In the present article, several parasitological features of mice, each experimentally infected with 75 metacercariae of *Echinostoma caproni* (Trematoda: Echinostomatidae), were studied during the first 12 wk postinfection. Moreover, the early pathological responses also were analyzed and compared with data previously published on other host species of *E. caproni* to gain further insight into the factors determining worm rejection or establishment of chronic infections. The results obtained show that the pattern of *E. caproni* infection in mice is consistent with a highly compatible host–parasite system. This combination is characterized by a high worm establishment, high egg output, and long survival of the worms. However, some differences with respect to other highly compatible hosts have been observed, particularly in relation to the survival of the adult worms. Histological studies suggest that the kinetics of goblet cells, mucosal neutrophils, and mononuclear inflammatory cells in the mesentery seem to be essential in determining the course of *E. caproni* infection in mice.

Infections with intestinal trematodes are widespread in humans and other animals. However, despite the frequency of these infections, the relationships between intestinal trematodes and their final hosts have received little attention in experimental parasitology. *Echinostoma caproni* (Trematoda: Echinostomatidae) is an intestinal fluke that does not undergo tissue migration in its definitive host. After infection of the definitive host with *E. caproni*, the metacercariae excyst in the duodenum and the juvenile parasites migrate to the posterior third of the intestine where they attach to the mucosa by the ventral sucker (Fried and Huffman, 1996).

Although *E. caproni* has a wide range of definitive hosts, its compatibility differs considerably between rodent species. *Echinostoma caproni* infection shows different patterns depending on the host species, which makes these host–parasite systems highly suitable for elucidating aspects of the host-specific components that determine the course of infections with intestinal trematodes (Toledo and Fried, 2005). For example, hamsters and mice show a high level of compatibility with this echinostome species, whereas rats and jirds are the opposite (Odaibo et al., 1988, 1989; Christensen et al., 1990; Hansen et al., 1991; Mahler et al., 1995; Toledo et al., 2004). This classification is based mainly on worm establishment and survival observed in each host species. However, the highly compatible hosts (hamsters and mice) show a markedly different pattern of infection. In mice, low level infections are rapidly expelled, whereas heavy infections are not rejected and they develop considerable resistance to reinfection (Odaibo et al., 1988, 1989). In contrast, golden hamsters develop only a limited capacity to expel *E. caproni* associated with primary infections and limited resistance to reinfection (Christensen et al., 1990). In this context, the early local lesions induced by *E. caproni* in each host species seem to be of great importance (Toledo, Esteban et al., 2006). Comparison of the histological features in *E. caproni*-infected hamsters and rats showed marked differences that may be related to differences in worm survival in each host (Toledo, Monteagudo et al., 2006). The analysis of the parasitological features of *E. caproni* infections in mice combined with the pathological features seems to be of great interest for the understanding of the factors determining the worm rejection or the parasite establishment.

The aim of this research was to further elucidate host–parasite relationships within the *E. caproni*-rodent systems. For this purpose, we have examined in detail several parasitological features of mice experimentally infected with *E. caproni* during the first 12 wk postinfection (PI). Moreover, we have studied the intestinal lesions induced by *E. caproni* in mice, and these results are compared with our results previously published on other rodent hosts with differing degrees of compatibility with *E. caproni* infection (Toledo, Monteagudo et al., 2006). This information may be useful to determine the factors involved in *E. caproni* worm expulsion, or, in contrast, the development of chronic infections.

MATERIALS AND METHODS

Parasite, host, and experimental infections

The strain of *E. caproni* has been described previously by Fujino and Fried (1993). Encysted metacercariae of *E. caproni* were removed from the kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used to infect ICR male mice. Fifty-two mice, weighing 30–35 g, were each infected by gastric gavage with 75 metacercariae of *E. caproni*. Mice were randomly allocated to group A (10 mice), B (36 mice), and C (6 mice). Group A was used to study the kinetics of egg release of *E. caproni* adults in the definitive host. Group B was used to analyze worm recovery and its variations over the course of the infection and the morphological features. Group C was used to examine the intestinal lesions induced by *E. caproni* in mice. Three mice were left uninfected and used as controls in the study of pathology. All the animals were maintained under conventional conditions with food and water ad libitum.

Worm recovery

This experiment was designed to compare the worm establishment of *E. caproni* adults in mice. Three mice of group B were necropsied each week PI, and the number of worms recovered per host was recorded.

Kinetics of egg release

This experiment was designed to examine the kinetics of egg output of *E. caproni* adults during the course of the infection in mice. For this purpose, the egg output was determined in mice in group A for each week PI.

Fecal samples were examined to determine the number of eggs re-

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TABLE I. Range and means and their standard deviations for each metrical variable of *Echinostoma caproni* analyzed in experimentally infected mice over the complete course of the experiment. See text for explanation of variable abbreviations.

Variable	Range	Mean	SD
BA (mm^2)	3.742–16.328	12.263	3.903
CW (μm)	290.7–554	480.3	23.4
OSA (μm^2)	18,162.2–50,884.8	39,625	10,005.9
PL (μm)	64.4–181.3	90.1	34.4
PHA (μm^2)	19,600.3–34,279.2	24,678.7	5,490.2
OL (μm)	169.3–426	301.3	64
CSA (μm^2)	18,461.7–59,541.2	36,229.5	13,493.7
VSA (μm^2)	179,589.4–586,226.9	470,635	123,656.9
OVA (μm^2)	62,091.9–196,281	137,274.9	39,682.9
ATA (μm^2)	160,027–867,674.7	523,392.3	197,775.7
PTA (μm^2)	168,969–1,144,754.9	651,699.1	269,921.5

leased per animal (EPA) as described in a previous study (Toledo et al., 2003). Briefly, 24-hr fecal productions were collected and weighed individually from each animal. The individual samples were emulsified in the ratio of 1 g/30 ml in a solution of 0.1 M NaOH. The mixture was shaken at room temperature for 2 hr, and the sediment was resuspended in 2 ml of the same solution. The eggs contained in 200 μl were counted. Five replicated samples were analyzed each day from each animal to determine the number of eggs released daily, and the average ± standard deviation was considered for each week of the experiment.

Morphological analysis

To evaluate the effect of aging on adult worms in experimental infections with *E. caproni* in mice, different morphometrical features were studied (Table I). Ten of the adult worms collected each week from group B mice were fixed in Bouin's fluid under coverslip pressure and mounted in Canada balsam. The following features (variables) were subjected to analysis: body area (BA), collar width (CW), oral sucker area (OSA), prepharynx length (PL), pharynx area (PHA), esophagus length (OL), cirrus sac area (CSA), ventral sucker area (VSA), ovarian area (OVA), anterior testis area (ATA), and posterior testis area (PTA). Each area was calculated as the product of the maximum length and width of the respective morphological feature.

Histology

Histological responses to *E. caproni* infections in mice were evaluated at 15 and 30 days PI. At each time interval, 3 mice were necropsied, and intestinal sections of 0.7–1 cm in length from the sites where the worms were located were obtained from each animal and fixed in 4% buffered formalin. After embedding in paraffin, 4-μm serial sections were cut. Intestinal sections were stained with hematoxylin and eosin, periodic acid-Schiff, Alcian blue, and toluidine blue.

Sloughing of the villi tips was considered as a histological criterion of *E. caproni* induced mucosal damage as described in a previous study (Bindseil and Christensen, 1984). Ten randomly selected low-magnification (×100) fields of each section were examined, and the numbers of destroyed or eroded villi and the total number of villi were recorded. Three sections of the intestine from each mouse were examined, and the results are expressed as the percentage of destroyed or eroded villi.

All the cell counts (goblet cells, neutrophils, mast cells, eosinophils, and mononuclear cells in the mesentery) were expressed as the number of cells per villus-crypt unit (VCU), except those of the mesenteric cells, which are expressed in number of cells per high-power field (HPF) (×400), studied over 10 selected HPFs. Results are expressed as the mean number of cells per VCU or HPF ± standard deviation.

Statistical analysis

The results obtained in this study on the lesions induced by *E. caproni* in mice have been compared with those obtained by Toledo, Monteagudo et al. (2006) on rats and hamsters. For this purpose, a 2-factor ANOVA with interaction was used with the time PI and host species as independent variables, using the data published previously (Toledo, Monteagudo et al., 2006). Moreover, when a significant time–host species interaction was detected, the Bonferroni *t*-test of the difference between means was performed as a post hoc analysis to determine whether there were significant differences due to the host species within the same time PI. $P < 0.05$ was considered as significant. Before analyses, data were log transformed to achieve normality.

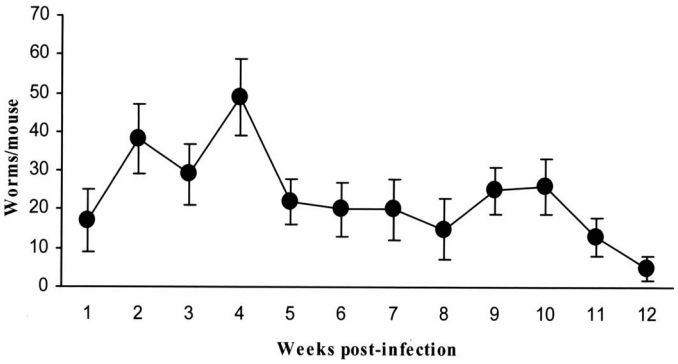

FIGURE 1. *Echinostoma caproni* worm recovery at increasing age (weeks) in infections with 75 metacercariae/mouse. Vertical bars represent the standard deviation.

RESULTS

Patent period and worm recovery

All mice individually exposed to 75 metacercariae of *E. caproni* became infected as determined by egg examination. The duration of the prepatent period studied on the mice in group A was rather uniform. Egg release began 9–12 (10.1 ± 0.4) days PI. All the mice remained positive by egg examination until the end of the experiment at 12 wk PI.

All mice in group B were positive at necropsy, except 1 mouse that was found negative at 12 wk PI. The number of worms recovered weekly per mouse during the first 12 wk PI is shown in Figure 1. Worms were recovered each week from 1 to 12 wk PI, and the number ranged from 5 to 49 (23.3 ± 11.6) worms/mouse. The number of worms recovered weekly progressively increased during the first period of the infection to reach a maximum at 4 wk PI. Thereafter, the number of worms suddenly decreased, and the decrease continued during the last period of the experiment. The minimum worm number was observed at 12 wk PI.

Kinetics of egg release

The egg release was continuous from the first day of the patent period until the end of the experiment. During this period, eggs were found in all the fecal samples analyzed. However, the egg output was not uniform over time (Fig. 2). The egg output rapidly increased during the first period of the infection to reach a period of high release from 5 to 11 wk PI. The egg output declined at 12 wk PI. The maximum egg output was observed at 10 wk PI.

Morphological analysis

The ranges and the means and their standard deviations of each metric variable of *E. caproni* in mice are shown in Table I. The variability of each morphological feature is shown in

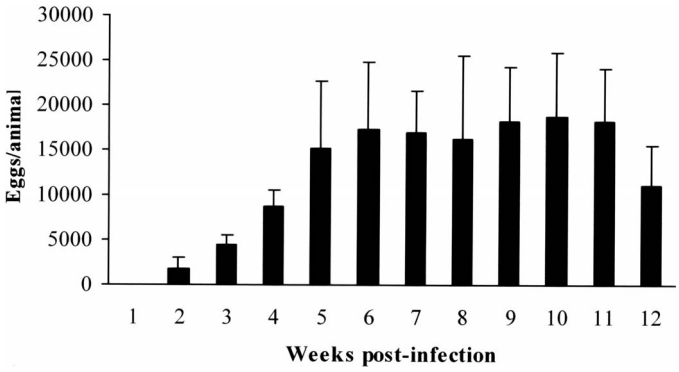

FIGURE 2. Weekly egg output of *Echinostoma caproni* at increasing age in infections with 75 metacercariae/mouse. Vertical bars represent the standard deviation.

Figure 3. All the morphological variables analyzed showed a progressive increase during the first period of the infection. Thereafter, the values became stable until the end of the experiment, except for CSA, ATA, and PTA, for which a significant decrease was observed from 10 wk PI and beyond (Fig. 3).

Pathology of the infection

All data reported in this section on the lesions of *E. caproni* infections in rats and hamsters were obtained from results in Toledo, Monteagudo et al. (2006), and they have been used for comparison with the results in mice as mentioned above.

Gross examination of the intestine of mice at necropsy showed a marked dilation of the intestine during *E. caproni* infection. Associated with these dilations were large groups of worms. Histological examination of the intestines did not include the changes at the worm attachment sites because they are traumatic (Simonsen et al., 1989). The main foci of interest were the areas that surrounded the attachment sites. *Echinostoma caproni*-infected mice did not have villi destruction. Villi erosion was not evaluated due to the high percentage of eroded villus observed in the control mice.

All the cell counts were variable over the course of the infection (Fig. 4). Application of 2-way analysis of variance (ANOVA) showed that all the cell counts were subjected to time-related changes. Thus, the number of neutrophils in *E. caproni*-infected mice was significantly lower than in hamsters at 15 and 30 days PI ($P < 0.001$) and higher than in rats at 30 days PI ($P < 0.05$). The number of goblet cells observed in *E. cap-*

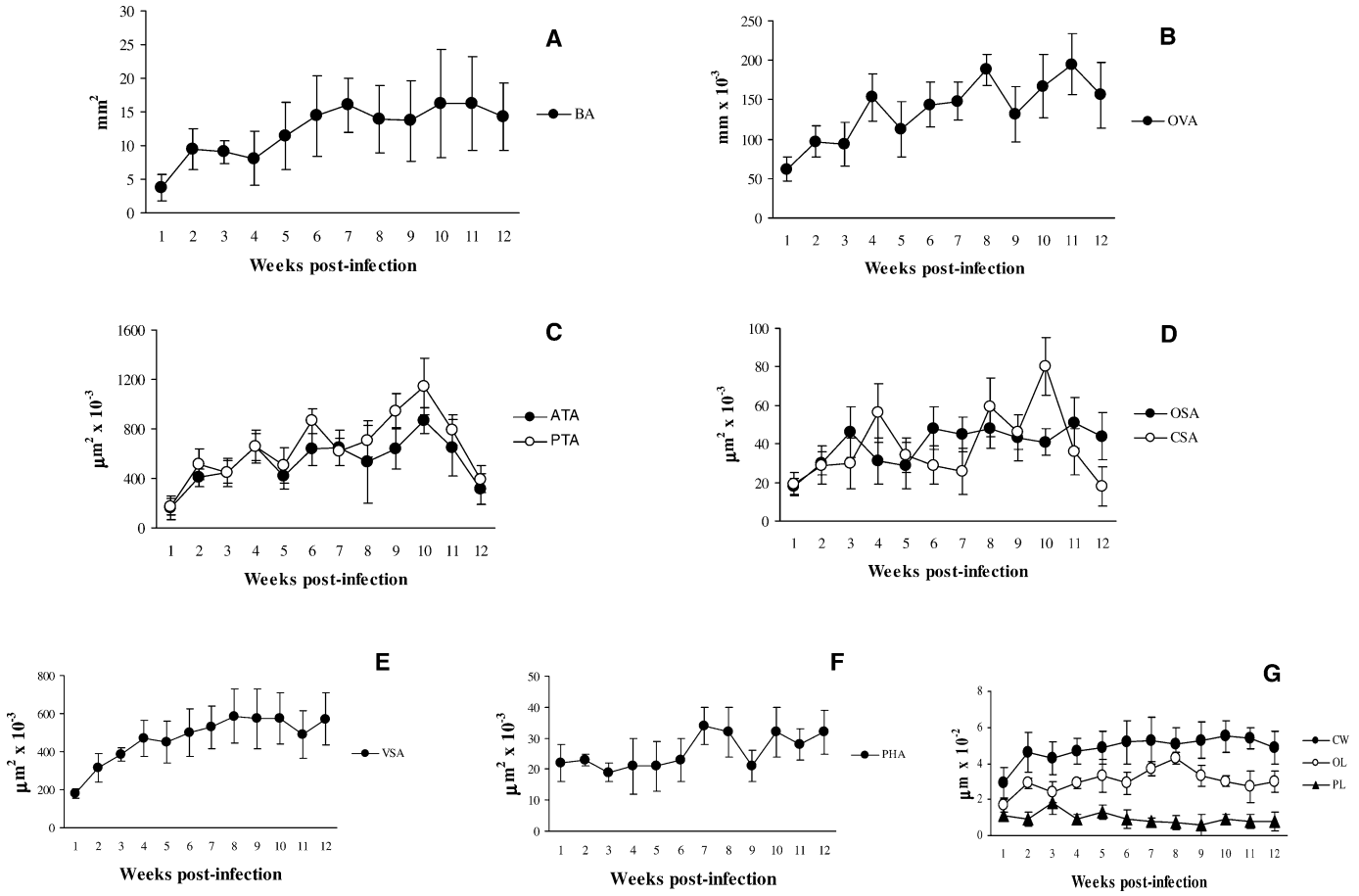

FIGURE 3. Morphological characteristics of *Echinostoma caproni* at increasing age in infections with 75 metacercariae/mouse. (**A**) Mean body area. (**B**) Ovarian area. (**C**) Anterior (closed circles) and posterior (opened circles) testis area. (**D**) Oral sucker (closed circles) and cirrus sac area (opened circles). (**E**) Ventral sucker area. (**F**) Pharynx (triangles) area. (**G**) Collar width (closed circles), esophagus length (opened circles) and prepharynx length (closed triangles).Vertical bars represent the standard deviation. See text for explanation of variable abbreviations.

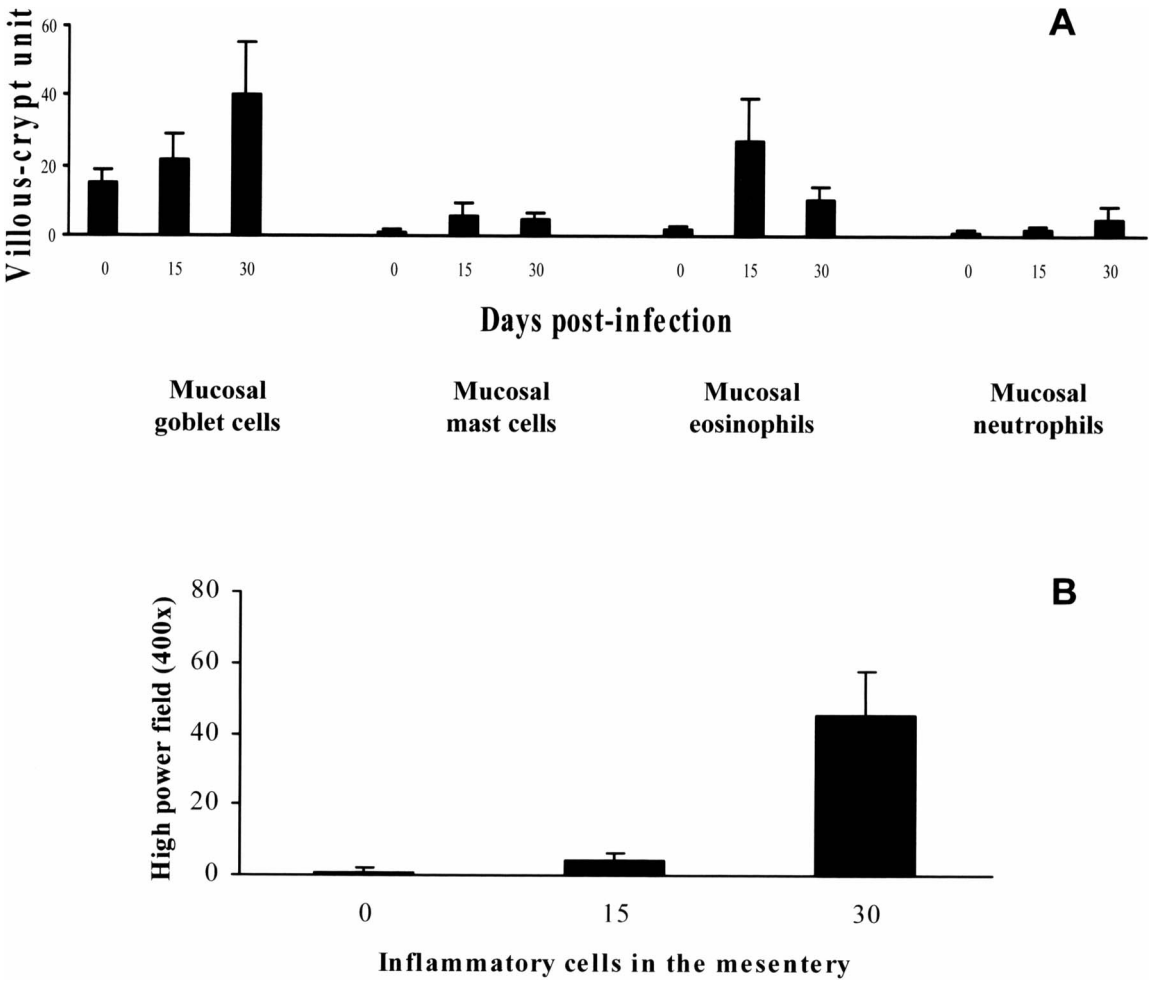

FIGURE 4. Numbers of goblet cells, mast cells, eosinophils and neutrophils in the mucosa of the small intestine (**A**) and inflammatory cells in the mesentery (**B**) of mice at 0, 15, and 30 days PI with 75 metacercariae of *Echinostoma caproni*. Vertical bars represent the standard deviation.

roni-infected mice over the course of the infection is shown in Figure 4. A progressive increase in the counts was observed, reaching a maximum at 30 days PI. The number of goblet cells was significantly higher than in hamsters at 15 and 30 days PI ($P < 0.05$). No significant differences were observed with respect to rats according to the study by Toledo, Monteagudo et al. (2006). Mast cell counts showed an increase at 15 and 30 days PI with respect to control mice. No significant differences with respect to hamsters and rats were detected over the course of the infection in each host species.

Probably the most striking observations of the present study were in relation to the numbers of eosinophils and the populations of the mononuclear inflammatory cells in the mesentery of the small intestine of mice. The number of eosinophils rapidly increased, reaching a maximum at 15 days PI. At 30 days PI, the values decreased, but they were higher than in control mice. Application of 2-way ANOVA and Bonferroni test showed that the number of eosinophils was significantly lower than in hamsters at 15 and 30 days PI ($P < 0.001$) and lower than in rats at 30 days PI ($P < 0.001$). However, the differences with respect to rats were not considered due to the marked variability in the counts of the control rats in the study by Toledo, Monteagudo et al. (2006). The presence of inflammatory cells in the mesentery of the small intestine of *E. caproni*-infected mice was investigated by light microscopy, and the counts are shown in Figure 4. The values at 15 days PI were slightly higher than in control mice. However, an increase was observed at 30 days PI. The number of inflammatory cells was significantly lower than in hamsters at 15 days PI ($P < 0.001$) and higher than in rats at 30 days PI ($P < 0.001$).

DISCUSSION

Echinostoma caproni has a wide range of definitive hosts, although the compatibility may differ considerably between host species. The differences are reflected mainly in worm establishment and survival (Odaibo et al., 1988, 1989; Christensen et al., 1990; Hansen et al., 1991; Mahler et al., 1995; Toledo et al., 2004; Toledo and Fried, 2005). Highly compatible hosts, such as hamsters and mice, develop a limited capacity to expel primary infections, resulting in long-lasting infections. In hosts of low compatibility, such as rats and jirds, the infection is rapidly expelled. In our study, the course of the infection of *E. caproni* in mice was examined by presenting quantitative data on worm establishment, egg output, and worm morphology, and their respective variations over time. Moreover, the intestinal

lesions induced by *E. caproni* in mice and the possible consequences in the course of the infection were analyzed.

The general pattern of *E. caproni* infection in mice is consistent with that of a highly compatible host–parasite system. This combination is characterized by a high worm establishment and egg output and a long survival of the worms. All mice experimentally exposed each to 75 metacercariae of *E. caproni* became infected; only 1 mouse became negative in terms of worm recovery during the course of the experiment. In contrast, all the worms were expelled in rats at 7–8 wk PI (Hansen et al., 1991; Toledo et al., 2004). In ICR mice, the kinetics of egg release and the worm growth curves were also similar to those described in other host species of high compatibility with *E. caproni*. The number of eggs released weekly is similar to that in hamsters and markedly higher than that in rats (Toledo et al., 2004). The first period of the infection was characterized by a progressive increase in egg output, probably in relation to the maturation of the adult worms, to reach a period of high release between 5 and 11 wk PI. Interestingly, a decrease in the egg output was observed by 12 wk PI. This decrease may be associated with the reduction in the worm burden, but the contribution of other factors cannot be dismissed. A marked reduction in the areas of the testes was observed in the last period of the experiment. This decrease has not been observed in other *Echinostoma* sp.–rodent combinations (Isaacson et al., 1989; Fried et al., 1997; Kostadinova et al., 2000; Toledo et al., 2003, 2004; Muñoz-Antoli et al., 2004). In this sense, the infective dose used in our study should be considered. Yao et al. (1991) suggested that the testicular area of *E. caproni* was subjected to a 'crowding effect.' They infected hamsters with 15, 50, and 200 metacercariae/hamster. In the animals infected with higher doses of metacercariae, a significant reduction in testicular size was observed.

According to the biological features of *E. caproni* infections, mice can be considered as highly compatible hosts. However, some differences with respect to other hosts of high compatibility have been observed. For example, the capacity of mice to expel primary *E. caproni* infections is greater than hamsters. Toledo et al. (2004) showed that the survival of *E. caproni* infection in hamsters is at least 20 wk PI without significant changes in the worm burden during this period. Herein, we have observed that infections in mice show a persistence of at least 12 wk PI, but a marked decrease in the worm burden was observed by 5 wk PI. Factors causing the earlier worm expulsion in mice are difficult to elucidate. In this context, we have examined the intestinal lesions induced by *E. caproni* in mice to compare the results with those described previously in other hosts of different compatibility with *E. caproni*. Our results show that the particular kinetics of *E. caproni* worm burden in mice is determined by a complex set of reactions in which goblet cells, mucosal neutrophils, and mesenteric mononuclear inflammatory cells seem to play a major role.

The increase in the number of all the cell types analyzed probably reflects on the importance of the local cellular mechanisms in the response against *E. caproni* in mice. However, the contribution of each cell population in worm expulsion or chronic worm establishment does not seem to be the same. Although the role of mast cells on the course of echinostomiasis is not well understood (Toledo, Esteban et al., 2006), our results suggest that they are not determinants in *E caproni* infections in mice. Infected mice and hamsters develop similar mast cell kinetics compared with rats, although worm survival differs greatly among these host species. This is supported by previously published studies on the *E. caproni*–mouse system that showed that reductions in mast cells counts did not affect the kinetics of worm expulsion (Fujino et al., 1998). Eosinophilic infiltration in *E. caproni*- and *E. trivolvis*-infected mice has been reported previously, and their involvement on worm rejection has been suggested (Bindseil and Christensen, 1984; Fujino et al., 1996). Our results show that eosinophilic counts in *E. caproni*-infected mice are higher than in hamsters, suggesting a role in worm expulsion. However, the difficulty involved in the comparison of data obtained from low compatible hosts (rats) prevents us from making further suggestions (Toledo, Monteagudo et al., 2006).

Of considerable interest seems to be the kinetics of goblet cells, mucosal neutrophils, and mononuclear inflammatory cells found in the mesentery. The combination of these features seems to be essential to explain the differences on *E. caproni* survival in mice with respect to rats and hamsters. Goblet cell counts in *E. caproni*-infected mice were higher than in hamsters, whereas those of mucosal neutrophils and inflammatory cells in the mesentery were higher than in rats.

Goblet cells and secreted mucus may play a major role in the expulsion of intestinal helminths (McKay and Khan, 2003; Seo et al., 2003). Moreover, development of chronic *E. caproni* infections has been often related to reduced numbers of goblet cells, whereas worm expulsion coincides with hyperplasia of goblet cells (Bindseil and Christensen, 1984; Weinstein and Fried, 1991; Fujino and Fried, 1993; Toledo, Monteagudo et al., 2006). Our study suggests that the increased goblet cell numbers in mice and rats with respect to hamsters may be involved in the earlier *E. caproni* expulsion observed in those hosts. However, other immune mechanisms are likely to operate in mice to explain the higher worm survival than in rats. In this context, the kinetics of mucosal neutrophils and the mesenteric inflammatory cells seem to be essential. In contrast to rats, significant increases in the number of neutrophils and mononuclear inflammatory cells have been detected in mice and hamsters, showing the importance of local inflammatory responses in these latter species. This response may be a determinant in the establishment of chronic infections. In *Giardia lamblia*-infected mice, local inflammatory response is a factor that contributes to increased susceptibility of a host (Müller and Von Allmen, 2005; Von Allmen et al., 2006). In *E. caproni* infections, the development of chronic infections has been associated with early local inflammatory responses (Toledo, Monteagudo et al., 2006). It is, therefore, likely that differences in intestinal inflammatory responses are involved in the longer survival of *E. caproni* in mice than in rats, although other parameters, such as the goblet cell counts, are similar between both hosts. Interestingly, the numbers of mucosal neutrophils were significantly greater in hamster than in mice. Neutrophils are an important source of proinflammatory cytokines such as interleukin-12 and tumor necrosis factor-α as well as free radicals and several chemokines. This raises the possibility that neutrophil infiltration and the consequences in the inflammatory response are essential in the longer survival of *E. caproni* in hamsters. Accordingly, approaches on the relation between neutrophil infiltration and the development of chronic *Echinostoma* spp. infections

will provide novel information on the susceptibility of a host to intestinal helminths.

In summary, we have analyzed the development and pathological lesions of *E. caproni* in mice. The biological features of the infection are consistent with those of a highly compatible host–parasite system. However, differences with respect to other hosts of high compatibility have been observed. These differences seem to be related to the local immune response generated by *E. caproni* in mice. The kinetics of goblet cells, mucosal neutrophils, and mononuclear inflammatory cells in the mesentery seem to be essential in determining the course of *E. caproni* infection in mice.

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