Capillaria hepatica in rats: focal parasitic hepatic lesions and septal fibrosis run independent courses

Ana Thereza Gomes*, Liliane Monteiro Cunha, Carla Guimarães Bastos, Bruno Frederico Medrado**, Bárbara CA Assis, Zilton A Andrade/+**

Laboratório de Patologia Experimental, Centro de Pesquisas Gonçalo Moniz-Fiocruz, Rua Valdemar Falcão 121, 40295-001 Salvador BA, Brasil *Departamento de Medicina Interna, Faculdade de Medicina, Universidade Federal da Bahia, Salvador, BA, Brasil **Escola Bahiana de Medicina, Salvador, BA, Brasil

Capillaria hepatica *causes two main lesions in the liver of rats: multifocal chronic inflammation, directly related to the presence of disintegrating parasites and their eggs, and a process of systematized septal fibrosis. The comparative behavior of these two lesions was investigated in rats experimentally infected with 600 embryonated eggs, following either corticosteroid treatment or specific antigenic stimulation, in an attempt to understand the relationship between these two lesions, and the pathogenesis of septal fibrosis. The two treatments differently modified the morphological aspects of the focal parasitic-related lesions, but did not interfere with the presentation of diffuse septal fibrosis, although a mild decrease in the degree of fibrosis occurred in corticoid-treated animals. These findings indicate that although the two lesions are* C. hepatica *induced, they are under different pathogenetic control, the induction of septal fibrosis being triggered during early infection to follow an independent pathway.*

Key words: *Capillaria hepatica* - septal fibrosis - rats - corticosteroids - antigenic stimulation

The nematode *Capillaria hepatica* causes two distinct lesions in the liver of rats: a focal inflammatory lesion, directly determined by the presence of live, dying and dead worms and their eggs, and an associated systematized process of septal fibrosis (Ferreira & Andrade 1993). These two lesions appear almost at the same time, but they run different courses, the septal fibrosis persisting longer after the focal lesions have been extinguished and repaired (Souza et al. 2006a). However, these two lesions are related to a same cause. If the worms are destroyed by chemotherapy before egg lying or if the parasitic focal lesions are induced by the direct injections of immature eggs into the peritoneal cavity, portal vein or into the liver tissue itself, focal lesions are formed, but septal fibrosis fails to develop (Santos et al. 2001). Therefore, lesions produced by both worms and eggs are necessary for the development of septal fibrosis. How much such focal lesions are related to the process of systematized septal fibrosis is not yet completely known. However, that is an important issue, since *C. hepatica*-induced septal fibrosis in rats has became one important model for the study of many aspects of hepatic fibrosis (see review in Andrade et al. 2005).

An immunological basis has been suggested for the pathogenesis of septal fibrosis occurring in another model, the pig-serum model following studies with neonatal-induced tolerance (Bhunchet et al. 1996). Similar lines of evidences have been produced for the septal fibrosis associated with *C. hepatica* infection of rats (Lemos et al. 2003), but the immunology of *C. hepatica* infection itself is still poorly understood. Repeated experimental infections have resulted in considerable modifications in the constitution of the focal parasitic lesions, while the dynamics and structure of the septal fibrosis were not much altered (Oliveira et al. 2004). During this latter study, the levels of anti-parasite circulating antibodies progressively increased with repeated infections, but that did not prevent the worms to live longer and to produce more eggs, while the process of septal fibrosis maintained its usual course. To further investigate the relationship between the focal parasitic lesions and the process of septal fibrosis, the present study explores the manipulation of the host immune response by either depressing it with corticoid treatment, or stimulating it by means of antigen administration. It is hoped that a better understanding of these two processes would bring important data, not only to the experimental model itself but, ultimately, for the pathogenesis and treatment of hepatic fibrosis.

MATERIALS AND METHODS

Animals - Healthy young adult Wistar rats of both sexes, weighing around 150 g were maintained in ample metallic boxes, with sex separation, and free access to a commercial balanced feed and water, in a well ventilated room. The experimental Protocol was previously approved by the Gonçalo Moniz Research Center (Fiocruz) Ethical Committee.

Inoculum - The animals were submitted to infection with 600 mature *C. hepatica* eggs, administered by a gastric tube. The eggs were isolated from the livers of experimentally infected mice, through homogenization in saline, followed by repeated washing and sedimentation. The clean isolated eggs were kept in a humidified Petri dish at 25-28ºC during a period of 28 days for embryonation.

Financial support: Pronex-Fapesb
+Corresponding author: zilton@cpqgm.fiocruz.br
Received 28 June 2006
Accepted 23 August 2006

Corticoid treatment - Seven days after inoculation with *C. hepatica*, 10 rats were daily treated with 15 mg/kg/bw (Hawk & Leary 1999) of prednisone (Meticorten®-Schering Plough) administered by the orogastric route as saline solution. Another group of 10 saline-treated, non-infected rats, served as controls. After 30 days from inoculation and 23 of infection, the animals were anesthetized and killed by severing the abdominal aorta.

Antigenic treatment - Twenty rats were divided into four groups, of five animals each, being two experimental and two controls. They were submitted to (a) subcutaneous and intramuscular injections of 500 µl of soluble *C. hepatica* antigen; (b) similar injections of particulate *C. hepatica* antigen; (c) injections of saline solution (control-1); and (d) simple infection with *C. hepatica* (control-2). Thirty-five days after these procedures all the animals were submitted to infection with approximately 600 embryonated eggs. Serum antibody levels were detected by ELISA, immediately following the immunization protocol, and 30 days after infection.

Antigen preparation - Antigens were obtained from *C. hepatica* immature eggs. Soluble antigen was obtained from the supernatant of an immature egg concentration submitted to repeated freezing and thawing, followed by triturating with a hand homogenizer. Its protein concentration reached 0.576 µg/ml. Particulate antigen consisted of a suspension of immature eggs in saline (742 eggs/100 µl). Five-hundred microliters of incomplete Freund's adjuvant (Sigma, US) were added to antigens and saline. The control-2 group did not receive adjuvant.

Histopathology - Fragments of the liver were fixed either in Bouin's fluid or 10% neutral formalin, and routinely embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, and picro-sirius red for collagen.

Semiquantitative evaluation of septal fibrosis - The picro-sirius red stained coded slides were microscopically evaluated by two independent observers. Fibrosis was considered as mild (+), moderate (++), and severe (+++) as followed, mild when approximately half of the microscopic fields were free from septal fibrosis, and severe when a morphologic picture of cirrhosis was present; the grade was moderate for the situation in between these two extremes.

Hydroxyproline measurement - The colorimetric method of Bergman and Loxley (1963) was used for the corticoid-treated animals only. Briefly, the liver samples were hydrolyzed for 18 h in 5 ml 6 N HCl at 110ºC, and then filtered. One drop of 1% phenolphthalein in absolute alcohol was added to 2 ml of the filtrate as an indicator and neutralization was obtained with 10 N NaOH and 3 N HCl. After neutralization subsequent steps were made in duplicate for each sample. To a 200 µl of the above solution, 400 µl of isopropanol in citrate-acetate buffered Chloramine T were added. After 4 min, 2.5 ml of Ehrlich reagent were added. Tubes were wrapped in aluminum foil and incubated for 25 min in a water-bath at 60ºC. Readings of the samples were made twice for each sample on the range of 558 nm absorbance band in a Hitachi spectrophotometer, mod. U-2000. Results were analyzed by computer and expressed as µmol hydroxyproline/g of hepatic tissue. The average from the two readings was used for the following analysis.

Statistical analysis - The Mann-Whitney or Wilcoxon test was applied to evaluate the differences among groups. The p value < 0.05 was taken as statistically significant. The SPSS Program, version 9.0 was used for performing the analyses.

RESULTS

Septal fibrosis appeared in 100% of the rats included in this study. Its amount and particular disposition varied very little among the several groups (Fig. 1A). It consisted in long and thin connective tissue septa that connected portal to portal spaces and, eventually, portal to central veins. Corticoid treatment resulted in a certain decrease of the septal fibrosis, detected by semiquantitative histological estimation, in comparison with non-treated controls (p = 0.0037), although no clear cut difference occurred with the hydroxyproline levels among these groups (p = 0.07) (Table).

On the other hand, animals submitted to series of antigenic stimulation did not exhibit any variation in the amount of fibrosis by semiquantitative estimation. Fibrosis present in all animals from the two groups submitted to antigenic stimulation, and their controls, was graded as moderate (++).

The focal parasitic lesions presented marked differences between the experimental groups and their respective controls. Corticoid treatment permitted the blind separation of all treated animals from their controls at the microscope, mainly because of the presence of live worms within the focal lesions, and the large amount of associated eggs, which made an evident contrast with the findings present in non-treated controls (Fig. 1B). The latter presented instead many dead and dying worms, usually undergoing resorption and/or calcification, within focal necrotic lesions, surrounded by a thick fibrous capsule (Fig. 1C). However, no evident variation in the cellular composition of septal fibrosis was noted in the various groups, and their controls. After routine staining, the cells

TABLE

Semiquantitative evaluation of septal fibrosis and hydroxyproline determination in *Capillaria hepatica* infected liver of rats treated with either corticoid or saline

	Corticoid group (n = 10)	Control group (n = 10)
Hydroxyproline content (µmol/g)[a] mean ± SD	5.11 ± 0.87	5.72 ± 0.86
Septal fibrosis (grade)[b, c]		
+	1	-
++	6	1
+++	3	9

a: p = 0.07; *b*: semiquantitative analysis ; *c:* p = 0.0037.

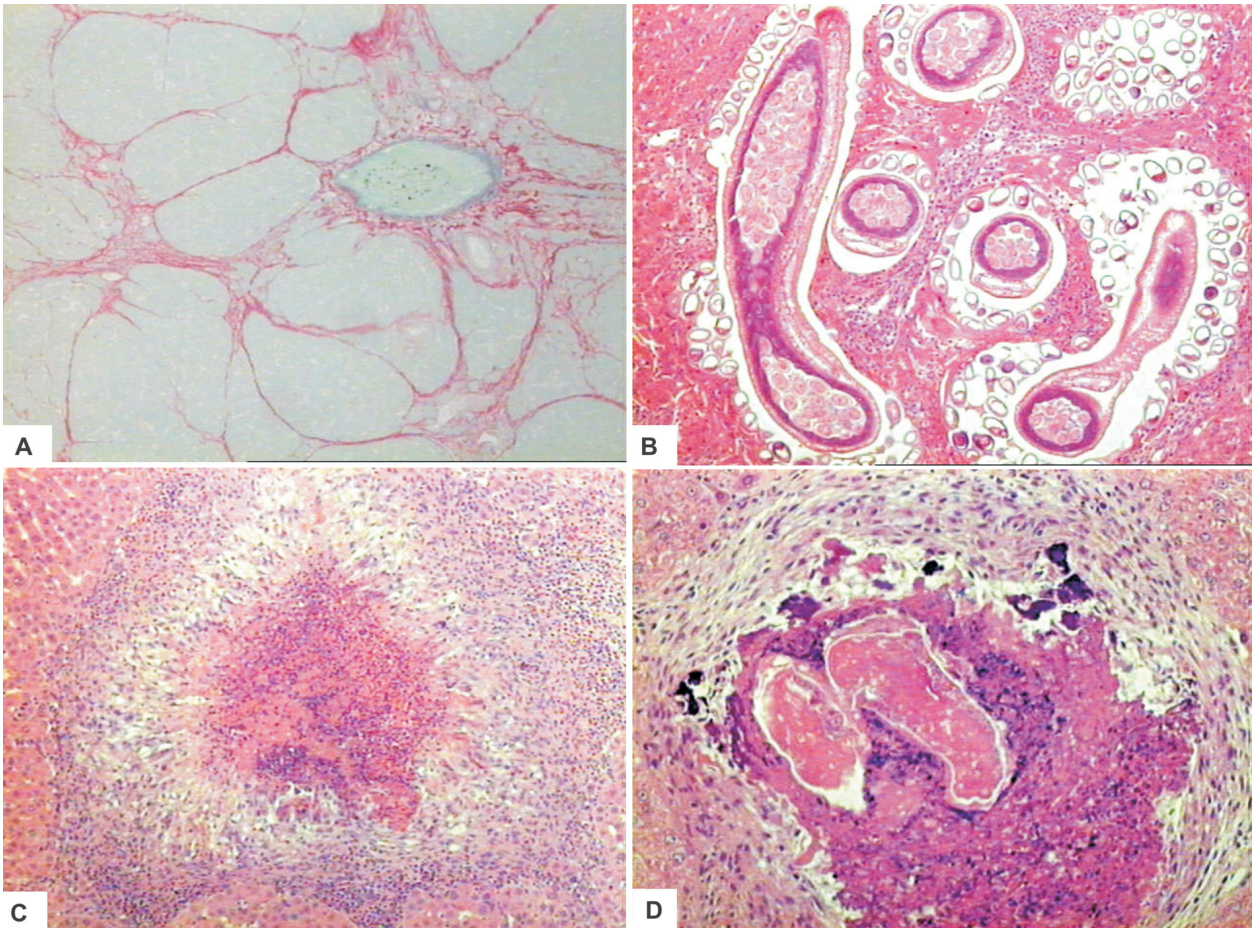

Fig 1A: the usual aspect of septal fibrosis represented by thin fibrous septa dissociating the hepatic parenchyma into irregular portions. Sirius red for collagen, × 100; B: focal parasitic lesion in a rat treated with corticoid. There is the striking presence of live worms surrounded by numerous parasite eggs; C: focal parasitic lesion in a rat treated with *Capillaria hepatica* antigen. There is focal hyaline necrosis delimited by a thick fibrous capsule and depicting an inner area of macrophage palisade; D: control submitted only to infection. There is a combination of necrosis, focal calcifications, dead worms, chronic inflammation, and fibrosis. B, C, D: hematoxylin and eosin, ×100.

present in the septa were predominantly lymphocytes and fibroblast-like, with a few sparsely distributed polymorph nuclear eosinophils.

The focal parasitic lesions from the animals treated with *C. hepatica* antigens were recognized at blind examination of the microscopic slides, especially by the presence of a thick fibrous capsule, abundant interstitial fibrosis, fewer eggs, and a predominance of necrotic worms, in comparison to controls (Fig. 1D).

The serum antibody levels did differ for the antigen-treated groups in comparison to controls, although all groups were infected and submitted to Freund's adjuvant, except for the only-infected group. These data appear on Fig. 2.

DISCUSSION

Septal fibrosis associated with *C. hepatica* infection has been considered as an experimental model for the study of hepatic fibrosis. Its usefulness for testing anti-fibrotic drug has been demonstrated (Souza et al. 2000, 2001). Several studies have been dedicated to the understanding of the pathogenesis of septal fibrosis. The model

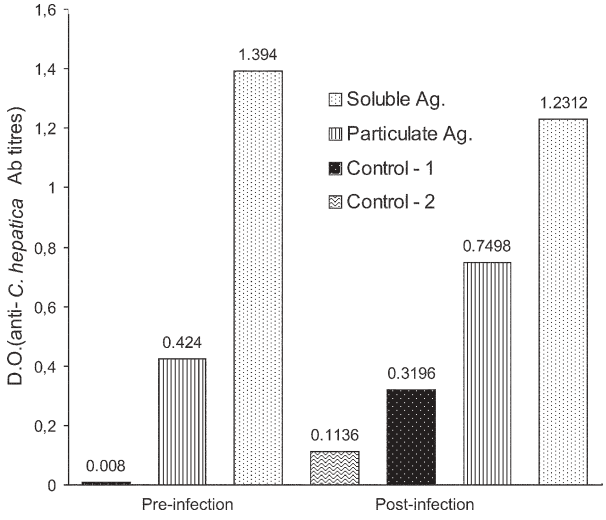

Fig. 2: rat anti-*Capillaria hepatica* antibody titres, before and 30 days following infection with 600 embryonated eggs of *C. hepatica* (titres are not represented for non-infected, intact, control - 2 group). The above numbers (in bars) represent the mean for each group.

presents advantages and disadvantages when compared to another similar model obtained in rats, the one produced by repeated intraperitoneal injections of pig-serum or its albumin fraction (Paronetto & Popper 1966, Andrade 1991, Bhunchet et al. 1996).

One advantage of the *C. hepatica* model is that septal fibrosis develops in 100% of the infected rats and its chronology can be predicted with a good margin of confidence. This has facilitated the timing when one is looking for the earliest changes leading to septal fibrosis, the details on its site of origin, and its routes of progression. These studies have revealed that septa takes origin from portal spaces, have a strong component of angiogenesis and portal mesenchymal cell proliferation, and do not show an evident stellate-cell involvement (Souza et al. 2006b).

On the side of the disadvantages stand the complexities of the inciting agents derived from the early larvae, worms, eggs and the focal inflammatory lesions they produce in the liver, in comparison with the relative simplicity of the pig-serum components.

The present investigation reveals a curious lack of correlation between the changes that can occur in the focal parasitic lesions and the systemic process of septal fibrosis associated with *C. hepatica* infection, indicating that the two lesions are related to the same etiology, but pathogenetically dissociated. Probably the chemical or molecular inciting cause or causes of septal fibrosis take origin from the focal parasitic lesions, act on a complex of portal mesenchymal cells that, from then on, follow an independent pathogenetic pathway. The direct lesions caused by *C. hepatica* are self-limited. During the vital cycle of this parasite, as soon as the adult worms develop and lay eggs, all of them will spontaneously die. However, the *C. hepatica*-induced septal fibrosis persists apparently active until 4 to 6 months following infection, remaining as such for at least over a year (Souza et al. 2006a). Future studies should be concentrated on the factors present within the active focal parasitic lesions in order to isolate the main factor or its molecules that stimulate the process of septal fibrosis in the rat. A whole extract of the lesions does not suffice, since repeated injections of such material into rats resulted in only 20% of septal fibrosis, while, under the same experimental conditions, whole pig-serum administration caused 44.4% of septal fibrosis (Gotardo et al. 2003). Finally, studies aiming at detecting the molecular pathogenesis of fibrosis may bring crucial data for the understanding and treatment of fibrosis, and the *C. hepatica* model stands as a good candidate.

REFERENCES

Andrade SB, Andrade ZA 2004. Experimental hepatic fibrosis due to *Capillaria hepatica* infection (differential features presented by rats and mice) *Mem Inst Oswaldo Cruz 99*: 399-406.

Andrade ZA 1991. Contribution to the study of septal fibrosis of the liver. *Internat J Exper Pathol 72*: 533-562.

Andrade ZA, Assis BCA, Souza MM 2005. *Capillaria hepatica*: papel em patologia humana e potencial como modelo experimental. In Coura JR, *Dinâmica das Doença Infecciosas e Parasitárias*, Guanabara-Koogan, Rio de Janeiro, cap. 94, p. 1121-1132.

Bergman I, Loxley R 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Ann Chem 35*: 1961-1965.

Bhunchet E, Eishi Y, Wake K 1996. Contribution of immune response to the hepatic fibrosis induced by porcine serum. *Hepatology 23*: 811-817.

Ferreira LA, Andrade ZA 1993. *Capillaria hepatica*: a cause of septal fibrosis of the liver. *Mem Inst Oswaldo Cruz 88*: 441-447.

Gotardo BM, Andrade RG, Oliveira LF, Andrade ZA 2003. Production of septal fibrosis of the liver by means of foreign protein injections into rats. *Rev Soc Bras Med Trop 36*: 577-580.

Hawk CT, Leary SL 1999. *Formulary for Laboratory Animals*, 5th ed., Iowa State University Press, Iowa, 168 pp.

Lemos QT, Magalhães-Santos IF, Andrade ZA 2003. Immunological basis of septal fibrosis of the liver in *Capillaria hepatica*-infected rats. *Braz J Med Biol Res 36*: 1201-1207.

Oliveira L, Souza MM, Andrade ZA 2004. *Capillaria hepatica*-induced hepatic fibrosis in rats (Paradoxical effect of repeated infections). *Rev Soc Bras Med Trop 37*: 123-127.

Paronetto F, Popper H 1966. Chronic liver injury induced by immunologic reactions. Cirrhosis following immunization with heterologous sera. *Am J Pathol 49*: 1087-1101.

Santos AB, Tolentino Jr M, Andrade ZA 2001. Pathogenesis of hepatic septal fibrosis associated with *Capillaria hepatica* infection of rats. *Rev Soc Bras Med Trop 34*: 503-506.

Souza MM, Paraná R, Trepo C, Barbosa Jr AA, Oliveira I, Andrade ZA 2001. Effect of interferon-α on experimental septal fibrosis of the liver - Study with a new model. *Mem Inst Oswaldo Cruz 96*: 343-348.

Souza MM, Silva LM, Barbosa Jr A, Oliveira IR, Paraná R, Andrade ZA 2000. Hepatic capillariasis in rats: a new model for testing anti-fibrosis drugs. *Braz J Med Biol Res 33*: 1329-1334.

Souza MM, Tolentino Jr M, Assis BCA, Gonzalez ACO, Silva TMC, Andrade ZA 2006a. Significance and fate of septal fibrosis of the liver. *Hepatol Res 35*: 31-36.

Souza MM, Tolentino Jr M, Assis BCA, Gonzalez ACO, Silva TMC, Andrade ZA 2006b. Pathogenesis of septal fibrosis of the liver. (An experimental study with a new model). *Pathol Res Pract* (in press).