Immunological Tolerance to Pig-serum Partially Inhibits the Formation of Septal Fibrosis of the Liver in *Capillaria hepatica*-infected Rats

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Systhematized septal fibrosis of the liver can be induced in rats either by repeated intraperitoneal injections of pig-serum or by Capillaria hepatica *infection. The relationship between these two etiological factors, as far as hepatic fibrosis is concerned, is not known, and present investigation attempts to investigate it.*

C. hepatica*-induced septal fibrosis of the liver was considerably inhibited in rats previously rendered tolerant to pig-serum. Pig-serum-tolerant rats developed antibodies against pig-serum when infected with* C. hepatica*, but this did not happen when the infection occurred in normal rats. On the other hand, anti-*C. hepatica *antibodies failed to recognize any epitope in pig-serum, by Western blot. However, no evidence of an immunological cross reactivity was found, at least at the humoral level. Alternatively, cell-mediated mechanisms may be involved, and further investigations are warranted.*

Key words: pig-serum model - *Capillaria hepatica* - hepatic septal fibrosis

Rats infected with the helminth *Capillaria hepatica* invariably develop a peculiar type of hepatic fibrosis, 30-40 days after inoculation. By the time the worms are usually dying and disintegrating, and while the focal necro-inflammatory lesions around them are exhibiting evidences of resorption, septal fibrosis starts throughout the liver (Ferreira & Andrade 1993). Morphologically, it has close similarity to the fibrosis experimentally induced in rats by repeatedly intraperitoneal injections of pig-serum or by its albumin fraction (Paronetto & Popper 1966, Ballardini et al. 1985, Andrade 1991, Bhunchet et al. 1996). In both cases the histological picture is dominated by fine and long fibrous septa, that crisscross the hepatic parenchyma, forming a mosaic pattern, sometimes reminiscent of the pig liver, that progressively may evolve toward a final morphological picture of cirrhosis (Paronetto & Popper 1966, Ferreira & Andrade 1993). Fibrosis first connects portal spaces to portal spaces and runs within and along the peri-sinusoidal zone III area of the liver acinus, but is preceded neither by hepato-cellular necrosis, nor by overt chronic inflammation (Rubin et al. 1969). Such characteristics strongly suggest the existence of a stimulus to non-parenchymal hepatic cells, probably through an immunological mechanism (Nakano 1980, Tsukamoto et al. 1990, Santos et al. 2001).

Bhunchet et al. (1996) demonstrated that rats turned tolerant to pig-serum failed to develop septal fibrosis or antibodies against it, when repeatedly injected during adult life. As a control to their experiments, they showed that the pig-serum tolerant animals still maintained their capacity to respond with hepatic fibrosis when subjected to treatment with carbon-tetrachloride. However, it would be more interesting in such case, to use another model of septal fibrosis, with similar characteristics as those of the pig-serum model, but dependent upon a different etiology. Since specificity is a hallmark of immunological reactions, such attempt would be of crucial importance. On this regard, it is herein indicated the *C. hepatica*-model as an adequate candidate. In as much as the *C. hepatica*-induced fibrosis can be partially suppressed by a neonatal toleration method similar to that used for the pig-serum model (Lemos et al. 2003).

The present investigation checks on the similarities between the pig-serum and *C. hepatica* models of septal hepatic fibrosis, by analyzing the behavior of the parasite infection in pig-serum tolerant rats. This would test whether there is specificity for the pig-serum model of hepatic fibrosis and would eventually contribute to the concept that some types of hepatic fibrosis indeed have an immunological basis.

MATERIALS AND METHODS

Experimental groups

Group I - Thirteen neonatal Wistar rats of both sexes, received intraperitoneal injections of whole pig-serum, twice a week, from the first day of life up to adult life. They received a total of 36 injections, starting with the dose of 0.05 ml, which was gradually increased up to 1 ml by the 10th day of injection onward. Immediately in sequence, these 18-week old animals were submitted to a liver biopsy, followed by the administration, in five of the animals, of 300 embryonated eggs of *C. hepatica*, suspended in saline, and placed directly into the stomach by a gastric tube. The remaining eight animals continued to be injected

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with pig-serum up to the end of experiment. Forty days after infection, a new hepatic biopsy was performed in all animals. They were sacrificed 30 days later, 70 days after *C. hepatica* infection. Details about the obtaining of eggs and their counting appear elsewhere (Ferreira & Andrade 1993).

Group II - a) control for fibrosis induced by pig-serum - Ten 8-week old intact Wistar rats, males and females, weighting approximately 200 g, received intraperitoneal injections of 1 ml of whole pig-serum, twice a week, totaling 26 injections. After that period they were submitted to a surgical liver biopsy. At the same time blood was taken for serological tests; *b) control of C. hepatica infection* - Four 8-week old Wistar rats, weighing 200 g, males and females, were infected with approximately 300 embryonated eggs of *C. hepatica*, administered by gavage, and were sacrificed 40 days later.

All the animals were maintained in good housing conditions, with controlled temperature and humidity, in separated boxes according to sexes, with free access to a commercial balanced diet and water. The size of the inoculum was arbitrarily chosen within the range expected to regularly produce septal fibrosis (Oliveira & Andrade 2001).

Pig-serum - The serum was obtained from the blood of one recently killed adult healthy pig. After blood coagulation at room temperature and centrifugation, the serum was collected, frozen in liquid nitrogen, and stored at –70^{o}C in several vials, each one being thawed at the moment of use. Only one batch was utilized throughout the experiments.

C. hepatica eggs - They were obtained from the livers of experimentally infected rats, around the 40th day after inoculation. The livers were washed in order to get rid of excess blood, homogeinized in an electrical blender, followed by several turns of washing with tap water and decantation, until the liquid above the sediment was completely clear. The clean immature eggs were kept moisturized with 0.5% formalin solution in a Petri dish, at room temperature (26-28^{o}C), for 28-30 days, in order to embryonate. They were counted under the microscope and used in the dose of 300 eggs suspended in 0.5 ml of distilled water per each animal.

Liver biopsy - With the animals under sodium pentobarbital general anesthesia, their abdomens were shaved and aseptically opened at the midline. The liver was exposed and a fragment of approximately 0.25 to 0.30 g was tied and removed. Recovery of the operated animals was uneventful.

Tissue preparation - Fragments obtained from biopsies and at the time of sacrifice of the animals were immediately fixed in neutral 10% formalin and routinely processed for paraffin embedding and cutting. Sections were stained with hematoxylin and eosin, picro-sirius-red method for collagen, PAS method, with and without previous diastase treatment, Gomori's silver impregnation for reticulin and the Perl's method for iron.

ELISA - This method was used to evaluate serum antibody levels either against pig-serum and *C. hepatica*. Sera were colleted from the tail vein at several occasions. For the animals injected since the first neonatal day with pig-serum, sera were obtained at the end of the toleration period and at the final period of *C. hepatica* infection. As for the control groups, sera were taken before and after either pig-serum treatment or *C. hepatica* infection. Detection of total Ig antibodies was made by using a goat anti-rat IgG conjugated to peroxidase (Sigma, St. Louis, MO, US). The plates were sensitized with 10 µg/ml/per well of either *C. hepatica*-egg antigen or pig-serum, diluted in carbonate buffer, pH 9.9. Lecture was made on a microplate reader "Molecular devices-Thermomax" spectrophotometer (Sunyvalle, CA, US) under wave-length 450 mm, connected to a computer with MDS-Soft Max with MDS-Soft Max. All sera were used at 1:1000 dilution in ELISA assays.

Western blot - *C. hepatica* antigens were prepared either from adult worms or from immature eggs. These materials were separately collected from the livers of experimentally infected mice. They were washed several times in distilled water, concentrated by centrifugation, and then homogenized, and suspended in saline. The samples utilized included these antigens, pig-serum, normal rat serum and sera from *C. hepatica*-infected rats. The samples were diluted in buffer (2% sodium dodecyl sulfate – SDS, 10% glycerol, 50 mM Tris pH 6.8) plus 100 mM mercaptoethanol, 0.1% bromophenol blue, and submitted to electrophoresis in 15% polyacrylamide gel and 5% stacking gel (Amresco Inc., Solon, OH, US). The gel was transferred to a nitrocellulose membrane for 2 h at 100 V (Novex – Novel Experimental Technology, San Diego, CA, US). The membranes were blocked with phosphate-buffered saline containing 0.3% tween and 5% skimmed milk during 1 h, under constant agitation. The membrane was then incubated with 1:650 *C. hepatica*-infected rat serum in blocking buffer, during 1 h under agitation. This was followed by incubation with rabbit peroxidase-conjugated anti-rat IgG (Sigma), diluted 1:650. Staining was accomplished by means of a peroxidase substrate-chromogen kit (Vector Laboratories, Inc., Burlingame, CA). The reaction was stopped with water.

Statistical analysis - The Kruskal-Wallis and the Dunn multiple comparison nonparametrical tests were used to compare serological data obtained from different experimental groups, considering $P < 0.05$ as significant.

RESULTS

All pig-serum tolerant animals developed infection when inoculated with embryonated *C. hepatica* eggs. The liver sections exhibited both disintegrating and well-preserved worms and eggs, within encapsulated focal lesions, which were sparsely distributed throughout the hepatic parenchyma. When compared with infected controls, these parasitic lesions were less numerous (Table), with diminished inflammatory cellular infiltration and peri-focal fibrosis. These lesions also contained several well-preserved worms, which were exceptionally seen in non-tolerant rats by the 40th day of infection. However, a clear-cut difference was noted in the degree and distribution of septal fibrosis. The livers of *C. hepatica*-infected pig-serum tolerant rats presented septal fibrosis in some focal areas of the liver only. Septa were thin and long, segregating areas of parenchyma of different sizes and appearance. Most of the time septa fibrosis was limited to a few

TABLE

Semi quantitative evaluation of hepatic fibrosis and inflammation in rats treated with pig serum and/or infected with *Capillaria hepatica*

Groups	Description	N	Fibrosis			Inflammation
			Present	Absent	Grade	
I	Pig-serum tolerant *C. hepatica*-infected	5	5	0	+/++	+
I	Non-infected pig-serum tolerant	8	0	8	0	0
II a	Pig-serum treated	9	4	5	++	0
II b	*C. hepatica*-infected	4	4	0	+++	++/+++

+ mild; ++ moderate; +++ marked

spurs radiating from the capsule of the focal parasitic lesions, while the rest of the liver parenchyma maintained its normal appearance (Fig. 1A). On the other hand, the livers from infected control rats exhibited septal fibrosis throughout the parenchyma, being more accentuated around focal parasitic lesions. Frequently, a morphologic picture of cirrhosis was formed, with round hepato-cellular nodules delimited by fibrous septa (Fig. 1B).

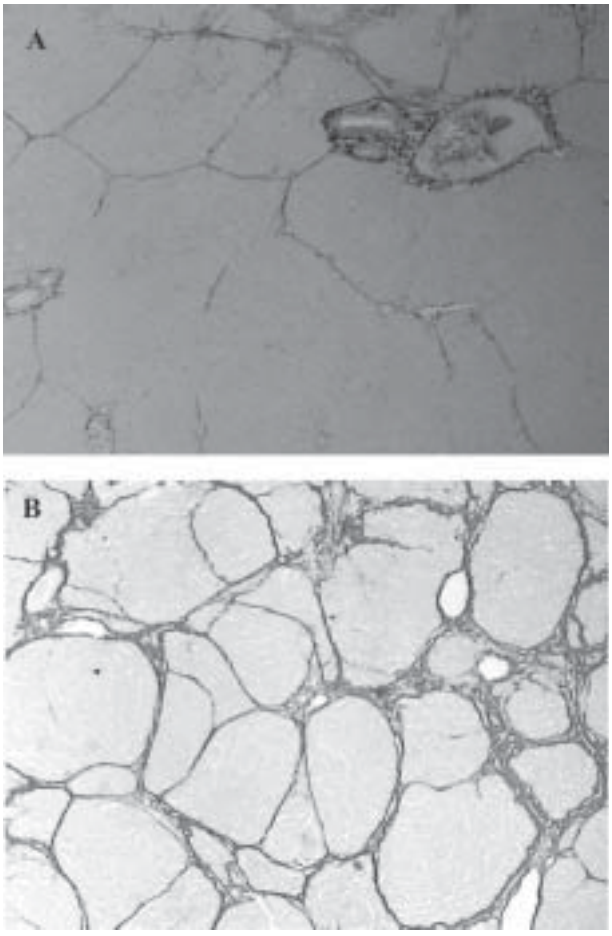

Fig. 1: septal fibrosis of the liver in a rat infected with *Capillaria hepatica*, as seen 40 days after infection. A: pig-serum tolerant rat. Septa are few, thin, and discontinued; B: non-tolerant rat. Septa are numerous and encircle irregular and nodular portions of the hepatic parenchyma, simulating a picture of cirrhosis. Picro-sirius-red staining method for collagen. X100

Animals subjected to immunological tolerance with pig-serum did not exhibit serum antibodies, neither against pig-serum or *C. hepatica* antigens (Figs 2, 3). However, 40 days after being infected with *C. hepatica*, the pig-serum tolerant animals presented serum antibodies against both pig-serum and *C. hepatica*. (Fig. 2). On the other hand, both antibodies were detected in the sera of normal adult rats after they were repeatedly treated with pig-serum (Figs 2, 3). However, the presence of antibodies against pig-serum was not detected in the sera of rats infected with *C. hepatica* only, as measured 40 days after inoculation (Fig. 2).

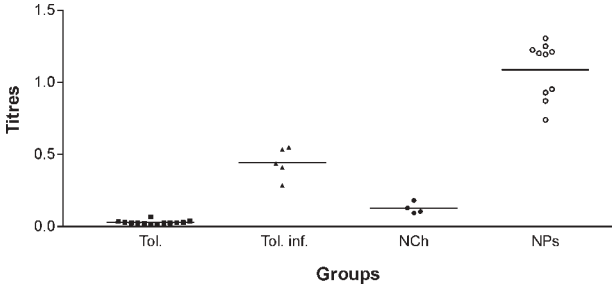

Fig. 2: total Ig against pig-serum (ELISA) in pig-serum tolerant rats: before (Tol.) and after *Capillaria hepatica* infection (Tol. inf.); and in normal rats: after *C. hepatica* infection (NCh) and following repeated pig-serum administration (NPs) $P > 0.05$ Tol. vs Tol. inf.; $P > 0.05$ Tol. vs NCh; $P < 0.001$ Tol. vs NPs

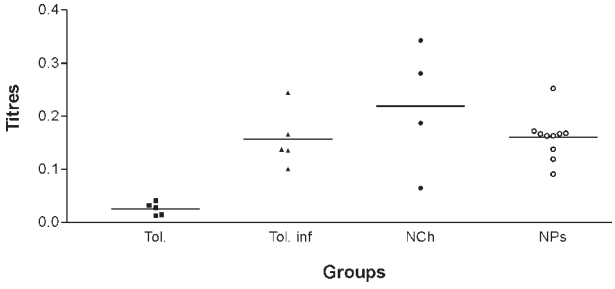

Fig. 3: total Ig against *Capillaria hepatica* (ELISA) in the sera of pig-serum tolerant rats (Tol.: tolerant non-infected), tolerant post-*C. hepatica* infection (Tol. inf.), in normal non-tolerant rats (NCh: non-tolerant *C. hepatica*-infected rats) and in normal rats repeatedly injected with pig-serum (NPs) $P > 0.05$ Tol. vs Tol. inf.; $P < 0.01$ Tol. vs NCh; $P < 0.05$ Tol. vs NPs

The antibodies present in the sera of *C. hepatica*-infected rats (Group II b) failed to recognize any antigenic fraction from the pig-serum by Western-blot.

As observed by histopathology, not a single animal receiving pig-serum injections from the first neonatal day until adult life, developed septal fibrosis of the liver. However, a moderate to severe degree of septal fibrosis did develop in four out of nine normal rats treated with pig-serum since adult life. This group was reduced to nine animals because one of them died spontaneously near the end of the experiment and was not autopsied. Antibodies were present in the serum of all these treated animals, but no correlation was found between their levels and the degree of septal fibrosis, even when animals with and without fibrosis were compared.

All animals inoculated with embryonated *C. hepatica* eggs developed infection.

DISCUSSION

The present investigation confirmed and extended previously described observations concerned with the induction of liver fibrosis in adult rats receiving multiple injections of pig-serum (Paronetto & Popper 1966, Ballardini et al. 1985, Bhunchet et al. 1996), and its absence when pig-serum administration started in the neonatal period (Bhunchet et al. 1996). In addition to maintaining their capacity to respond with hepatic fibrosis to carbon tetrachloride administration, as demonstrated by Bhunchet et al. (1996), pig-serum tolerant rats were still able to develop septal hepatic fibrosis, when subjected to a different etiologic agent, although to a reduced degree. In addition, it was also shown that long-term pig-serum administration, starting in the neonatal period, could profoundly influence the development of liver fibrosis, which usually accompanies the infection of rats with *C. hepatica*. The animals presented a less intense inflammatory infiltrate in response to the parasitic infection and an inhibition in the development of septal fibrosis of the liver. However, *C. hepatica* infection also raised up the levels of antibodies to pig-serum components in previously unresponsive rats. Higgins and Weiner (1988) have called attention to the fact that disease manifestations can be partially or completely suppressed in tolerant host in a dose-dependent manner, indicating that the state of tolerance is not a static one.

The upsurge of anti-pig-serum antibodies in previously tolerant rats infected with *C. hepatica* suggests that this helminth may share common antigenic epitopes with pig-serum. This primary hypothesis was ruled out by the demonstration that *C. hepatica*-infected rats do not produce specific antibodies to pig-serum components, although adult rats injected multiple times with pig-serum showed an antibody response to *C. hepatica* antigens. Also, in both experimental approaches rats produced specific antibodies to immunizing antigens. This set of experimental data strongly suggests that these different sets of antigens (*C. hepatica* and pig-serum) do not share cross-reactive epitopes at the level of antibody recognition, as far as IgG antibodies are concerned. However, one may not rule out the possibility that such set of antigens may share T cell epitopes. Reactivity to immunoglobulin isotypes other that IgG should also be considered. Therefore, breaking of tolerance to pig-serum antigens by *C. hepatica* infection, as suggested in other experimental systems (Costalonga et al. 2002, Roep et al. 2002), may still be the explanation for this finding. Yet, the raise in antibody levels to pig-serum antigens in previously unresponsive mice, induced by infection with *C. hepatica* in the absence of further specific antigen administration is intriguing, and suggests that the rats have been, in fact, silenced primed for an immune response. In addition, the set of data showed in this study argues against a major role for antibodies in the genesis of liver fibrosis in this model, since animals presenting similar levels of antibodies, either to pig-serum components or *C. hepatica*, presented distinct histological aspects in relation to liver fibrosis and inflammatory response to the parasitic agent. Taken together, these provoking findings may stimulate the formulation of an alternative hypothesis to be considered. First of all, one should point out that the immune response to pig-serum antigens has a conventional aspect, which is the production of specific antibodies to pig-serum components, which apparently has no major impact in the modulation of liver fibrogenesis and a very peculiar, local or regional liver immune response that may lead to major changes in liver fibrogenesis without affecting the hepatocytes. The liver harbors conventional T cells, NK cells, gamma-delta T cells, and a special T cell lineage known as NK T cells (Kmiec 2001). It was shown that NK T cells secrete a number of different cytokines upon stimulation, including high amounts of IFN-γ and IL-4 (Burdin et al. 1999, Takeda et al. 2000). More importantly, the pattern of NK T cell cytokine production may be modulated by chronic antigen stimulation (Yoshimoto & Paul 1994). In addition, liver NK T cells have been related to liver injury in a mouse model of autoimmune hepatitis (Chen & Paul 1997) and in one study, NK T cells were involved with liver fibrosis in a mouse model of infection (Carvalho et al. 2002). Therefore, experiments aiming at characterizing the role of NK T cells and conventional T cells are urgently required to better define their role in liver fibrogenesis and, in particular, in this experimental model.

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