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SCHISTOSOMIASIS MANSONI: EVIDENCE FOR A MILDER RESPONSE IN GERMFREE MICE

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ABSTRACT

Germfree (GF and conventional (CV) mice were infected intraperitoneally with GF cercariae of **Schistosoma mansoni** and kept for six weeks. Twenty four hours before killing, they were injected with [^{3}H]-thymidine. **Schistosoma** worms, harvested after perfusion of portal system, were counted as well as eggs from liver and intestines. Liver was also used for DNA, protein, and collagen determinations. [^{3}H]-Thymidine incorporation and collagen determinations were used to establish the indices given by the difference between their contents in infected and control animals and expressed per thousand eggs in liver. The recovery of worms in GF mice was around twice as much as in CV ones, and the total number of eggs was higher in the liver of GF animals. No hypertrophy of liver cells was observed by the ratio protein/DNA, but [^{3}H]-thymidine incorporation into DNA was higher than in controls in both GF and CV infected animals. The [^{3}H]-thymidine and collagen indices were lower in GF animals which indicate a more discrete cellular replication and smaller collagen content in relation to the number of eggs present in livers of these mice. It was concluded that the disease seems to be less severe in GF animals.

KEY WORDS: Germfree mice; Schistosomiasis mansoni; Axenic cercariae.

INTRODUCTION

The worm **Schistosoma mansoni** is a blood fluke that inhabits the portal system of some mammals. The female lays thousands of eggs in the mesenteric veins, around 50% of which may become trapped in the host's tissues, mainly the liver and intestines [23]. These eggs elicit the formation of a granulomatous inflammation, characterized by the infiltration of macrophages, T and B lymphocytes, eosinophils, fibroblasts, plasmocytes, neutrophils, and mastocytes [1]. The granuloma leads to formation of scar tissue in the region, as a consequence of collagen synthesis by fibroblasts which have been shown to replicate **in vitro** when exposed to products from **S. mansoni** eggs or from granuloma [25].

Previous data had shown that germfree (GF) mice respond to schistosomiasis in a milder degree when compared with conventional (CV) mice. Granulomas were less numerous, hepatomegaly and splenomegaly less acentuated and portal hypertension was virtually absent in the GF animals [22].

The objective of this work was to study liver reaction of GF and CV mice infected with **S. mansoni** comparing collagen content,

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local cell replication or migration, number of eggs, and worm recovery from portal system.

MATERIALS AND METHODS

Germfree cercariae were obtained from germfree **Biomphalaria glabrata** snails, as previously described [22,6,21]. They were used to infect, by intraperitoneal injection, GF and CV LOB: (CFW) mice of 70 days of age. GF mice were kept in individual cages inside plastic isolators (Standard Safety Equipment Co., Palatine, USA) [15]. CV mice were kept in an open room in individual cages and fed the same autoclaved diet given to GF mice. After six weeks of infection, mice were injected intraperitoneally with 10 μCi of [^{3}H]-thymidine (New England Nuclear, Boston, MA). Mice were killed 24 hours later and the hepatic portal system was perfused with citrate containing saline [14]. The perfusion liquid was collected in conical flasks and worms were harvested from the bottom, counted and expressed by geometric means. Small intestines and livers were removed. Each liver was finely chopped and approximately 1/3 of it was separated for egg counting and the remainder was kept frozen for future assays. Small intestine and the 1/3 of the finely chopped liver were digested with 5% KOH and the eggs were counted [4]. Non-infected control mice were injeted with [^{3}H])-thymidine and killed 24 hours later. Their portal systems were also perfused and livers were removed. Liver egg counts were corrected for the whole liver.

Protein and DNA were assayed in properly diluted liver homogenates [11,16]. [^{3}H]-Thymidine incorporation into DNA was evaluated after perchloric acid extraction from liver homogenate and the counting (Beckman LS 150 Spectrometer, Beckman Inst., Palo Alto, CA, USA) was corrected for the total radioactivity in the homogenate, Collagen was assayed in digested samples of liver [2] by hydroxyproline determination [20]. [^{3}H]-Thymidine and collagen indices were calculated, respectively, by the difference between the mean of the determination in infected and in control animals, being expressed per thousand eggs found in livers. A similar index has already been described [5].

Adult worm recovery was statistically analyzed by the chi-square test, Student t test and analysis of variance followed by comparison t test were performed to compare the remaining data [19].

RESULTS

The absolute and percentual recoveries of adult worms in relation to the amount of GF cercariae injected is shown in table I. GF mice harboured more adult worms than the CV animals and the percentual recovery was consequently higher in GF mice.

TABLE I

Adult **Schistosoma mansoni** worm recovery after perfusion of the portal system of germfree (GF) and conventional (CV) mice. (Mean ± SEM)

Group[1]	Cercariae Injected	Worms Recovered	Recovery[2] %
CV (12)	47	5.7 ± 0.5	12.1 a
CV (4)	52	7.5 ± 0.6	14.4 a
GF (4)	51	13.5 ± 1.1	26.5 b
GF (9)	38	9.2 ± 0.8	24.3 b

1. The number of animals used in each group is in parentheses. Two independent infections were performed in CV and two in GF.
2. Different letters stand for statistically significant differences by the chi-squase test ($P < 0.01$).

The total number of eggs found in livers was higher in GF animals; however, the number of eggs per worm pair was similar in both groups, as well as the number of eggs trapped in the small intestine. (Fig. 1).

Protein and DNA contents in livers of GF and CV mice are shown in Table II. The protein content of livers from infected GF mice was significantly higher than their uninfected counterparts. This difference, however, was not observed in CV animals. Livers from infected GF mice showed a significantly higher DNA content than their uninfected controls. CV animals either infected or uninfected showed same values for DNA content in livers. The ratios protein/DNA were similar in all groups.

Table III shows the values for [^{3}H]-thymidine incorporation in liver DNA. Thymidine incorporation per liver was higher in infected animals than in controls in both GF and CV

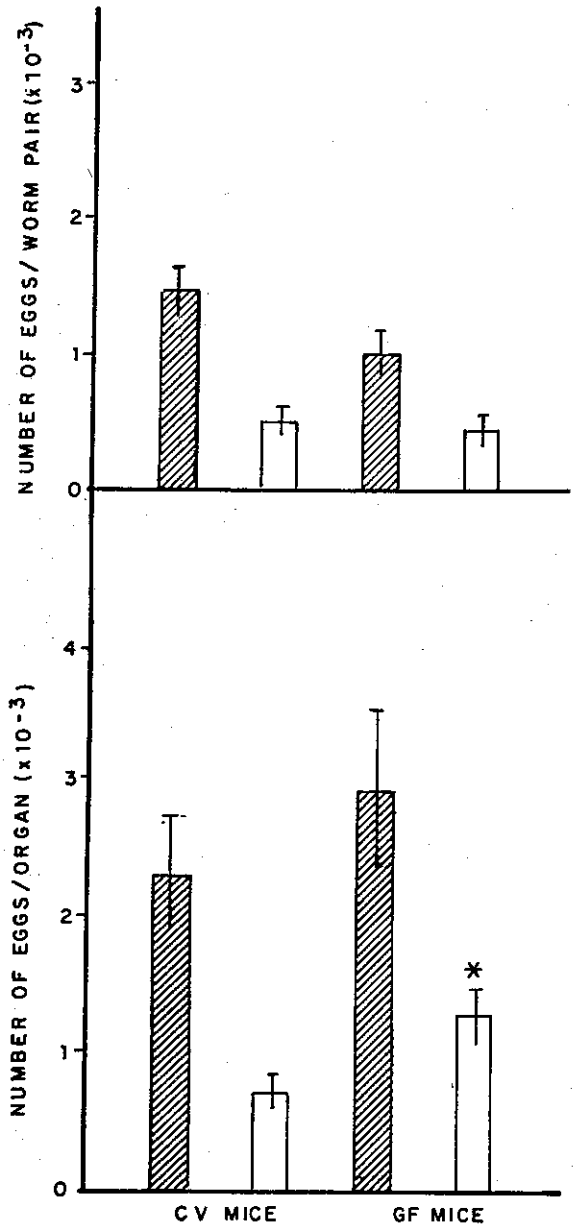

Fig. 1 — Number of eggs per organ and number of eggs per worm pair found in livers (□) and small intestines (□) of germfree (GF) and conventional (CV) infected mice. *Number of eggs per liver in GF mice is statistically different from CV ones ($P < 0.05$). Bars represent mean ± SEM.

groups. GF control animals showed higher incorporation of [^{3}H]-thymidine per liver than CV controls.

Table IV shows the [^{3}H]-thymidine and collagen indices for GF and CV mice. The [^{3}H]-Thymidine index indicates cell multiplication or migration to the site of granuloma formation. The collagen index indicates that this protein has been formed as a consequence of granulomatous lesions. Both indices were higher in CV animals.

T A B L E II

Protein, DNA, and the ratio Protein/DNA in livers of control (c) and Schistosoma mansoni infected (i) conventional (CV) and germfree (GF) mice. *

Group	Protein (mg/organ)	DNA (mg/organ)	Protein/DNA
iCV	214.0 ± 8.5^{a,b} (16)	4.94 ± 0.31^{a,b,c} (15)	45.91 ± 3.33^{a} (15)
cCV	196.5 ± 7.2^{a} (15)	4.15 ± 0.19^{b} (14)	49.86 ± 3.20^{a} (14)
iGF	235.8 ± 9.3^{b} (13)	5.24 ± 0.36^{c} (12)	46.37 ± 3.95^{a} (12)
cGF	199.2 ± 5.9^{a} (15)	4.18 ± 0.24^{b,d} (13)	49.52 ± 3.27^{a} (13)

* Mean ± SEM. Numbers of animals used in each assay are in parentheses. Means bearing the same letter are not stastistically different ($P < 0.05$).

T A B L E III

[^{3}H]-Thymidine incorporation in DNA of livers of control (c) and Schistosoma mansoni infected (i) germfree (GF) and conventional (CV) mice. *

Group	CPM % of the total	CPM/mg DNA % of the total
iCV	77.9 ± 2.4^{a} (16)	16.6 ± 1.1^{a,c,d} (15)
cCV	52.1 ± 2.0^{b} (15)	12.8 ± 0.7^{b} (14)
iGF	87.8 ± 3.0^{c} (11)	18.2 ± 1.3^{c} (11)
cGF	64.2 ± 0.4^{d} (15)	14.5 ± 1.3^{b,d} (12)

* Mean ± SEM. Number of animals used in each assay are in parentheses. Means bearing the same letter are not statistically different ($P < 0.05$).

DISCUSSION

The highest recovery of cercariae, as adults worms, which was obtained in CV animals (15%) has been lower than that reported in the literature which is around 40% [18]. This may be due to the use of extensively manipulated GF cercariae to infect GF and CV mice. It might also be possible that axenic cercariae are less resistant than conventional ones.

TABLE IV

Indices related to granuloma formation in conventional (CV) and germfree (GF) mice infected with **Schistosoma mansoni**

Groups	[^{3}H]-Thymidine*	Collagen**
CV	5.63	20.9
GF	2.79	16.7

Indices:

* $\frac{\text{mean [}^3\text{H]-thymidine in DNA} - \text{mean [}^3\text{H]-thymidine in DNA}}{\text{mg DNA in infected livers} \quad \text{mg DNA in normal livers}} \times 10^3$

** $\frac{\text{mg hydroxiproline in infected livers} - \text{mg hydroxiproline in normal livers}}{\text{mean number of eggs in infected livers}} \times 10^3$

On the other hand, GF mice harboured more worms than CV group, a phenomenon perhaps ascribable to the immunological mechanisms which are involved in the killing of the S. **mansoni** larvae during migration. The reason for this is unkwown, since the mechanism of death of worms during migration is obscure. Macrophages from GF animals seem to be less reactive than from CV ones [24]. It has been demonstrated [12] that non-specifically activated macrophages can kill S. **mansoni** larvae in vitro more efficiently than non-activated controls. If macrophages are responsible for the death of migrating schistosomula **in vivo**, we could assume that the cells derived from GF mice had never been exposed to the effects of the normal flora or its products and should be less active than conventional macrophages. If death of migrating larvae occurs in the lungs [13], then CV animals being more exposed to hazards of a contaminated environment should be more efficient in killing invaders. In addition in has been shown [10] that non-specifically activated lymphocytes, kill more schistosomula **in vitro** than non activated cells. If these cells are involved in the mechanism of death during worm migration, GF animals should be less reactive, as it has been shown that their organs harbour more naive lymphocytes than their CV counterparts [17]. On the other hand, egg laying per couple did not seem to be altered by the microbial status of the host, although other authors have shown that it is markedly influenced by host's immunonological status in CV animals [8].

Average liver cell size seems not to be altered in murine schistosomiasis which suggests that liver hypertrophy is not due to an increase in cell size. Liver cell replication or infiltration seems to be occurring as shown by [^{3}H]-thymidine incorporation. These cells could be lymphocytes, fibroblasts, or eosinophils, as factors from eggs and granulomas, that induce cell replication **in vitro**, have been isolated [1,25,3].

It has been demonstrated that granulomas are formed only when mature eggs, or egg products, are present in livers [9]. So we took the [^{3}H]-thymidine incorporation index to show that infected CV animals have more infiltration or replication of cells in the liver, which should be responsible for a more drastic pathological effect of schistosomiasis in those animals than in GF ones. This conclusion is reinforced by the collagen index, which was also higher in CV mice and the production of collagen is related with granulomatous reaction [7]. The small difference in collagen content betwen CV and GF infected livers could be due to an earlier decline in granuloma formation in GF livers with a possible higher scar tissue production in GF than in CV animals at the time of the sacrifice [23]. Still, our hydroxyproline results were quite consistent with those already reported [5]. There may be at least two reasons why GF mice reacted less to S. **mansoni** eggs: 1) They may have less T lymphocytes than CV animals which could reduce the reaction induced by the eggs. It has already been shown that GF rats respond less to phytohemmaglutinin than their CV counterparts [17]; 2) T Lymphocytes from CV animals might be stimulated by the flora nonspecifically and react more promptly against eggs, enhancing the response in CV animals. It is supposed that, due the absence of nonspecific stimulation by the normal micro flora, GF mice would show a milder reaction to eggs in liver than CV mice. For this reason, disease would be less severe in GF animals.

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RESUMO

Esquistossomose mansônica: evidência de menor resposta nos camundongos isentos de germes

Camundongos isentos de germes (GF) e convencionais (CV) foram infectados intraperitonealmente com cercárias GF de **Schistosoma mansoni** e mantidos por seis semanas. Vinte e quatro horas antes do sacrifício, eles foram injetados com [^{3}H]-timidina. Vermes de **Schistosoma**, recolhidos através de perfusão do sistema porta, foram contados, assim como os ovos no fígado e intestino delgado. O fígado foi também usado para determinações de DNA, proteínas e colágeno. A incorporação de [^{3}H]-timidina e as determinações de colágeno foram usadas para calcular os índices dados pela diferença entre seus conteúdos nos animais infectados e controles e expressos por mil ovos no fígado. A recuperação de vermes nos camundongos GF foi cerca de duas vezes aquela dos CV. O número total de ovos foi maior no fígado dos animais GF. Nenhuma hipertrofia das células hepáticas foi observada pela relação proteína/DNA mas a incorporação de [^{3}H]-timidina em DNA foi maior que nos controles em ambos animais infectados (GF e CV). Os índices de [^{3}H]-timidina e colágeno foram menores nos animais GF indicando uma replicação celular mais discreta e um conteúdo de colágeno menor em relação ao número de ovos presentes nos fígados destes camundongos. Concluiu-se que a doença parece ser menos severa em animais GF.

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