Some patients concurrently infected with *Schistosoma mansoni* and Salmonella develop a peculiar clinical picture recognized as chronic septicemic salmonellosis\(^2\)\(^3\)\(^1\). The pathogenesis of this entity is not completely understood but it has been shown that the decreased ability of macrophages to ingest and kill Salmonella may play a part in the prolonged survival of this bacteria\(^1\). Several factors interfere with the efficiency of phagocytosis, including the effect of specific antibodies and the load of bacteria exposed to macrophages. The role played by these intervening factors in schistosomiasis is not clear.

In some experimental models, such as mice infected with Salmonella, specific antibodies are recognized to increase phagocytosis of this bacteria\(^4\)\(^5\)\(^6\), via the receptors for the Fc portion of IgG\(^8\). It is not known whether similar antibodies are capable to increase phagocytosis of otherwise deficient macrophages as occurs in schistosomiasis. Some other class of opsonin-independent receptors, not as yet properly characterized, but unrelated to immunoglobulin or complement, which bind oligosaccharides as mannose or glucan, also mediate phagocytosis\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\). Phagocytosis via these later non-specific receptors would function as a first line defence mechanism, being primarily influenced by the load of bacteria. The importance of this non-immune load dependent phagocytosis in schistosomiasis has not been assessed.

The present work aimed at evaluating the influence of specific antibodies and the bacterial load on the phagocytosis of *Salmonella typhimurium* by peritoneal macrophages from *Schistosoma mansoni*-infected mice.

**MATERIAL AND METHODS**

To evaluate the influence of specific antibodies and the bacterial load on the phagocytosis of *Salmonella typhimurium* by macrophages from *Schistosoma mansoni*-infected mice, four groups of 5-7 months old
male Charles River CD1 mice (Wilmington, USA) were used. Group Sm, consisted of 25 mice infected by percutaneous route at the 30th day of life with 50 ± 5 (mean ± SD) cercariae of Schistosoma mansoni per mouse; macrophages were collected 4 to 6 months later. Group St, with 17 mice intravenously inoculated with 1.5x10^5 colony forming units of Salmonella typhimurium per mouse one week before macrophages were tested. Group Sm + St, with 8 mice infected with Schistosoma mansoni, as in group Sm, and inoculated 4 to 6 months later with Salmonella typhimurium, as in group St; macrophages were obtained one week after this second infection. Group C, with 33 non-infected mice used as normal controls. Schistosoma mansoni infection was confirmed in all animals from groups Sm and Sm + St by the detection of eggs in liver specimens squashed between microscopic slides.

Mouse macrophages obtained by washing the peritoneal cavity with 5ml of cold phosphate-buffered saline (PBS), pH 7.2, were washed twice with cold PBS (400x g for 5min), individually quantified in a hemocytometer, suspended in complete RPMI 1640 medium (Gibco, Grand Island, USA), pH 7.2, and 2x10^5 viable cells (viability always higher than 95%) allowed to adhere onto 13mm circle cover glasses in 24-well plastic plates (Linbro/Flow, McLean, USA) for two hours. After rinsing the cover glasses with PBS, adherent cells (> 99% macrophages) were incubated with a suspension of Salmonella typhimurium (1x10^6 or 4x10^6 bacteria per well, obtained from 14h cultures, quantified by spectrophotometry standardized) in RPMI 1640 with 20% fetal calf serum (Gibco) in the presence or absence of 10% anti-Salmonella typhimurium immune serum, in a wet chamber at 37°C and an atmosphere of 5% CO2 in air. Forty minutes later, cover glasses were washed with RPMI 1640 to get rid of non-phagocytosed bacteria, fixed with absolute methanol, and stained with 20% buffered Giemsa solution. The number of attached and/or ingested Salmonella typhimurium per 200 macrophages in duplicate preparations was microscopically evaluated. Microscopic fields distributed throughout the cover glass were randomly selected, and all macrophages in each field were assessed. Microscopic examination was blindly performed by the same observer (MIM-J).

The phagocytic index was calculated by the average number of bacteria ingested by macrophages multiplied by the percentage of these cells engaged in phagocytosis.

Immune serum was obtained by subcutaneous inoculation of New Zealand white rabbits with 4mg dry-weight formol-killed Salmonella typhimurium in complete Freund adjuvant. A booster dose of 1 mg was given 15 days after and the blood obtained 8 days later. The immune serum was inactivated at 56°C for 30 min before used, and its potency evaluated by an agglutination test. The concentration used was selected on the basis of laboratory trials of phagocytosis using successive dilutions of the serum. Salmonella typhimurium was obtained from 14h culture in Brain Heart Infusion (Difco, Detroit, USA), killed by 0.5% formaldehyde, washed three times with NaCl 0.9% (400 x g) and dried for 6 days in a vacuum dry chamber at 37°C.

Statistical analysis of the data were performed by the Mann-Whitney test for comparison of two unrelated non-normally distributed samples, and the Student t test when two samples showed a normal distribution, by using the SigmaStat Jandel's software package, at a significancy level of 5% (p < 0.05).

RESULTS

Macrophages from Schistosoma mansoni-infected mice, both without or with concurrent Salmonella typhimurium infection, were unable to increase the phagocytosis when exposed to a four-fold increment in the number of Salmonella typhimurium. The median of phagocytic index from group Sm was 442 to the low bacteria load and 431 to the high bacteria load (p > 0.05) and for group Sm + St, 421 and 615, respectively (p > 0.05) (Figure 1c). Neither the number of ingested bacteria (p > 0.05) (Figure 1a), nor the proportion of macrophages engaged in phagocytosis showed modifications (p > 0.05) (Figure 1b). Differently, macrophages from mice infected exclusively with Salmonella typhimurium presented a high median of phagocytic index, which was subsequently increased by the exposition of these cells to an augmented load of bacteria. The median of phagocytic index was 680 to the low bacteria load and 932 to the high bacteria load (p = 0.014) (Figure 1c). This increase in the phagocytic index was due both to an
enhancement in the number of phagocytosed bacteria per macrophage (Figure 1a) and to an increase in the proportion of macrophages engaged in phagocytosis (p = 0.033) (Figure 1b). The same increase occurred in normal control mice (Figure 1a, b, c).

Addition of anti-\textit{Salmonella typhimurium} immune serum to the system caused a significant increase in phagocytosis of macrophages from \textit{Schistosoma}-infected mice subjected to a high load of bacteria (Figure 2c). This effect was due exclusively to an increase in the median of the number of phagocytosed bacteria (7.8 to 12.5; p = 0.001) (Figure 2a), no modification being detected in the proportion of macrophages involved in phagocytosis (p > 0.05) (Figure 2b). In the presence of a low load of bacteria, immune serum caused an increment in phagocytosis exclusively in macrophages from \textit{S. typhimurium}-infected mice (p = 0.002) (Figure 3c). This occurred mainly due to an increase in the number of phagocytosed bacteria (8.5 to 12.6; p = 0.006) (Figure 3a), no modification being detected in the proportion of macrophages engaged in phagocytosis (Figure 3b).

**Figure 1** - Influence of the bacterial load on the phagocytosis of \textit{Salmonella typhimurium} by macrophages from mice infected with \textit{Schistosoma mansoni} (Sm), \textit{Salmonella typhimurium} (St), both (Sm + St) and normal controls (C), submitted to $1 \times 10^6$ (low load) or $4 \times 10^6$ (high load) \textit{Salmonella typhimurium} per well (open bars) or $4 \times 10^6$ (high load) \textit{Salmonella typhimurium} per well (shaded bar). Values are expressed as median, quartiles and extremes. A) Number of bacteria ingested by phagocytosing macrophage; B) Proportion of macrophages engaged in phagocytosis (M); C) Phagocytic Index (PI).

\* statistically different. Group C: p < 0.005 (Mann-Whitney test); Group St: p = 0.01, PI (low load x high load) (Student t test); p = 0.03, M (low load x high load) (Mann-Whitney test).

**Figure 2** - Influence of antibodies against \textit{Salmonella typhimurium} on the phagocytosis of this bacteria by peritoneal macrophages from mice infected with \textit{Schistosoma mansoni} (Sm), \textit{Salmonella typhimurium} (St), both (Sm + St) and normal controls (C). Macrophages were submitted to $4 \times 10^6$ bacteria per well (high load); open bar - without immune serum; shaded bar - with immune serum. Values are expressed as median, quartiles and extremes. A) Number of bacteria ingested by phagocytosing macrophage; B) Proportion of macrophages engaged in phagocytosis; C) Phagocytic Index.

\* statistically different. Group Sm: p < 0.001 (Student t test)
DISCUSSION

It has been recognized that macrophages play an important role in the recovery from infection by Salmonellae\textsuperscript{11}. This function is enhanced by T-lymphocytes in association with the development of immunity\textsuperscript{9,11,28}. Macrophages from mice chronically infected by Schistosoma mansoni showed a deficient ability to ingest and kill Salmonella typhimurium, and this can explain the prolonged survival of this bacteria in chronic salmonellosis associated with schistosomiasis\textsuperscript{15}.

The present data showed that macrophages from normal mice and from those infected with Salmonella typhimurium presented a wide functional reserve in such way that they have their phagocytic capacity enhanced upon an increase in the exposition to bacteria. Infection by Schistosoma mansoni causes an evident narrowing of this reserve, since no significant alteration of phagocytosis was detected after a four-fold increase of the bacteria load. This, is in keeping with the findings of Rocha et al\textsuperscript{23} who found an increased susceptibility of Schistosoma mansoni-infected mice to a high Salmonella load, while Collins et al\textsuperscript{5} showed normal susceptibility to a low load of Salmonella.

We found that the incapacity of macrophages from Schistosoma mansoni-infected mice to tackle with an increased demand was associated both to their inability to ingest more bacteria per cell and to increase the number of macrophages engaged in phagocytosis. It is possible that this deficiency was caused by an overall immaturity of macrophages due to the increased input of newly formed cells to the peritoneal cavity in schistosomiasis\textsuperscript{15,29}. Alternatively, soluble antigens released from the worm\textsuperscript{6,21} or circulating immunocomplexes\textsuperscript{27} could occupy or internalize macrophage receptors involved in phagocytosis\textsuperscript{2}. Accordingly, it has been demonstrated the presence of immunoglobulins on the surface of peritoneal macrophages collected from animals with chronic schistosomiasis\textsuperscript{3,4} while macrophages isolated from granulomas from infected mice showed a reduced phagocytic activity via Fc receptors\textsuperscript{33}.

We showed that anti-Salmonella typhimurium antibodies enhanced the phagocytosis of this bacteria by macrophages from Schistosoma mansoni-infected mice, mainly due to an increase in the number of phagocytosed bacteria per macrophage. This finding indicates that these macrophages were expressing more Fc receptors on their surface, suggesting that they were elicited\textsuperscript{30} or that they displayed a certain degree of activation\textsuperscript{13,34}. However, even in the presence of antibodies to the bacteria, the phagocytic capacity of macrophages from Schistosoma mansoni-infected mice was smaller than that of non-infected animals.

We demonstrated the occurrence of a reduced phagocytic reserve of macrophages during chronic schistosomiasis, which can be partially compensated by the action of opsonizing antibodies. However, we also have previously shown in human schistosomiasis an impaired capacity to produce specific antibodies.
to Salmonella\textsuperscript{16}. Therefore, the association in schistosomiasis of a reduced functional reserve of macrophages with the impaired production of specific antibodies\textsuperscript{16,20,24} and the deficient T-lymphocyte function\textsuperscript{17} appear to be insufficient to overcome the Salmonella infection as seems to be the case of chronic salmonellosis associated with schistosomiasis.

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