

FACTORS INFLUENCING PHAGOCYTOSIS OF *SALMONELLA TYPHIMURIUM* BY MACROPHAGES IN MURINE SCHISTOSOMIASIS

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We investigated the influence of Salmonella typhimurium load and specific antibodies on phagocytosis in schistosomiasis. Macrophages from Schistosoma mansoni-infected mice showed depressed capacity to increase the phagocytosis in the presence of a high bacterial load, due to a reduced involvement of these cells in phagocytosis and to a deficient ability to increase the number of phagocytosed bacteria. Normal and Salmonella-infected mice increased their phagocytic capacity when exposed to a high bacterial load. Antibody to Salmonella increased the phagocytic capacity of macrophages from Schistosoma-infected mice due to an increase in the number of bacteria phagocytosed but caused no modification in the number of macrophages engaged in phagocytosis. Our data indicate that macrophages from Schistosoma-infected mice work close to their functional limit, since no increase in phagocytosis was observed after increasing the bacterial load. Specific antibodies can improve their phagocytic capacity and, therefore, could help clearing concurrent infection.

Key-words: Schistosomiasis. Salmonella typhimurium. Macrophages. Phagocytosis. Chronic salmonellosis associated with schistosomiasis.

Some patients concurrently infected with *Schistosoma mansoni* and *Salmonella* develop a peculiar clinical picture recognized as chronic septicemic salmonellosis^{24,31}. 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 bacteria¹⁵. 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 bacteria¹², via the receptors for the Fc portion of IgG⁸. 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^{7,18,26,30}. 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

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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 \pm SD) cercariae of *Schistosoma mansoni* per mouse; macrophages were collected 4 to 6 months later. *Group St*, with 17 mice intravenously inoculated with 1.5×10^5 colony forming units of *Salmonella typhimurium* per mouse one week before macrophages were tested¹⁵. *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 slides²².

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 2×10^5 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^{14, 32}. After rinsing the cover glasses with PBS, adherent cells (> 99% macrophages) were incubated with a suspension of *Salmonella typhimurium* (1×10^6 or 4×10^6 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 phagocytosis²⁵.

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°C¹⁰.

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 significance 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-*Salmonella typhimurium* immune serum to the system caused a significant increase in phagocytosis of macrophages from *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 *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).

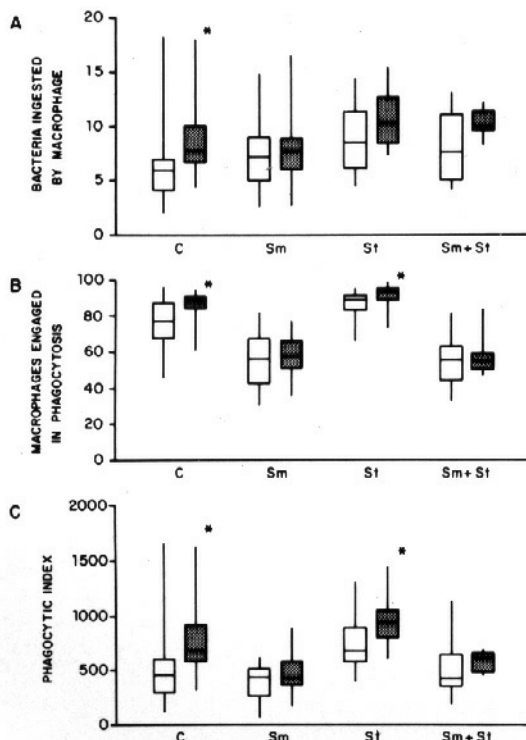


Figure 1 - Influence of the bacterial load on the phagocytosis of *Salmonella typhimurium* by macrophages from mice infected with *Schistosoma mansoni* (Sm), *Salmonella typhimurium* (St), both (Sm + St) and normal controls (C), submitted to 1×10^6 (low load) *Salmonella typhimurium* per well (open bar) or 4×10^6 (high load) *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).

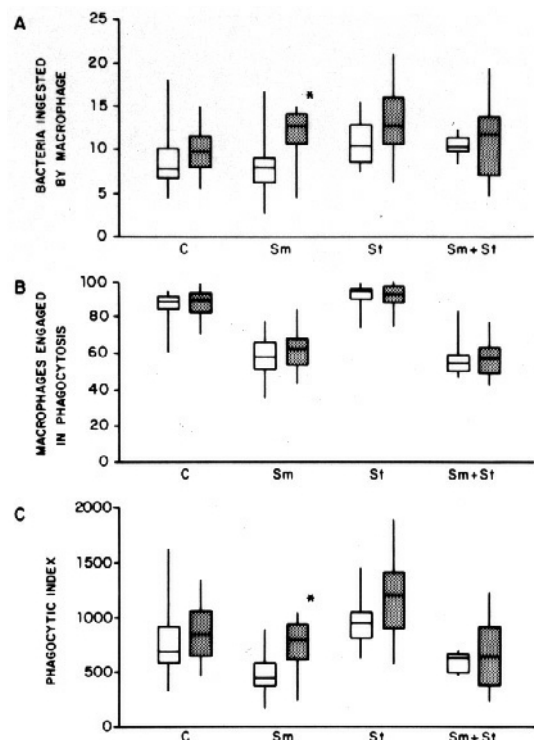


Figure 2 - Influence of antibodies against *Salmonella typhimurium* on the phagocytosis of this bacteria by peritoneal macrophages from mice infected with *Schistosoma mansoni* (Sm), *Salmonella typhimurium* (St), both (Sm + St) and normal controls (C). Macrophages were submitted to 4×10^6 bacteria per 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)

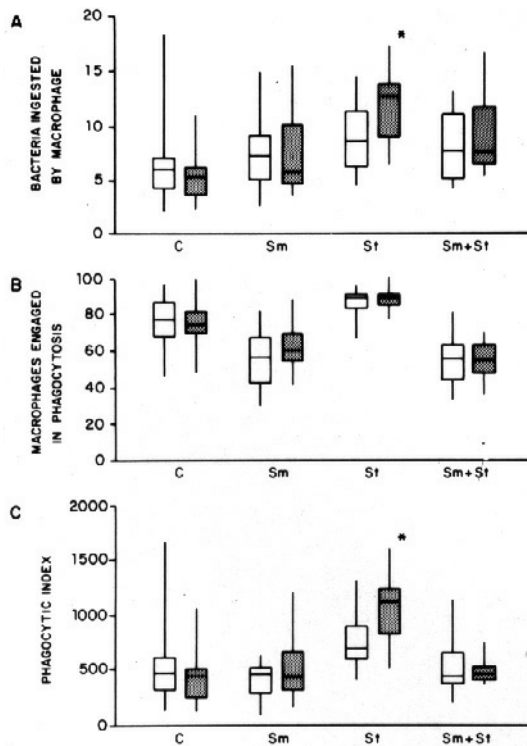


Figure 3 - Influence of antibodies against *Salmonella typhimurium* on the phagocytosis of this bacteria by peritoneal macrophages from mice infected with *Schistosoma mansoni* (Sm), *Salmonella typhimurium* (St), both (Sm + St) and normal controls (C). Macrophages were submitted to 1×10^6 bacteria per well (low load); open bar - without immune serum; shaded bar - with immune serum. Values are shown as median, quartiles and extremes. A) Number of bacteria ingested by phagocytosing macrophage; B) Proportion of macrophage engaged in phagocytosis; C) Phagocytic Index;

* statistically different. Group St: $p < 0,006$ (Student t test)

DISCUSSION

It has been recognized that macrophages play an important role in the recovery from infection by *Salmonellae*¹¹. This function is enhanced by T-lymphocytes in association with the development of immunity^{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 schistosomiasis¹⁵.

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 al²³ who found an increased susceptibility of *Schistosoma mansoni*-infected mice to a high *Salmonella* load, while Collins et al⁵ 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^{15 29}. Alternatively, soluble antigens released from the worm^{6 21} or circulating immunocomplexes^{19 27} could occupy or internalize macrophage receptors involved in phagocytosis². Accordingly, it has been demonstrated the presence of immunoglobulins on the surface of peritoneal macrophages collected from animals with chronic schistosomiasis^{3 4} while macrophages isolated from granulomas from infected mice showed a reduced phagocytic activity via Fc receptors³³.

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 elicited³⁰ or that they displayed a certain degree of activation^{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*¹⁶. Therefore, the association in schistosomiasis of a reduced functional reserve of macrophages with the impaired production of specific antibodies^{16 20 24} and the deficient T-lymphocyte function¹⁷ appear to be insufficient to overcome the *Salmonella* infection as seems to be the case of chronic salmonellosis associated with schistosomiasis.

RESUMO

*Investigamos a influência da carga bacteriana e de anticorpos específicos sobre a fagocitose na esquistossomose. Macrófagos de camundongos esquistossomóticos mostraram uma menor capacidade para aumentar a fagocitose mesmo na presença de alta carga bacteriana, devido a um reduzido envolvimento destas células na fagocitose e a uma incapacidade para aumentar o número de bactérias fagocitadas por macrófago. Camundongos normais e infectados por *Salmonella typhimurium* aumentaram sua capacidade fagocitária quando expostos a uma alta carga bacteriana. Anticorpos para *Salmonella typhimurium* significativamente aumentaram a capacidade fagocitária de macrófagos de camundongos esquistossomóticos devido ao aumento do número de bactérias ingeridas mas não causaram nenhuma modificação no número de macrófagos envolvidos na fagocitose. Nossos dados indicam que macrófagos de camundongos esquistossomóticos trabalham próximo ao seu limite funcional, desde que nenhum aumento significativo na fagocitose foi observado após aumentar a carga bacteriana. Anticorpos específicos podem melhorar sua capacidade fagocitária e, portanto, poderiam auxiliar na eliminação da infecção concomitante por *Salmonella*.*

Palavras-chaves: Esquistossomose. Salmonelose septicêmica prolongada. Salmonella typhimurium. Macrófagos. Fagocitose.

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