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EFFECTS OF IMMUNE AND HYPERIMMUNE SERUM ON THE DYNAMICS OF PHAGOCYTOSIS OF PLASMODIUM BERGHEI-INFECTED ERYTHROCYTES

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SUMMARY

Macrophages obtained by implanting epoxy discs into the peritoneal cavity of normal rats were used for assessing the phagocytosis **in vitro** of erythrocytes from **Plasmodium berghei**-infected rats, in the presence of immune (IS) and hyperimmune (HS) serum. Phagocytosis of parasitized erythrocytes was significantly higher both in HS and in IS, as compared with normal serum (NS). However, HS caused a more dynamic phagocytosis than IS, parasitized erythrocytes being rapidly interiorized after attachment to macrophages. The opsonic effect of HS appears to be due, at least in part, to the presence of macrophage-cytophilic antibodies since macrophages incubated with HS followed by extensive washings exhibited significantly increased phagocytosis over that found with NS. These results indicate that during the process of reinfection with **P. berghei** there is an enhancement of the opsonic effect of the serum towards parasitized erythrocytes due, presumably, to macrophage-cytophilic antibodies.

INTRODUCTION

Plasmodium berghei infection in adult rats leads to an acute attack of malaria followed by spontaneous recovery and resistance to reinfection by the homologous strain. This system therefore provides a good model to study the factors involved in the acquisition of immunity. The bulk of available evidence indicates that antibodies play an important role in antimalarial resistance. It has been shown that serum from rats which have recovered from **Plasmodium berghei** infection is capable of transferring immunity, and that a greater degree of protection is associated with serum from animals which have been repeatedly infected (BRIGGS et al.[2], STECHSCHULTE et al.[12]). However, the mechanisms of action of protective antibodies have not yet been conclusively established, although it has been recognized that antibodies can enhance **in vitro** phagocytosis of free **P. berghei** (CHOW & KREIER[4]) and parasitized erythrocytes (TOSTA[14], HUNTER et al.[7]). The present investigation was undertaken to establish whether the ability of immune serum to enhance phagocytosis of **P. berghei** — infected erythrocytes increases after repeated infections and whether the dynamics of phagocytosis are different in 'hyperimmune' and 'immune' serum.

MATERIALS AND METHODS

Animals — 200-250 g R/A female rats bred in our Animals Unit were used as donors of serum and peritoneal macrophages. Young rats (7-10 days old) were used to obtain blood with a high parasitaemia following infection with **P. berghei.**

Parasites — Plasmodium berghei was obtained from the Institute Pasteur (Paris) and maintained by blood passage in SW mice. It is lethal for mice and young rats. Adult rats

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recover spontaneously after which they are resistant to reinfection by the homologous strain.

Parasitized erythrocytes — Young rats were inoculated intraperitoneally (ip) with 10^6 **P. berghei**-infected homologous erythrocytes and were bled one week later. Blood was collected by cardiac puncture in Hanks' balanced salt solution (HBSS) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10 U/ml of heparin. Suspensions containing about 50% of infected cells were obtained by low speed centrifugation (100 xg, 10 min), washed twice in cold HBSS and made up to contain 300 x 10^6 erythrocytes/ml.

Sera — Immune serum was collected from adult rats 2 weeks after ip infection with 10^6 **P. berghei**-infected erythrocytes, after parasites had been cleared from the blood. Hyperimmune serum was obtained from rats submitted to an acute infection with 10^6 infected erythrocytes, followed by 3 challenges with 5 x 10^5 parasitized red cells at 30 day intervals. Blood was collected by cardiac puncture 15 days after the last reinfection. Normal serum was collected from age-matched rats. All sera were heat-inactivated (56°C, 30 min) and 0.5 and 1 ml samples stored at -20°C. In some experiments, hyperimmune serum was absorbed twice with either normal or **P. berghei**-infected erythrocytes (60 min at 37°C followed by 60 min at 4°C). Antiplasmodial antibodies were detected by the indirect immunofluorescence technique (VOLLER [46]) using fluorescein-labelled rabbit anti-rat immunoglobulins. Negative results were observed when **Leishmania braziliensis** strain 49 was used as antigen instead of **P. berghei**.

Collection of macrophages — Macrophages were obtained by implanting sterile epoxy resin discs (5 mm diameter, thickness 0.3 mm) into the peritoneal cavity of normal adult rats. Animals were killed after 24 hours by air embolism, the discs removed and washed in HBSS. The average number of cells per disc was 9,400 (range 6,300 to 13,500): 85% were macrophages, 12% lymphocytes, 1% eosinophils, 0.5% mast cells and occasional neutrophils and mesothelial cells. Macrophages were characterized by the presence of acid phosphatase using the technique of Gomori modified by BARKA & ANDERSON [1] and also by their ability to phagocytose polystyrene beads of 0.81 μm diameter (BD-Merieux, France); 97% of the macrophages were found to phagocytose these particles.

Phagocytic assay — Discs with adhering macrophages were incubated at 37°C for 60 min a moist chamber with 0.2 ml of a suspension of parasitized erythrocytes (300 x 10^6/ml, 50% parasitaemia) in HBSS containing 30% v/v of the serum under study. After incubation, discs were vigorously rinsed twice with HBSS at 37°C and processed. For optical microscopy discs were fixed in absolute methanol, stained with 20% Giemsa solution, mounted on slides, and erythrocytes associated with macrophages were classified as attached, ingested or digested, as proposed by CARR [3]. They were scored as ingested when surrounded by a vacuole (phagosome), but still showing structural integrity, as digested when found inside phagosomes but showing signs of destruction, and as attached when associated with macrophages but not included in the above categories. In addition, cells scored as attached or ingested were further differentiated as parasitized or non-parasitized erythrocytes (PE and NPE). The microscopical assessment was always performed by the same observer, on unidentified duplicate preparations.

Some preparations were also studied by electronmicroscopy after fixation in 3% glutaraldehyde in 0.1M sodium cocadylate buffer pH 7.2, final osmolarity 280 mOsm, post-fixation in osmium tetroxide, embedding in Epon and staining with uranyl acetate and lead citrate.

The possible occurrence of macrophage-cytophilic antibodies in hyperimmune serum was tested by incubating discs containing phagocytic cells with 30% serum, either at 37°C or at 4°C, for 60 min. Discs were rinsed with HBSS at 37°C and reincubated with a suspension of PE in HBSS containing 30% bovine calf serum for 60 min.

The presence of opsonic antibodies directed towards PE in hyperimmune serum was tested by incubating the suspension of PE with a 30% dilution of serum at 37°C for 2 h. Erythrocytes were washed 3x with cold HBSS, suspended in 30% bovine calf serum, and reincubated with macrophages for 60 min.

Statistical analysis — The data were analysed by the Student's t test.

RESULTS

Characterization of the phases of phagocytosis

A series of experiments was carried out in order to determine the criteria for characterization of the different phases of phagocytosis. It was found that attached erythrocytes appeared forming rosettes to the macrophage and frequently blurring its outline (Fig. 1A). To assess the reliability of the method of microscopical characterization of attached cells, preparations were submitted to hypotonic shock (45 sec in Hanks' solution diluted 1:5 with distilled water) and stained with Giemsa solution. The number of erythrocytes associated with macrophages were counted and compared with unlysed preparations. Attached erythrocytes are lysed by this treatment, whereas red blood cells interiorized by macrophages are not affected (OLIVEIRA LIMA et al.[9]). The difference between the numbers of erythrocytes associated to 100 macrophages in lysed and unlysed preparations corresponded, approximately, to the number of cells characterized as attached in the latter preparations, which seems to validate the method of assessment. As shown by electronmicroscopy, attachment of

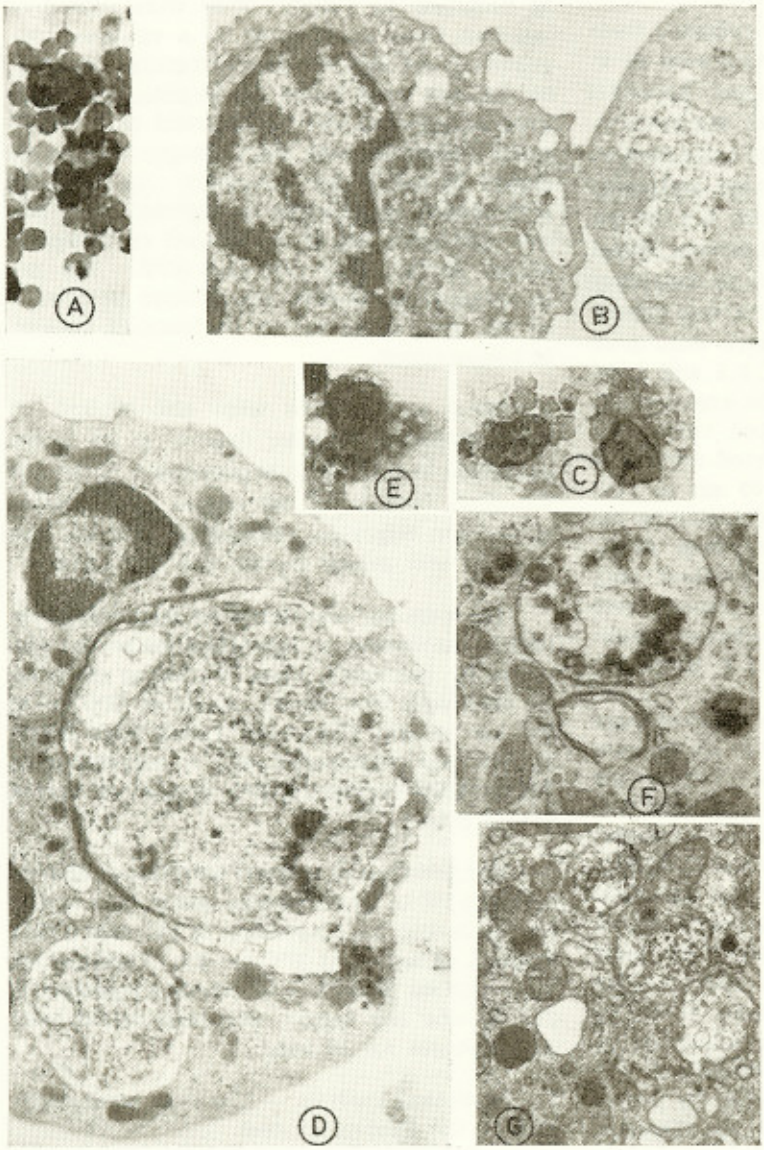

Fig. 1 — The different phases of phagocytosis of **P. berghei**-infected erythrocytes from by macrophages. (A) Parasitized and non-parasitized erythrocytes, mainly attached to macrophages, after incubation in the presence of immune serum. Giemsa-stained preparation, x1,250. (B) The attachment process involves a close association between the plasma membranes of the erythrocyte and macrophage. The deformity of the parasitized erythrocyte suggests that it is pulled towards the macrophage. Electronmicrograph, x10,750. (C) Ingested erythrocytes lose their normal refringence (compare with the cells attached to the macrophage in the left), and are circumscribed to the limits of the macrophage. Incubation with hyperimmune serum, x1,250. (D) Two parasites ingested by a macrophage are localized into phagosomes and show some degree of destruction of their plasmalemmas. Electronmicrograph, x10,750. (E) During the phase of digestion macrophages exhibit large phagosomes containing debris of parasites. Hyperrimmune serum, x1,250. (F) Phagosomes presenting semi-digested parasites and malarial pigment. Electronmicroscopy, x22,500. (G) As digestion proceeds, parasitic material, including pigment, can be found in association with residual bodies. Electronmicrograph, x22,500

erythrocytes to macrophages involved a close association of plasma cell membranes (Fig. 1B).

Ingested erythrocytes were distinguished from attached cells by the different refringence of their outline (Fig. 1C). In addition, they were always circumscribed to the limits of the macrophage, almost invariably surrounded by a clear halo, and exhibiting structural integrity (Fig. 1D). The loss of integrity characterized the last phase of phagocytosis, namely the digestion (Fig. 1E). At this stage, parasitized and non-parasitized erythrocytes showed a variable degree of destruction, and phagosomes frequently contained malarial pigment (Fig. 1F). Incubation times longer than 60 min was avoided in order to minimize the effects of digestion, which makes cells difficult to be characterized (Fig. 1G).

Effect of serum on the dynamics of phagocytosis of parasitized erythrocytes

Phagocytosis of erythrocytes from **P. berghei**-infected rats was measured in the presence of normal, immune and hyperimmune serum and the total number of erythrocytes attached, ingested and digested was assessed. In comparison to normal serum, both immune and hyperimmune serum caused a significant enhancement of phagocytosis (Table I), the latter being more effective than the former. Figure 2 shows the effect of the same serum pools on different phases of phagocytosis. Hyperimmune serum caused the greatest increase in the number of cells which were in the process of being digested by macrophages at the end of the incubation period, and also in the number of erythrocytes ingested by macrophages. However, once interiorized the speed of the phagocytic process ending with the digestion and destruction of ingested cells appears not to depend on the type of serum used. 71% of the cells engulfed in the presence of hyperimmune, and 65% in immune serum, were found in the phase of digestion. The number of erythrocytes found still attached to macrophages, rather than interiorized, was on the other hand, significantly greater when immune serum was used.

TABLE I

Number of erythrocytes from **P. berghei**-infected rats phagocytosed by macrophages in the presence of normal (NS), immune (IS) and hyperimmune serum (HS)

serum (a)	erythrocytes phagocytosed/ 100 macrophages mean ± s.e. (b)	number of experiments	titre of anti-plasmodial antibody (e)
HS	420 ± 26 (c)	6	640
IS	230 ± 7 (d)	3	320
NS	97 ± 21	3	20

(a) erythrocytes (50% parasitaemia) suspended in HBSS containing 30% serum and incubated with macrophages for 60 min
(b) includes parasitized and non-parasitized erythrocytes attached, ingested and being digested by macrophages
(c) $P < 0.01$ as compared with NS and $P < 0.02$ with HS
(d) $P < 0.01$ as compared with NS
(e) indirect immunofluorescence

Specificity of the opsonizing effect — Immune and hyperimmune serum increased the phagocytosis of both parasitized (PE) and non-parasitized erythrocytes (NPE). Using a 50% suspension of PE, 83% of the cells ingested after incubation with immune serum, and 87% with hyperimmune serum, were parasitized erythrocytes (Table II). Furthermore, the total number of PE ingested was significantly greater ($p < 0.05$) in the presence of hyperimmune serum than with immune serum. In the presence of the latter serum a considerable number of NPE were attached, but not ingested. Absorption of hyperimmune serum with normal rat erythrocytes caused a decrease, although not statistically significant, of its opsonizing effect towards parasitized erythrocytes.

TABLE II

Discrimination by macrophages between **P. berghei**-parasitized (PE) and non-parasitized erythrocytes (NPE) in the presence of normal (NS), immune (IS) and hyperimmune serum (HS)

serum (a)	cells attached/100 macrophages (mean ± s.e.)				cells ingested/100 macrophages (mean ± s.e.)			
	PE	NPE	total	%PE	PE	NPE	total	%PE
HS	20±5	3±1	23±5	87	99±12	15±3	114±10	87
IS	30±9	25±8	55±13	55	52±11	11±3	63±8	83
NS	5±2	1	6±2	83	25±8	1	26±7	96

(a) see footnote (a) Table I

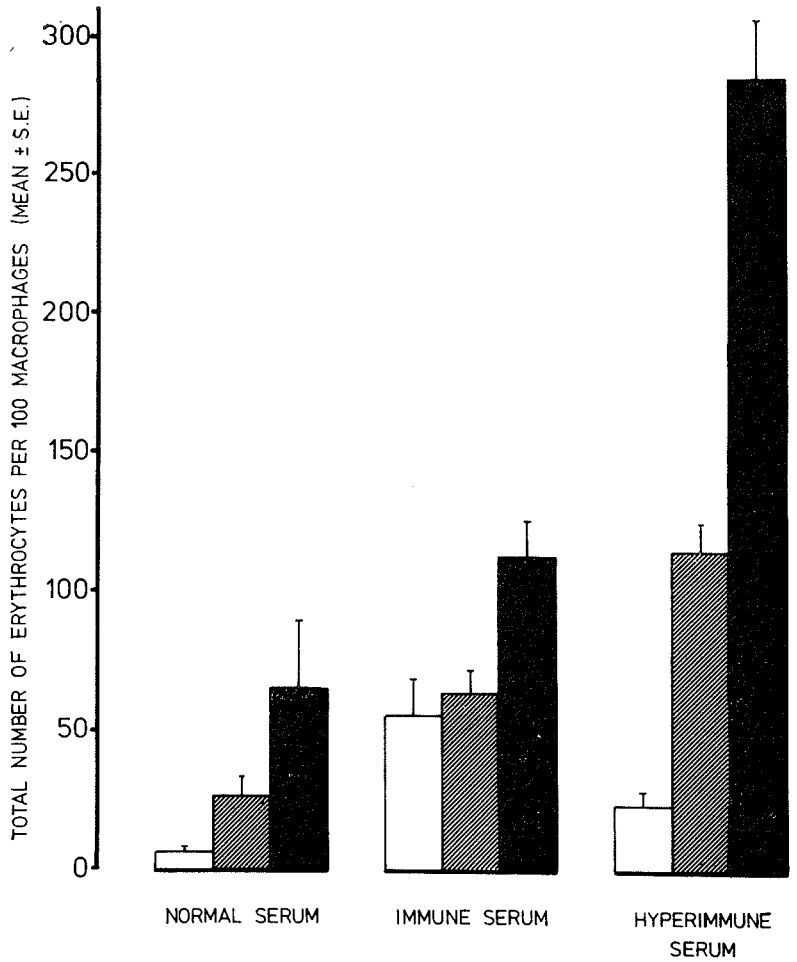

Fig. 2 — Total number of **P. berghei**-parasitized, and non-parasitized erythrocytes attached (▭), ingested (▨), and being digested (■) by 100 peritoneal macrophages after 60 min incubation in the presence of either normal, immune or hyperimmune serum

Nature of the opsonizing effect of hyperimmune serum

A series of experiments was designed to investigate the nature of the opsonizing effect of hyperimmune serum. The phagocytic assays described above were carried out by incubating macrophages with erythrocytes from infected rats suspended in the serum under study. The opsonizing effect observed could have been due to the presence of either an opsonic antibody acting on parasitized erythrocytes and/or to a macrophage-cytophilic antibody with an opsonizing effect. These possibilities were tested by incubating either macrophages or PE with hyperimmune serum, followed by extensive washing, before the phagocytic assay was performed. Macrophages preincubated with hyperimmune serum showed an increased ability to phagocytose PE, as compared with that observed when cells were incubated with normal serum (Table III). Apart from a significant opsonizing effect (a 2.4-fold increase in phagocytic ability), phagocytosis by macrophages preincubated with hyperimmune serum was highly specific since 89% of the cells attached to and ingested by macrophages were parasitized erythrocytes.

The temperature of incubation of macrophages with hyperimmune serum appeared to be critical for phagocytosis. At 4°C there was a 1.2-fold increase in phagocytosis with hyperimmune as compared with normal serum under the same conditions, but this was not statistically significant. Incubation at 37°C led to a significant increase in phagocytosis as shown in Table III.

TABLE III

Effect of preincubation of macrophages with normal (NS) and hyperimmune serum (HS) on their phagocytic ability towards parasitized (PE) and non-parasitized erythrocytes (NPE)

serum (a)	PE attached + ingested/10 macrophages (mean ± s.e.)	NPE attached + ingested/100 macrophages (mean ± s.e.)	% PE attached + ingested	total erythrocytes phagocytosed/100 macrophages (mean ± s.e.) (b)	number of experiments
HS	106 ± 3 (c)	13 ± 2	89	290 ± 6 (d)	3
NS	45 ± 19	5 ± 1	90	176 ± 22	4

(a) macrophages incubated with HBSS containing 30% serum at 37°C for 60 min, washed and re-incubated with erythrocytes (50% PE) in bovine calf serum for 60 min

(b) see footnote (b), Table I

(c) $P < 0.05$ compared with NS

(d) $P < 0.01$ compared with NS

The possible contribution of opsonic antibodies directed towards parasitized erythrocytes, to the effect of hyperimmune serum, was tested by incubating PE with serum, followed by through washing and then reincubation with macrophages, in the presence of bovine calf serum. This treatment caused a 1.3-fold increase in phagocytosis of PE as compared with erythrocytes treated with normal serum, but this enhancement was not statistically significant (data not shown).

DISCUSSION

These results confirmed previous observations that serum from **P. berghei**-recovered rats exhibited a considerable ability to cause enhancement of the phagocytosis **in vitro** of parasitized erythrocytes (TOSTA [14]). In addition, it was demonstrated that immunity developed after repeated reinfections is accompanied by an increase in the opsonic effect of serum towards parasitized erythrocytes, acting, at least in part, as macrophage-cytophilic antibodies.

The titre of antiplasmodial antibody, as assessed by immunofluorescence, was 640 in the hyperimmune serum, and 320 in the immune serum. It is therefore possible that their different effects on phagocytosis was mainly due to qualitative, rather than quantitative, differences. Indeed, STECHSCHULTE et al. [12] have shown that serum collected from rats recovered from **P. berghei** infection contained mainly IgM, and exhibited less protective activity than serum from reinfected animals containing predominantly IgG and IgA. Our attempts to characterize the relative contribution of IgM to phagocytosis were unsuccessful, since sera after treatment with 2-mercapto-ethanol (Sigma) followed by extensive dialysis against iodoacetamide (Aldrich) and again against 0.15M NaCl solution, caused degenerative alterations in macrophages.

The greater number of erythrocytes found attached to macrophages in the presence of immune rather than hyperimmune serum, strongly suggests that IgM predominates in the former. Although the question whether IgM can act as an opsonin in the absence of complement factors has not been fully elucidated (GRIFFIN [5]), there are some studies indicating that this immunoglobulin can mediate binding of erythrocytes to macrophages (LAY & NUSSENZWEIG [8]), but not their ingestion (RABINOVITCH [11]). Thus, the considerable number of erythrocytes found in the phase of attachment after incubation with immune serum, rather than indicating that this serum caused a slower rate of engulfment in comparison with hyperimmune serum, was probably due to the presence of cells bound to macrophages by IgM molecules in the absence of complement.

The present data support the concept that antigens from the parasite can be expressed in the membrane of parasitized erythrocytes providing the sites for combination with opsonizing antibodies. The finding that erythrocytes infected by late trophozoites and schizonts of **P. berghei** (HUNTER et al. [7]) and **P. yoelii** (TOSTA & WEDDERBURN [15]) are more susceptible to the opsonizing effect of immune serum than cells infected by less mature forms suggests that an alteration of the erythrocyte cell membrane due to the growth of the parasite may contribute to the action of opsonizing antibodies. Some authors, however, have been unable to demonstrate an opsonic effect of immune serum towards parasitized erythrocytes, claim-

ing that only free parasites are capable of being opsonized (see HAMBURGER & KREIER [6]). The ability of opsonizing antibodies to act not only upon free parasites but also on intracellular organisms would widen considerably their possible role in resistance. Enhancement of phagocytosis could be associated with the presence of either opsonic or macrophage-cytophilic antibodies. PARISH [10] has shown that cytophilic and opsonic antibodies can exhibit different electrophoretic mobilities, and different susceptibility to treatment with 2-mercaptoethanol. Furthermore, cytophilic antibodies could be absorbed by macrophages in the absence of antigen, while opsonic antibodies could not, unless they were combined with antigen. In our system a slight but not significant increase in phagocytosis of parasitized erythrocytes previously incubated with hyperimmune serum was observed. However, we cannot rule out the possibility that opsonic antibodies were eluted from parasitized erythrocytes during washing particularly since, as STEWARD & VOLLER [13] have shown, malaria infection causes a decrease in antibody affinity. Our findings indicate that the major part of the opsonic effect of serum from rats submitted to repeated infections is due to macrophage-cytophilic antibodies. The fact that these antibodies are capable of sensitising macrophages at 37°C supports the possibility that they may also have an **in vivo** effect.

RESUMO

Efeito de soro imune e hiperimune sobre a dinâmica da fagocitose de eritrócitos parasitados por Plasmodium berghei

Macrófagos obtidos através de implante de discos de epoxi na cavidade peritoneal de ratos normais foram utilizados em testes de fagocitose **in vitro** de eritrócitos parasitados por **P. berghei**, na presença de soro immune (SI) e hiperimune (SH). Tanto SI quanto SH causaram significante aumento da fagocitose de eritrócitos parasitados, em comparação ao soro normal (SN). Entretanto, SH causou maior incremento da dinâmica da fagocitose que SI e em seguida à aderência aos macrófagos os eritrócitos parasitados eram rapidamente interiorizados. O efeito opsonizante do SH foi devido, pelo menos em parte a presença de anticorpos citofílicos para macrófagos já que, quando estas células foram incubados com SH e, em seguida submetidas a exaustiva lavagem, elas apresentaram significante aumento da capacidade fagocitária em comparação a SN. Estes resultados indicam que durante o processo de reinfecção por **P. berghei**, ocorre um incremento da capacidade opsonizante do soro em relação à eritrócitos parasitados, devido, presumivelmente à anticorpos citofílicos para macrófagos.

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