INFECTION AND IMMUNITY, Nov. 1983, p. 733–739
0019-9567/83/110733-07$02.00/0

Vol. 42, No. 2

Malaria-Induced Lymphokines: Stimulation of Macrophages for Enhanced Phagocytosis

CHRISTIAN F. OCKENHOUSE* AND HANNAH L. SHEAR

Division of Parasitology, Department of Microbiology, New York University School of Medicine, New York, New York 10016

Received 23 May 1983/Accepted 25 August 1983

Culture supernatants from antigen-pulsed spleen cells of mice infected previously with either BCG or *Plasmodium chabaudi* were used to study macrophage activation as judged by phagocytosis of immunoglobulin G-sensitized erythrocytes and *Plasmodium berghei*- and *P. chabaudi*-infected erythrocytes. Resident peritoneal macrophages were incubated in vitro with spleen cell factor and then assayed for ingestion of immunoglobulin G-sensitized or parasitized erythrocytes. Macrophages activated with BCG-induced lymphokine bound and ingested two- to threefold more *P. berghei* parasitized erythrocytes than macrophages incubated with control spleen cell factor. Similarly, *Plasmodium*-stimulated spleen cells from mice infected with malaria produced a lymphokine(s) capable of activating macrophages for enhanced Fc receptor-mediated phagocytosis. The stimulation of phagocytosis by the lymphokine is nonspecific in nature, since phagocytosis of parasitized erythrocytes from one species of murine malaria is enhanced by the lymphokine prepared from a heterologous species. Nylon wool-nonadherent, malaria-sensitized spleen cells elaborated a lymphokine which stimulates macrophages for enhanced phagocytosis, whereas anti-θ-treated spleen cells failed to produce the phagocytosis-promoting lymphokine. Consequently, this lymphokine appears to be elaborated by sensitized T lymphocytes. Interestingly, enhanced phagocytosis of opsonized trophozoites and schizonts, but not ring stage parasites of *P. chabaudi*, was displayed by macrophages activated with the lymphokine(s) prepared from *P. chabaudi*-recovered mice. Preincubation of the malaria parasitized erythrocytes with hyperimmune serum raised against the parasites greatly facilitated both binding and ingestion by the stimulated macrophages.

Cell-mediated immunity plays a crucial role in the development and maintenance of protective immunity against malaria. T lymphocytes from malaria-infected mice are highly activated and respond in vitro to malarial antigen (15, 35). In T cell-deprived mice, the infection is often more severe (14), resulting in decreased blood monocyte responses (13), accumulation of fewer macrophages in the spleen (36), and decreased phagocytic function (26). Antibody undoubtedly contributes to the control of acute infections (8); however, functional B lymphocytes are not essential for resistance to secondary malarial infections with *Plasmodium yoelii* (12, 27).

In intact animals, malarial infection is associated with pronounced splenomegaly and hyperplasia of the mononuclear phagocyte system (33). Parasitized erythrocytes (PE) have been observed in the phagocytic vacuoles of splenic macrophages of birds and monkeys (32), and in vitro studies have demonstrated the phagocytosis of free parasites (merozoites) (6, 17) and intraerythrocytic parasites by both rodent macrophages (34) and human monocytes (5). Recent studies have suggested that spleen macrophages are activated during malaria, as detected by enhanced phagocytosis of opsonized PE and immunoglobulin G (IgG)-coated erythrocytes (30).

This study examines the role of T cells in the activation of macrophages during malaria. We found that a spleen cell factor or lymphokine (LK) produced by BCG- or malaria-sensitized T lymphocytes stimulates resident peritoneal macrophages. Such LK-stimulated macrophages non-specifically phagocytize higher levels of both PE and IgG-coated erythrocytes. Immune serum enhances the ingestion of late trophozoite or schizont stages but not the ring stage of *Plasmodium chabaudi*. The importance of T

cell-mediated macrophage activation, in terms of both phagocytosis and cytotoxicity against the malaria parasite, is discussed.

MATERIALS AND METHODS

Animals. Female SW mice (Taconic Farms, Inc., Germantown, N. Y.), 4 to 8 weeks old, were used in all experiments.

Malaria parasites. The NK65 strain of *Plasmodium berghei* was used. It is maintained by passage in hamsters with malaria-infected blood, alternating with mosquito-induced infections. Mice were inoculated intravenously with 10^4 PE.

A lethal strain of *P. chabaudi* parasites was maintained in mice by injection of 10^4 parasites intravenously. Infections in mice are synchronous, with a periodicity of 24 h. Groups of infected mice were maintained on an inverse light schedule from which late trophozoite and schizont stages of the parasite were collected in the afternoon. The ring stage of the parasite was collected in the afternoon from infected mice kept on a normal light pattern. Parasitemias of infected animals were determined by microscopic examination of Giesma-stained smears.

Sera. Hyperimmune serum was obtained from infected mice which were treated with 0.5 ml of chloroquine (400 μg/ml) injected intraperitoneally for 3 consecutive days. The mice were then rechallenged three times with 10^6 PE at weekly intervals. PE were incubated in heat-inactivated hyperimmune sera (IS) (1:10 dilution) for 30 min at 37°C, washed, and resuspended to 2×10^8 PE+IS per ml. Antibody binding to PE was determined by the indirect fluorescent antibody technique by using a wet mount of PE with fluorescein-labeled goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.) by the method of Hommel et al. (11).

Preparation of mouse EIgG. Mouse erythrocytes sensitized with IgG (EIgG) were prepared as described previously (23). Mouse erythrocytes were washed three times, resuspended to 2×10^8/ml, and incubated with an equal volume of an IgG fraction of rabbit anti-mouse erythrocyte antibody (subagglutinating titer) for 30 min at 37°C. The erythrocytes were washed and adjusted to 2×10^8 cells per ml.

Macrophages. Cell preparations were essentially those described previously (9). Mice were sacrificed, and the peritoneal cavity was washed with phosphate-buffered saline (GIBCO, Grand Island, N.Y.). The peritoneal cells were centrifuged once and resuspended to 10^6/ml in Dulbecco modified Eagle medium (DMEM) (GIBCO) containing 10% fetal bovine serum (D10) plus 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin (GIBCO) per ml. Peritoneal cells (100 μl) were pipetted onto 12-mm round glass cover slips, incubated in 7.5% CO_2 at 37°C for 45 min, and rinsed in DMEM. Macrophage monolayers were either assayed immediately for phagocytosis or reincubated for up to 48 h in 16-mm Falcon 24-well plates (Falcon Plastics, Oxnard, Calif.) in D10 plus spleen cell supernatants before the phagocytic assay. In some experiments, macrophages were obtained from mice previously infected intravenously with 2×10^7 viable BCG (Trudeau Institute, Saranac Lake, N.Y.). Peritoneal cells were harvested 3 days after a secondary challenge of 10^7 heat-killed BCG (hKBCG) from mice infected for 3 to 4 weeks.

Antigens. hkBCG was obtained by autoclaving a suspension of 5×10^6 viable BCG per ml at 15 lb/in^2 for 15 min.

Malarial antigen was prepared from PE which were enriched by centrifugation of erythrocytes in DMEM at $750 \times g$. The upper layer was removed and contained >90% PE. A 40-μl sample of enriched PE pellet was lysed with 0.3 ml of 0.2% NaCl solution. Isotonicity was restored with the addition of an equal volume of 1.6% NaCl solution.

Preparation of antigen-stimulated spleen cell supernatant. Unfractionated spleen cells (1.5×10^7/ml) from either individual mice infected for 3 weeks with BCG or individual malaria-infected mice (30 to 60% parasitemia) were incubated with either 5×10^6 hkBCG or 15 μl of lysed PE suspension per ml, respectively, for 48 h at 37°C in 7.5% CO_2. The cells were incubated in D10 plus 5×10^{-5} M mercaptoethanol. Control supernatants were prepared by culturing spleen cells from normal mice for 48 h with either hkBCG or lysed PE suspension. Additionally, control LKs were prepared from malaria-sensitized spleen cells not stimulated with specific antigen or stimulated with antigens prepared from uninfected erythrocytes. LKs prepared in such a manner did not significantly increase ingestion of IgG-coated erythrocytes by LK-treated macrophages. Immune T lymphocytes used to prepare spleen cell supernatants were isolated from whole spleen cells by passage through nylon wool by the method of Julius et al. (16). Spleen cell lymphocytes depleted of T cells were isolated by treating 10^7 cells per ml with anti-mouse T cell serum (Accurate Chemical and Scientific Corp., Westbury, N.Y.) for 60 min at 4°C. Cells were washed and suspended to the original volume in rabbit complement for 60 min at 37°C. Viable cells were washed, resuspended to 10^7/ml, and incubated with the malaria antigens for 48 h. Supernatants were collected and centrifuged at $500 \times g$ for 15 min, sterilized by filtration, and stored at −70°C. Before use, supernatants were thawed and diluted 1:5 in D10.

Phagocytic assay. Cover slips containing macrophage monolayers were rinsed, overlaid with 0.1 ml of either 2×10^8 EIgG per ml or 2×10^8 PE or PE+IS per ml, and incubated for 45 min. The cover slips were rinsed in DMEM to assess attachment or in phosphate-buffered saline diluted 1:5 with distilled water to assess ingestion. After glutaraldehyde fixation, 200 macrophages on triplicate cover slips were counted under phase-contrast optics, and the results were expressed as either the percentage of macrophages ingesting or attaching one or more erythrocytes or the mean number of erythrocytes ingested by or attached to 100 macrophages.

RESULTS

Enhanced phagocytosis of PE by macrophages activated in vivo with BCG. Since injection of BCG into normal mice can afford protection against the *Plasmodium* and *Babesia* protozoan parasites (7), we undertook preliminary experiments to assess the ability of BCG-activated macrophages to phagocytize PE. Peritoneal mac-

TABLE 1. Phagocytosis of *P. berghei*-infected erythrocytes by macrophages activated in vivo with BCG

Macrophage	Phagocytic index[a]			
	PE[b]		PE+IS[c]	
	Attachment	Ingestion	Attachment	Ingestion
Resident	207 ± 17	27 ± 3	325 ± 10	46 ± 14
BCG	318 ± 21	41 ± 9	520 ± 26	98 ± 21

[a] Data are expressed as PE or PE+IS ingested or attached per 100 macrophages.
[b] *P. berghei* PE (60% parasitemia).
[c] *P. berghei* PE preincubated for 30 min at 37°C with a 1/10 dilution of hyperimmune serum.

rophages harvested from mice previously infected with BCG bound and ingested more *P. berghei* PE than did resident macrophages (Table 1). Greater than 90% of the ingested erythrocytes were parasitized. In this and subsequent experiments, erythrocytes from uninfected mice were not phagocytized by either resident or LK-stimulated macrophages.

BCG-activated macrophages also ingested threefold more EIgG than did resident macrophages (results not shown). We therefore investigated whether Fc receptor-mediated phagocytosis contributed to the ingestion of PE. The phagocytic activity of both activated and resident macrophages was greatly enhanced when PE were preincubated in hyperimmune serum raised against *P. berghei* (Table 1). No role for cytophilic antibody was evident, since macrophages preincubated with hyperimmune serum did not show enhanced phagocytosis (not shown).

Enhancement of phagocytic activity by a factor(s) from antigen-stimulated, sensitized spleen cells. The exposure of resident peritoneal macrophages to a lymphocyte product(s) from BCG-sensitized spleen cells incubated with hkBCG resulted in increased attachment and phagocytosis of *P. berghei* PE (Fig. 1). Immune serum augmented the phagocytosis of PE by both resident and LK-stimulated macrophages. The phagocytic activity of the macrophages was proportional to the LK concentration incubated with macrophage monolayers (data not shown).

Since spleen cells from malaria-infected mice respond to specific antigen in vitro (35), we investigated whether such cells could produce a phagocytosis-promoting LK. Macrophages treated with the LK produced by spleen cells of malaria-infected or recovered mice ingested more EIgG per 100 macrophages than resident macrophages incubated with control LK (Fig. 2). In addition, a larger percentage of LK-activated macrophages than resident macrophages contained at least one ingested EIgG.

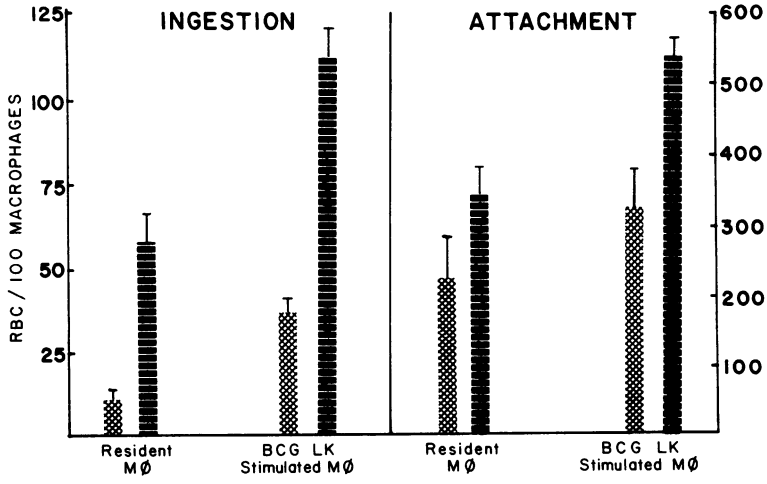

FIG. 1. Effect of BCG-induced LK on the phagocytosis of *P. berghei* PE by resident peritoneal macrophages. LK was prepared as described in the text. LK (1:5) was incubated with macrophage (MØ) monolayers for 24 h. The macrophages were overlaid with either PE (▒) or PE + IS (■). Results are expressed as the mean number of erythrocytes (RBC) ingested or bound per 100 macrophages on triplicate cover slips ± the standard error of the mean and are representative of three experiments.

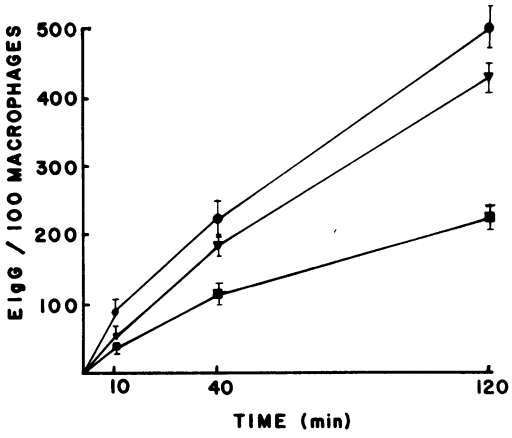

FIG. 2. Effect of malaria-induced LK on the phagocytosis of EIgG. LK was prepared from spleens of control uninfected mice (■), spleen cells of mice which had recovered from a *P. chabaudi* infection (▼), or from spleens of mice acutely infected with *P. chabaudi* (60% parasitemia) (●). The respective LK was incubated with macrophage monolayers for 24 h before the assay for phagocytosis of EIgG. The abscissa represents the time at which EIgG were exposed to macrophages. Results represent the mean ± the standard error of the mean of triplicate cover slips.

The LK-treated macrophages appeared to be highly spread and contained numerous pinocytic vesicles.

Stage specificity of LK-stimulated phagocytosis of *P. chabaudi* erythrocytes. The synchronous development of *P. chabaudi* parasites in blood cells allowed us to study the phagocytosis of specific stages (i.e., ring, trophozoite, and schizont) of the parasite. Mouse erythrocytes infected with ring forms (35% parasitemia) were poorly phagocytized by either resident macrophages or by macrophages exposed in vitro to supernatants of *P. chabaudi*-sensitized lymphocytes (Fig. 3). In contrast, schizont-enriched populations of infected erythrocytes (52% parasitemia) were more readily ingested by LK-treated macrophages compared to normal peritoneal cells. Although no increase in phagocytosis was noted when ring stage PE were preincubated in hyperimmune serum, phagocytosis of opsonized, schizont-infected erythocytes was considerably enhanced.

Species specificity of LK-stimulated phagocytosis of malaria PE. Although the induction of macrophage activation depends on a specific immune response, the expression of effector functions is nonspecific (20). Therefore, we investigated whether macrophages stimulated by a malaria-induced LK could promote the phagocytosis of PE of another species of *Plasmodium*. LKs prepared from spleens of both acutely infected mice and mice which had been cured of either *P. berghei* or *P. chabaudi* infection by chloroquine treatment were effective in stimulating macrophages to ingest higher levels of the homologous and heterologous parasites compared to macrophage monolayers incubated with the respective control LK (Table 2).

Cellular origin of the malaria-induced LK. Spleen cell factor(s) was prepared from the

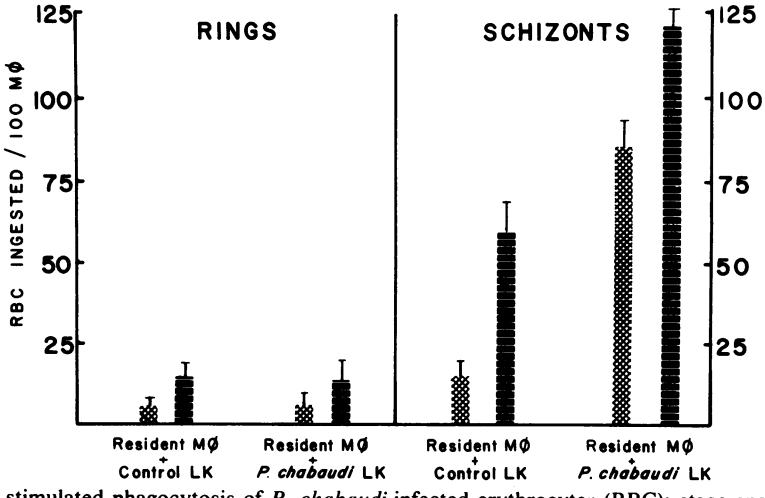

FIG. 3. LK-stimulated phagocytosis of *P. chabaudi*-infected erythrocytes (RBC): stage specificity and the role of immune serum. Resident peritoneal macrophages (MØ) were incubated for 24 h with LK prepared from either the spleens of normal mice (control LK) or mice cured of the infection (*P. chabaudi* LK). Macrophages were subsequently assayed for the ingestion of ring or schizont stages of *P. chabaudi* PE preincubated with normal mouse serum (1:10) (▒) or hyperimmune serum (1:10) (■). Results represent the mean ± standard error of the mean of triplicate cover slips.

TABLE 2. LK-stimulated phagocytosis of *Plasmodium*-infected erythrocytes: species specificity

Source of LK[a]	PE ingested/100 macrophages[b]	
	P. berghei	*P. chabaudi*
P. berghei-infected mice	98 ± 10[b] ($P < 0.05$)	56 ± 6 ($P < 0.05$)
P. berghei-recovered mice	102 ± 11 ($P < 0.05$)	57 ± 5 ($P < 0.05$)
Control (normal spleen + *P. berghei* antigen)	58 ± 7	24 ± 3
P. chabaudi-infected mice	52 ± 6 ($P < 0.05$)	56 ± 5 ($P < 0.05$)
P. chabaudi-recovered mice	82 ± 10 ($P < 0.025$)	53 ± 3 ($P < 0.05$)
Control (normal spleen + *P. chabaudi* antigen)	26 ± 4	31 ± 4

[a] LK was prepared as described in the text. LK was incubated in D10 with peritoneal macrophages for 24 h. Macrophages were subsequently allowed to phagocytize either *P. berghei* PE (56% parasitemia) or *P. chabaudi* PE (32% parasitemia).

[b] Mean ± standard error of triplicate samples. Results are representative of three experiments. Significance levels were determined by Student's *t* test.

spleens of *P. chabaudi*-infected mice stimulated in vitro with parasite antigen. The spleen cells were not fractionated, nonadherent to nylon wool, or depleted of T lymphocytes by anti-θ treatment plus complement. The LK was incubated with resident macrophages, and two parameters of phagocytosis were assessed: the percentage of macrophages ingesting at least one EIgG and the number of erythrocytes ingested per 100 macrophages. The LK, prepared from either whole spleen cells of *P. chabaudi*-infected mice or nylon wool-passaged T cells, was capable of enhancing phagocytosis of EIgG (Table 3). Both parameters of phagocytosis were increased three- to fivefold over macrophages incubated with control LK. In addition, there was a marked inhibition of the ability of such malaria-sensitized spleen cells to stimulate macrophages for increased phagocytosis when these cells were depleted of T lymphocytes. Macrophages incubated with an LK derived from T-depleted spleen cells ingested similar levels of EIgG as control macrophages.

DISCUSSION

The importance of T lymphocytes in the immune response to malaria is known; however, the key mechanism of T cell function has remained unclear. That helper T cells can participate in antimalarial antibody production was first proposed by Brown et al. (3) and later confirmed by Jayawardena et al. (13), who demonstrated high levels of IgM and IgG antibodies in T cell-deprived mice reconstituted with $Ly1^+$ cells. In addition, adoptive transfer of T cells of the $Ly1^+$ phenotype from immune animals to nonimmune recipients conferred protection. However, other experiments indicated that antibodies play little role in immunity to reinfection with malaria (12, 27).

Two additional correlates of cellular immunity to malaria, delayed-type hypersensitivity and blood monocyte responses, were detected in T cell-deprived mice reconstituted with $Ly1^+$ cells from immune mice (13). Other lines of evidence suggested that enhanced phagocytosis of colloidal particles (29), as well as splenomegaly, are thymus-dependent responses to malaria (26).

Until now, it was not known whether specific malaria-sensitized T cells (other than helper cells for antibody production) were required for the generation of nonspecific effector functions. Although macrophage activation was mediated by soluble factors or LKs prepared from anti-

TABLE 3. Characterization of cell type producing malaria-induced LK

Source of spleen cells[a]	Phagocytic index[b]	
	% Macrophage ingesting	EIgG ingested/100 macrophages
Normal uninfected mice	31 ± 7	53 ± 4
P. chabaudi-infected mice		
Whole spleen	99 ± 18 ($P < 0.05$)	303 ± 47 ($P < 0.025$)
Nylon wool-purified T cells	92 ± 9 ($P < 0.05$)	274 ± 21 ($P < 0.025$)
Anti-ϕ + complement-treated spleen cells	36 ± 15	67 ± 11

[a] LK was prepared from spleen cells incubated with *P. chabaudi* antigen for 48 h. Supernatant (1:4) was incubated with normal macrophage monolayers for 24 h before phagocytic assay.

[b] Mean ± standard error of triplicate samples. Results are representative of three experiments. Significance levels were determined by Student's *t* test.

gen-sensitized T cells in some systems (18, 22), macrophages from *Plasmodium*-infected mice deficient in T cells were shown to be activated, as assessed by the detection of plasminogen activator (24).

We therefore attempted to study the interaction of T cells and effector macrophages in immunity to malaria by focusing on the possibility that during malaria infection macrophages are activated by a T cell-derived LK from malaria antigen-sensitized spleen cells. Indeed, in this report we have demonstrated a T cell-dependent mechanism for macrophage activation in malaria. We have shown that LK-treated macrophages are activated nonspecifically to ingest malaria PE of both homologous and heterologous species from which the malaria-induced LK was prepared, as well as IgG-coated erythrocytes. When PE were preincubated with antiplasmodial hyperimmune serum, greater attachment and ingestion by both resident and LK-stimulated macrophages were observed.

The mechanism of the phagocytosis of PE not preincubated with immune serum is still unresolved. Ingestion is most likely Fc receptor mediated, since PE which were extensively washed displayed a substantial residue of bound immunoglobulin, as judged by immunofluorescence with fluorescein-conjugated goat anti-mouse IgG. It has been shown that parasitized as well as nonparasitized erythrocytes are coated with IgG and IgM in rodent (19) and human malaria infections (10). It is unlikely that the PE are phagocytized via the C3b receptor, since normal macrophages as well as LK-stimulated macrophages ingest the erythrocytes. It has been previously demonstrated that normal macrophages are unable to ingest C3b-coated erythrocytes, although they are quite capable of ingestion of IgG-coated erythrocytes (2). Our observations that higher levels of opsonized PE are ingested by LK-stimulated macrophages are consistent with a previously published study showing that Fc receptor-mediated phagocytosis by activated macrophages is approximately 1.5 to 2 times greater than phagocytosis by resident macrophages of IgG-coated sheep erythrocytes (2).

It has been suggested that phagocytosis of malaria parasites is not of importance to cell-mediated immunity but may only be a contributory factor (1). Although histological as well as in vitro studies indicate otherwise (4, 17, 33, 36), two investigations (25, 31) utilizing in vivo models to assess clearance of PE showed that rheological changes, leading to decreased deformability of PE, and not opsonization of PE by macrophages is a primary defense mechanism. In both studies, the clearance of ^{51}Cr-labeled PE by the spleen was not altered by preincubation of PE with hyperimmune serum. However, it is possible that the introduction of large numbers of labeled PE into the circulation (1×10^8 to 10×10^8 PE) mitigates against the detection of the clearance of antibody-coated PE due to a masking effect exerted by excessive trapping of parasites. Nonimmune factors undoubtedly contribute to the clearance of PE from the circulation by the spleen. Wyler et al. (37) have shown that the alteration of the splenic architecture at the onset of resolution of the infection increases circulation through the open pathway, providing greater clearance of poorly deformable PE. PE circulating through the open pathway may be trapped and cleared by cordal macrophages.

Recent studies in our laboratory suggest that a role for phagocytosis of malaria PE by LK-activated macrophages should not be readily dismissed. Although the trapping and clearance of parasites may occur via non-immunological mechanisms, the increased phagocytosis by activated macrophages reported here is an important precursor to the actual killing of the malaria parasite. It has been reported that H_2O_2 synthesis and release are closely related to the rates of opsonized particle ingestion via the Fc receptor (28). The increased level of ingestion of opsonized PE correlates with the oxidative killing of the parasite (C. F. Ockenhouse and H. L. Shear, submitted for publication). Although the effectiveness of activated macrophages in controlling the rising parasitemia in an acutely infected animal initially infected with malaria may be minimal due to immunosuppression, we propose that a sequence of events occurs in animals previously infected with malaria which leads to the cytotoxic mechanism against the intraerythrocytic parasite. Macrophages, which are activated either by a T-independent mechanism or by a malaria-sensitized, T cell-derived LK, recognize and bind trophozoite- and/or schizont-infected erythrocytes. Opsonizing antibody may aid in the recognition and binding to the effector cells, although human monocytes bind the knob-positive strain of *Plasmodium falciparum* in the absence of antibody (21). Phagocytosis triggers a respiratory burst, which results in the extracellular release of toxic oxygen products. We have recently observed that these products produced by malaria-induced LK-activated macrophages kill the malaria parasite (C. F. Ockenhouse and H. L. Shear, submitted for publication).

ACKNOWLEDGMENTS

This work was supported by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and by Public Health Service grant AI 15235 and National Research Service Award AI 07180 from the National Institutes of Health.

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