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Kupffer Cell Elimination Enhances Development of Liver Schizonts of *Plasmodium berghei* in Rats

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We investigated the development of exoerythrocytic forms (EEF) of *Plasmodium berghei* in livers of normal and macrophage-depleted Brown Norway rats. Macrophages were depleted by use of liposome-encapsulated dichloromethylene diphosphonate. Upon inoculation of sporozoites, macrophage-depleted rats had significantly larger numbers of EEF than untreated rats. We also investigated the effect of macrophage impairment by silica treatment on the development of EEF and confirmed that silica induces a significant reduction of EEF development. Intravenous administration of silica induced high levels of interleukin-6 in plasma within a few hours. The seemingly contradictory results for EEF development may be explained by our previous observation that interleukin-6 strongly inhibits sporozoite penetration and EEF development in vivo. We conclude that in experimental infections with sporozoites, Kupffer cells inhibit rather than enhance EEF development.

Malaria infection can be acquired by the inoculation of sporozoites into the skin by an infected mosquito taking a bloodmeal. Sporozoites are transported by the bloodstream to the liver, in which they invade hepatocytes and subsequently develop into mature exoerythrocytic forms (EEF). The hepatic sinusoids are lined with endothelial cells and Kupffer cells. To invade the liver parenchyma, sporozoites must escape these cells. The role of Kupffer cells in the clearance of sporozoites from the circulation and the subsequent development of sporozoites into mature EEF are subject to contradictory observations. Seguin et al. reported a minimal interaction in vitro of sporozoites with nonimmune mouse Kupffer cells, which could readily ingest latex particles and *Leishmania* amastigotes (12). Observations made by Verhave et al. indicated that silica treatment caused a drastic reduction in the number of EEF, a result that led to the hypothesis that Kupffer cells are helpful in if not obligatory for the in vivo penetration of sporozoites into hepatocytes (18). Ultrastructural studies have shown that sporozoites are present inside Kupffer cells from 10 min after inoculation into the portal vein and that they are able to escape Kupffer cells on the parenchymal side to subsequently invade hepatocytes (7). Finally, in a videomicroscopic study, Vanderberg and Stewart found that sporozoites both actively and passively enter Kupffer cells and also emerge from these cells (15). Whether Kupffer cells are essential for the establishment of a malaria infection needs further confirmation, since in vitro infection of isolated and plated primary hepatocytes has been achieved (8). For investigation of whether the depletion of functional macrophages has an effect on the penetration and development into liver schizonts of sporozoites, a recently developed method was used to selectively eliminate macrophages by allowing them to ingest liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP). The liposomes are ingested by the macrophages, and the drug is released into the interior of the cells as soon as the liposomal phospholipid bilayers are disrupted because of the influence of phospholipases present in the lysosomal compartments of the cells (16, 17). After a single treatment, macrophages in the liver and spleen of rats are eliminated. Endothelial cells do not seem to be affected (1). We studied the effect of Kupffer cell elimination in vivo on the development of EEF by using this method.

MATERIALS AND METHODS

Rats. Male Brown Norway rats were obtained from Harlan OLAC Ltd., Shaw's Farm, Blackthorn, Bicester, United Kingdom. Rats were housed in our animal laboratory and fed a standard diet and acidified water ad libitum. Six- to 10-week-old rats were used in all experiments. These rats were chosen because of their high susceptibility to sporozoite infection (7).

Production of *Plasmodium berghei* sporozoites. Sporozoites of *P. berghei* (ANKA strain) were harvested from homogenized infected *Anopheles gambiae* mosquitoes and purified on a biphasic gradient containing Urographin (Schering AG, Berlin, Germany) and bovine calf serum (6). Live sporozoites (3×10^5 to 5×10^5 in 1 ml of sterile phosphate-buffered saline [PBS]) were injected intravenously (i.v.).

Liposomes. Multilamellar liposomes containing Cl_2MDP (a kind gift from Boehringer GmbH, Mannheim, Germany) were prepared as described earlier (16). They were suspended in PBS at a concentration of 10 mg/ml. Rats were injected i.v. with 1 ml/100 g of body weight.

Silica. Silica particles with a diameter of <5 μm (Sigma Chemical Co., St. Louis, Mo.) were suspended in sterile PBS at a concentration of 17 mg/ml. Rats were injected i.v. with 1 ml/100 g of body weight.

ED2 monoclonal antibody. Mouse anti-rat macrophage monoclonal antibody ED2 was kindly provided by Christine Dijkstra, Department of Histology, Free University, Amsterdam, The Netherlands (3).

Evaluation of the number of liver schizonts (EEF). At 44 h after the injection of sporozoites, rats were killed by cervical

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dislocation and livers were removed. A few 1-cm-thick slices of the left liver lobe were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) and subsequently transferred to 70% ethanol. Paraffin sections (7 μm) were stained with hematoxylin and eosin. The number of EEF per square centimeter of a liver section from each rat was assessed microscopically on coded slides.

B9 cell bioassay for IL-6. Rats were bled from the tail vein by use of sodium citrate (final concentration, 0.38%), and plasma samples were stored at −80°C. Interleukin-6 (IL-6) was measured in plasma samples by the B9 cell bioassay (2). In brief, the IL-6-dependent murine hybridoma cell line B9 was grown to a density of 0.1×10^5 to 1×10^5/ml in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 50 μM β-mercaptoethanol (GIBCO), 40 μg of gentamicin (Essex, Amstelveen, The Netherlands) per ml, and 8 U of human recombinant IL-6 per ml. Before the assay, B9 cells were harvested by centrifugation and washed twice with IL-6-free medium. B9 cells were seeded at 5,000 cells per 200 μl in flat-bottom wells (Costar Europe, Badhoevedorp, The Netherlands) in the presence of plasma samples in appropriate dilutions. Proliferation was measured by use of a ^{3}H-thymidine pulse during the last 16 h of a 96-h culture period. ^{3}H-thymidine was added at 3.7×10^4 Bq per well (specific activity, 0.7×10^8 to 1.1×10^8 MBq/mmol; Amersham International, Amersham, United Kingdom).

Samples were tested in triplicate, and data were always compared with a standard curve included in each experiment. One unit of IL-6 per milliliter gives rise to half-maximal proliferation. The sensitivity of the assay is 0.3 pg/ml.

Immunoperoxidase staining of cryostat liver sections for the demonstration of the successful elimination of Kupffer cells. Immunoperoxidase staining was used to confirm the elimination of Kupffer cells from the liver in Cl_2MDP- or silica-treated rats. Portions of removed livers were frozen in liquid nitrogen and stored at −80°C. Cryostat sections of 5 μm were fixed in acetone and air dried. They were incubated for 45 min at room temperature with monoclonal antibody ED2 at a 1/400 dilution in PBS with 2% bovine serum albumin. After being washed thoroughly in PBS, the sections were incubated for 20 min with rabbit anti-mouse peroxidase (DAKO, Glostrup, Denmark) at a 1/200 dilution in PBS–1% rat serum. After another wash in PBS, the sections were incubated with 0.05% (wt/vol) diaminobenzidine (Sigma)–0.03% (vol/vol) hydrogen peroxide (E. Merck AG, Darmstadt, Germany) in Tris-sucrose buffer. Finally, the sections were washed in PBS and counterstained with hematoxylin. In this procedure, Kupffer cells can be recognized as golden brown structures between the parenchymal cells (Fig. 1).

Statistical analysis. Results were analyzed for significance by use of the two-tailed Wilcoxon rank sum test.

RESULTS

Effect of macrophage depletion by liposome-encapsulated Cl_2MDP on EEF development. For investigation of the effect of Cl_2MDP-induced elimination of macrophages on the development of EEF, Brown Norway rats were injected i.v. with either Cl_2MDP-containing liposomes or an equal volume of sterile PBS. After 72 h, all rats were injected i.v. with 2×10^5 sporozoites. At 44 h later, livers were prepared for EEF assessment. Cl_2MDP-treated rats developed significantly more EEF per square centimeter than control rats ($P < 0.05$; Table 1).

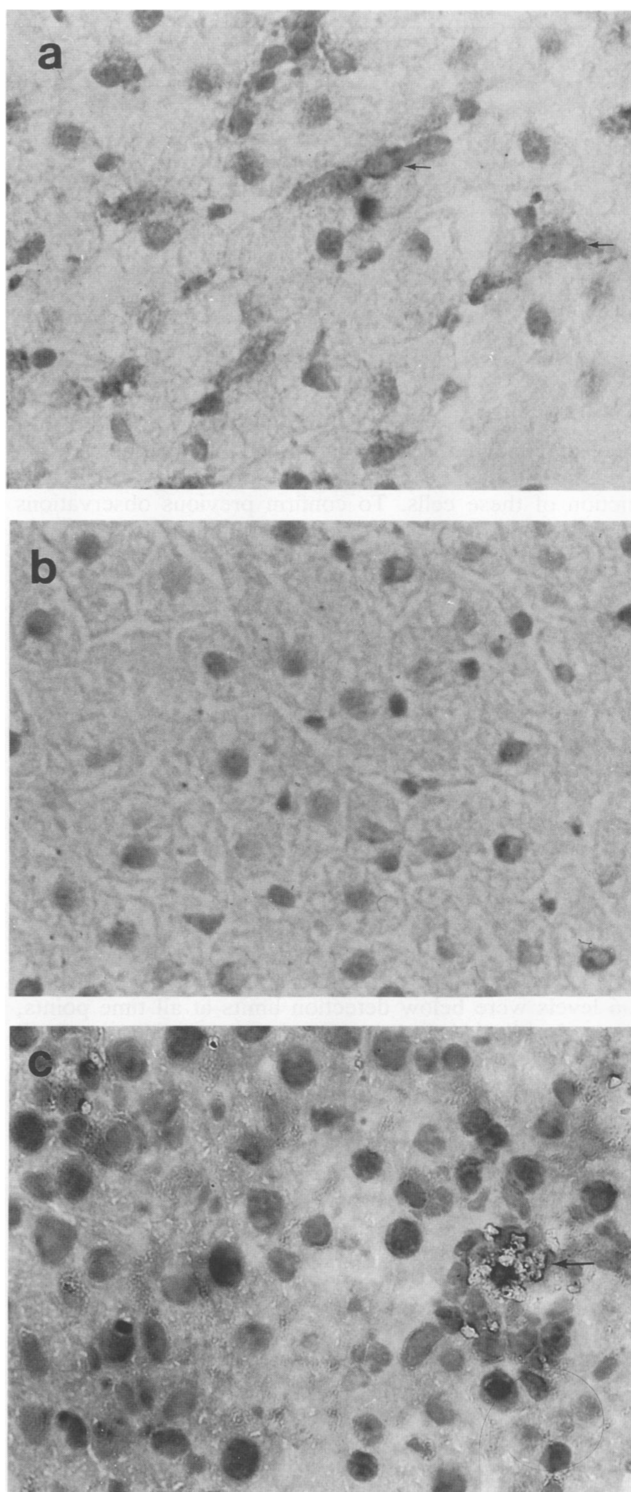

FIG. 1. Effect of liposomal Cl_2MDP or silica treatment on Kupffer cells. Cryostat sections from rats injected with PBS (a), Cl_2MDP (b), or silica (c) were incubated with monoclonal antibody ED2 and rabbit anti-mouse peroxidase. In the control rat, there was a normal distribution of Kupffer cells (a; arrows), which were absent in Cl_2MDP-treated rat (b). The section from the silica-treated rat shows Kupffer cells engorged with silica (c; arrow).

TABLE 1. Influence of liposomal Cl_2MDP and silica on EEF development[a]

Expt	No. of injected sporozoites	No. of rats	EEF density in the presence of:			P
			Cl_2MDP	PBS	Silica	
1	1.5×10^5	5	45 ± 11	8 ± 7	ND	<0.05
2	5×10^5	4	140 ± 35	34 ± 7	ND	<0.05
3	5×10^5	5	ND	25 ± 7	4 ± 1	<0.05

[a] In experiments 1 and 2, rats were injected i.v. with liposomal Cl_2MDP. After 3 days, sporozoites of *P. berghei* were administered i.v. In experiment 3, rats were injected i.v. with a silica suspension 24 h prior to the inoculation of sporozoites. In all experiments, the control group received an equal volume of sterile PBS. EEF density is expressed as the number of liver schizonts per square centimeter. ND, not done.

Effect of silica treatment on EEF development. Silica particles administered i.v. are absorbed by macrophages, a process that has been shown to impair the phagocytic function of these cells. To confirm previous observations indicating that this treatment led to a significant reduction of EEF development, we injected Brown Norway rats with either a silica suspension or sterile PBS. After 24 h, all rats were injected with sporozoites. Another 44 h later, livers were prepared for EEF assessment. We observed a significant reduction of EEF density in silica-treated rats ($P < 0.05$; Table 1).

Measurement of circulating IL-6 after silica treatment. Elimination of macrophages by Cl_2MDP-containing liposomes and macrophage impairment by silica had opposite effects on EEF development. Since in vitro IL-6 production by macrophages can be induced by silica (4) and also since preincubation of hepatocytes with IL-6 has been shown to inhibit EEF development (11), we tentatively concluded that the reduction by silica of EEF development was due to the induction of IL-6 production. Therefore, plasma samples were taken from rats at 0, 6, and 24 h after the injection of silica, Cl_2MDP, or PBS. In control and Cl_2MDP-treated rats, IL-6 levels were below detection limits at all time points, whereas in silica-treated rats, IL-6 levels were elevated in all samples (Fig. 2).

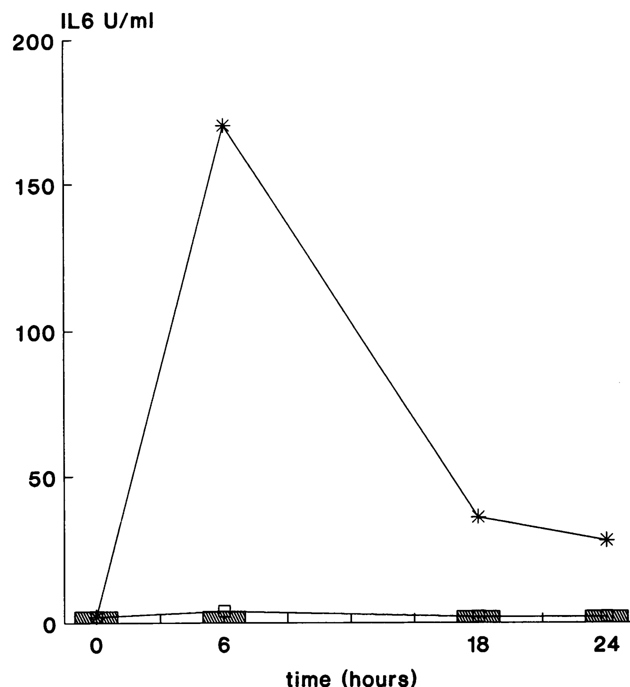

FIG. 2. Plasma IL-6 levels after silica or Cl_2MDP treatment. Plasma was collected at the indicated times after i.v. administration of silica (*), PBS (□), or Cl_2MDP (▨). IL-6 was measured in a B9 cell bioassay. In PBS (control)- and Cl_2MDP-treated rats, no circulating IL-6 could be detected, whereas silica-treated rats had high levels of IL-6 within 6 h after treatment.

DISCUSSION

The elimination of Kupffer cells from the liver of BN rats by Cl_2MDP-containing liposomes led to an increased number of EEF. This result sheds new light on the role of Kupffer cells in the initial phase of malaria infection. Sporozoites can only leave the bloodstream by penetration of the sinusoidal lining, which is formed by endothelial cells and Kupffer cells. The size of the fenestrae between the former probably does not allow sporozoite passage (20). There is evidence that sporozoites can enter Kupffer cells and subsequently leave these cells on the parenchymal side without being disrupted (7). The interaction of sporozoites of *P. berghei* with Kupffer cells in vitro shows a wide variety of patterns; sporozoites can actively enter and leave these cells and eventually destroy them (15). However, sporozoites can also be ingested and destroyed by Kupffer cells in vivo (5, 13). External killing of sporozoites during an interaction with macrophages has also been reported (15). The contribution of each of these phenomena to the overall successful development of sporozoites in vivo has not been elucidated to date. Since in our experiments the elimination of Kupffer cells led to a fourfold increase in the number of EEF, it is very likely that external killing or ingestion and subsequent destruction by Kupffer cells is the fate of the majority of sporozoites in experimental infections. Another possibility is that Kupffer cells inhibit the maturation of schizonts indirectly by the production of IL-6. Penetration of a Kupffer cell may activate this cell and induce IL-6 production. Elsewhere, we showed that IL-6 significantly inhibited EEF development even when administered 24 h after sporozoite inoculation (19).

The reductive effect of pretreatment with silica has been explained by the hypothesis that sporozoites can only enter the liver parenchyma by passing through Kupffer cells (18). When these cells are fully engorged with silica, they fail to internalize sporozoites. However, as we show here, phagocytosis of silica leads to IL-6 production within a few hours after administration. Therefore, the inhibitory effect of silica treatment on EEF development may be due to the induction of IL-6 secretion. Sinden and Smith demonstrated a significant reduction of Kupffer cell and EEF densities after silica treatment; they concluded that the reduction of Kupffer cell density was responsible for the lower EEF density (14). Our results with Cl_2MDP treatment show that this is not the case. Treatment with liposomal Cl_2MDP may also affect other liver cells, such as hepatocytes, to some extent. Ultrastructural studies of the effects on the liver are not available. However, it is not very likely that eventual hepatocyte damage will enhance schizont development.

The IL-6 levels in silica-treated rats had decreased to low levels at the time of sporozoite inoculation. However, by that time hepatocytes had been exposed to high levels of IL-6 for many hours. Pied et al. demonstrated that preincubation of hepatocytes with IL-6 in vitro prior to the inocu-

lation of sporozoites strongly inhibits sporozoite penetration and EEF development (11). If IL-6 is the factor that leads to the inhibition of EEF development after silica injection, one might expect that the simultaneous injection of antibody to IL-6 would prevent the reduction of EEF development. Using a polyclonal rabbit anti-mouse IL-6 antibody, we could not demonstrate this effect (data not shown). This result may have been due to the inadequate neutralization of IL-6 because of the unknown kinetics of silica-induced IL-6 production or the presence of other, as-yet-unknown inhibiting effector mechanisms activated by silica treatment.

The effect on hepatocytes of IL-6, which makes them resistant to sporozoite penetration and development, remains after the removal of IL-6. Several mediators have been shown to implement this prolonged refractoriness (9–11). Among these are acute-phase proteins, reactive oxygen, and L-arginine derivatives. Once induced, these mediators remain present for a prolonged time.

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