Relationship of Alterations in Splenic Clearance Function and Microcirculation to Host Defense in Acute Rodent Malaria

DAVID J. WYLER and THOMAS C. QUINN, *Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20205*

LI-TSUN CHEN, *Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

ABSTRACT During the course of *Plasmodium berghei* malaria in the rat, splenic clearance of damaged uninfected erythrocytes (heated or Heinz body-containing) underwent changes strikingly similar to those of infected erythrocytes. Splenic trapping of abnormal erythrocytes was impaired during the period of rising parasitemia but became supernormal just before the onset of resolution of the acute infection. These changes could be related to the development of splenomegaly and alterations in splenic cordal microcirculation during infection. The relative distribution of flow through the cords was decreased during rising parasitemia and was restored before the onset of resolution. Together, our observations support the hypothesis that altered rheologic properties of infected erythrocytes are a major determinant of their removal by the spleen. These data suggest that the alterations in splenic microcirculation that occur during malaria may have important implications for host defense.

INTRODUCTION

Although a major role for the spleen in defense against plasmodial infection is suggested by the strikingly deleterious effects of splenectomy (1), the mechanisms of host defense of malaria are incompletely defined. To more clearly understand how the spleen exerts a protective effect we examined its role in trapping parasitized erythrocytes by studying the intravascular clearance of ^{51}Cr-labeled *Plasmodium berghei*-infected erythrocytes in the rat (2). In this model infected erythrocytes were cleared from circulation more rapidly than uninfected erythrocytes as a result of splenic trapping. Trapping of parasitized erythrocytes seemed to be important in the host defense to acute malaria since clearance was markedly reduced during the period of rising parasitemia but increased to supernormal levels just before the onset of spontaneous resolution of the infection (2). Since clearance of these cells was not antibody-dependent (3) we proposed that it might be based upon the decreased deformability of infected erythrocytes (4). In the present study, we have attempted to test this hypothesis by comparing the clearance patterns of parasitized erythrocytes with those of heated or phenylhydrazine-damaged uninfected erythrocytes during acute *P. berghei* infection.

METHODS

Malaria infections. Male Wistar rats (Hemobartonella-free; Charles River Breeding Laboratories, Inc. Wilmington, Mass.) weighing 125 g were used throughout. Infections were initiated by the intraperitoneal inoculation of 10^7 *P. berghei* (NYU-2 strain) parasitized rat erythrocytes suspended in sterile isotonic saline. The magnitude of parasitemia was determined daily by counting the number of *P. berghei*-infected erythrocytes per 1,000 total erythrocytes on Giemsa-stained thin films prepared from tail vein blood. The course of this infection has been described (2).

Preparation of erythrocytes and clearance studies. P. berghei-infected erythrocytes and Heinz body-containing erythrocytes were prepared and tagged with ^{51}Cr as described (2). Briefly, *P. berghei*-infected erythrocytes were obtained by cardiac puncture from weanling rats with 70% parasitemia and enriched to a concentration of 85–90% infected cells by centrifugation (900 *g* for 5 min). Heinz-body containing erythrocytes were obtained from infected rats injected subcutaneously on two separate occasions (48 h apart) with phenylhydrazine hydrochloride (J. T. Baker Chemical Co., Phillipsburg, N. J.). Erythrocytes were labeled by incubation for 30 min at 20°C with $Na_2{}^{51}CrO_4$ (200 μCi/ml packed cells; sp act, 400 mCi/mg Cr; Amersham Corp., Arlington Heights, Ill.). Normal RBC

Dr. Wyler's present address is Division of Geographic Medicine, Department of Medicine, Tufts-New England Medical Center, Boston, Mass. 02111.

Received for publication 27 October 1980 and in revised form 14 January 1981.

were radiolabeled in the same manner and heated at 50°C for 20 min in a stationary water bath before use in some experiments. Cells were all washed once with phosphate-buffered saline (pH 7.4) and resuspended to 10^9 erythrocyte/ml in sterile isotonic saline before injection of 1 ml i.v.

Three groups of 40 rats were simultaneously infected intraperitoneally with 10^7 *P. berghei*-infected erythrocytes, and on alternate days thereafter studies of clearance of the three damaged erythrocyte populations were performed on four rats from each group. Splenic and hepatic uptake of radiolabeled erythrocytes were performed 24 h after injection of these cells by measuring the radioactivity present in 0.5 g samples of tissue, and calculating total uptake on the basis of the determined wet weight of the organ. Organ uptake was expressed as a percentage of the radioactivity present in the inoculum. The methods and calculations employed in these studies were the same as those described (2).

Determination of splenic microcirculation. The clearance of mildly heated and Heinz body-containing erythrocytes appears to depend upon the distinctive vasculature of the spleen (5). Slits between adjacent sinus endothelial cells restrict the movement of erythrocytes with decreased deformability (6), whereas normally deformable erythrocytes can pass through these spaces unimpeded. Blood flowing through the cordal pathways (open circulation) encounters these spaces in passing into the sinuses, while blood bypassing the cords (closed circulation) enters the sinuses without encountering this obstacle. Thus, the ability of the spleen to remove rigid erythrocytes depends in large part on the relative distribution of blood flow through the open and closed pathways.

To assess splenic microcirculation in rats with malaria, a technique was used that measures the distribution of plastic microspheres within the spleen after intravenous injection (7). This method is based upon the fact that the anatomic distribution of microspheres in a capillary bed is a function of relative blood flow to these areas (8). In our experiments, microspheres of 3–4 μm were used since they are small enough to move within the cordal meshwork, yet large enough to be detained in the cords by the sinus wall (7). The interendothelial spaces in the splenic sinus wall are in the range of 0.5–2.5 μm (6).

Uninfected rats and those at different stages of infections were anesthetized with pentobarbital (4 mg/100 g body wt i.p.), secured to a guillotine platform, and 2×10^8 carbonized plastic microspheres (3–4 μm; 3M Co., St. Paul, Minn.) suspended in 0.4 ml of saline were injected rapidly into the dorsal vein of the penis. The splenic blood was stopped 5–6 s later by dropping the guillotine through the thorax at a level between the diaphragm and the heart. The spleen was removed and fixed by interstitial perfusion with 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 10 min and then allowed to remain in this fixative for additional 12 h. Spleens were cut transversely into 1.5-mm thick slices, and the slices were washed in cacodylate buffer, dehydrated through graded ethanol, and embedded in a mixture of butoxylethanol and glycomethacrylate (Polysciences, Inc., Warrington, Pa.). 3-μm thick sections were cut with a JB-4 microtome (Dupont Instruments Sorvall-Biomedical Div., Newtown, Conn.) using glass knives and stained with hematoxylin and eosin. For each spleen, the percentage of the microspheres in the cords and the sinuses was determined by counting 1,000 microphores in several random tissue sections.

RESULTS

Parasitemia. After the inoculation of 10^7 *P. berghei*-infected erythrocytes parasitemia rose steadily (precrisis period) in all rats to a peak of 61.2±5.7% (mean ±SEM) by day 15 of the infection. During the next 3 d (crisis period) parasitemia declined rapidly to undetectable levels. After day 19, no circulating parasites could be detected on blood smears. 15 of 120 infected rats died (12.5% mortality).

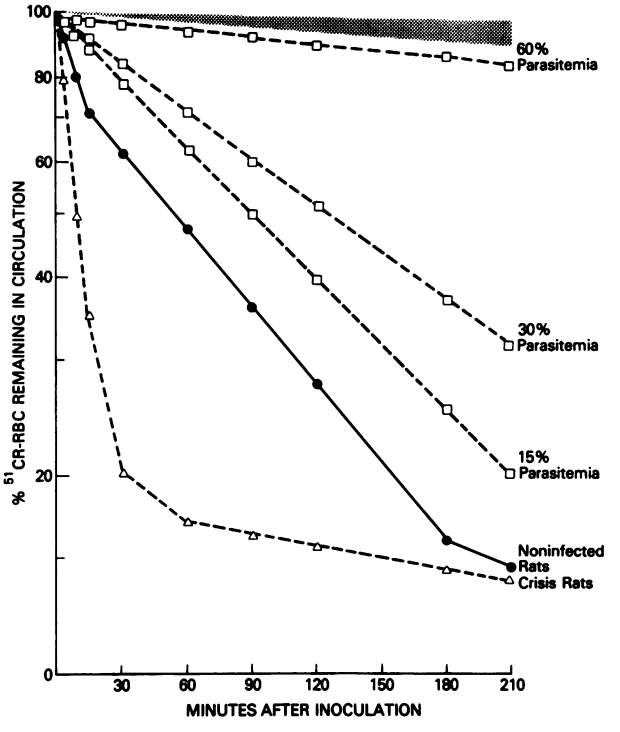

FIGURE 1 Clearance of ^{51}Cr-labeled heat-damaged erythrocytes (RBC) in noninfected rats and in *P. berghei*-infected rats, precrisis and during crisis. Each point represents the mean of determinations in six rats; SEM <10% of mean.

Clearance of ^{51}Cr-labeled erythrocytes during malaria. In this study we confirmed our previously published observations (2) which indicate that ^{51}Cr-labeled PRBC are cleared rapidly in uninfected rats. Clearance was characterized by a rapid phase that occurred in the first hour and attributable to splenic trapping. This was followed by a slower second phase most likely representing lysis of infected erythrocytes resulting from terminal intraerythrocytic parasite development (2). In rats experiencing rising parasitemia, the splenic phase of clearance was not observed, whereas in rats studied during the period of resolution of the infection (crisis period, days 16–18), the rate of clearance of infected erythrocytes was markedly accelerated with enhancement of both the first and second phase (2).

Like infected erythrocytes, heat-damaged and Heinz body-containing erythrocytes were cleared in uninfected rats with an initial rapid phase and a slower second phase (Figs. 1 and 2). In infected rats experiencing a rising parasitemia, these altered erythrocytes

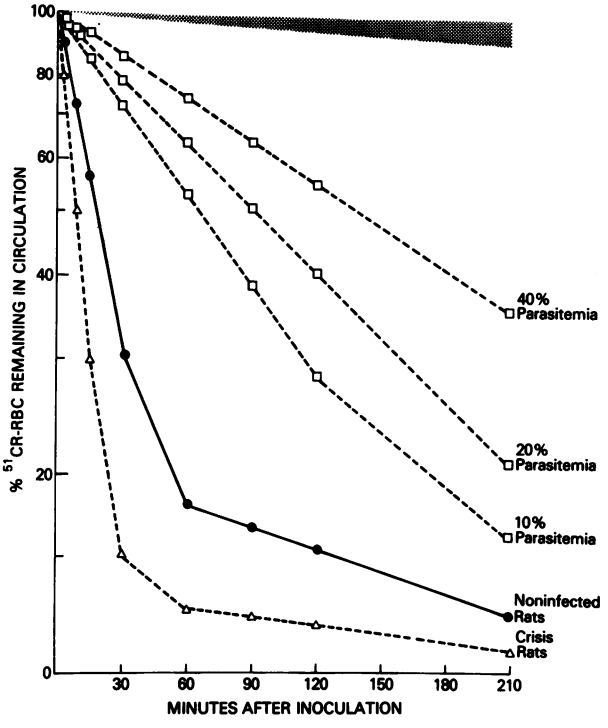

FIGURE 2 Clearance of ^{51}Cr-labeled Heinz body-containing erythrocytes (RBC) in noninfected rats and in *P. berghei*-infected rats, precrisis and during crisis. Each point represents the mean of determinations in six rats; SEM < 10% of mean.

were cleared slowly, with disappearance of the early rapid (splenic) phase. There appeared to be an inverse relationship between magnitude of parasitemia and clearance rates for these cells. At the time of crisis, when the acute infection began to resolve and parasitemia fell, clearance of altered uninfected erythrocytes was markedly increased to supernormal rates.

The reduction in clearance of infected and uninfected altered erythrocytes during the rising parasitemia could be explained on the basis of decrease in splenic uptake (Fig. 3). Decreased splenic uptake occurred despite the fact that splenomegaly developed during the early infection. Hepatic uptake of infected and damaged uninfected cells is shown in Table I. A reciprocal relationship between hepatic and splenic uptake of damaged uninfected erythrocytes was observed during the precrisis period and of all abnormal erythrocytes during the crisis period. This suggests that in the absence of normal splenic clearance, the liver can remove a greater percentage of damaged uninfected but not parasitized erythrocytes. The lung contained ≤6% of radioactivity at all times during the infection. With the onset of crisis, there was a rapid and sudden increase in the 24-h splenic uptake of all abnormal erythrocytes (Fig. 3) and accentuation of the early rapid clearance phases (Figs. 1 and 2).

Alterations in splenic microcirculation. In uninfected rats, microspheres distributed primarily to the cords (Table II), thus confirming in these rodents the previous studies in rabbits, which demonstrated the preeminence of the open pathway (7). Rising parasitemia was associated with a significant decrease in the relative blood flow through the cords and a relative increase in circulation through the closed pathways which bypass the cords. With the onset of crisis, the pattern of microcirculation reverted toward normal, with an increase in relative cordal flow above precrisis levels.

DISCUSSION

Previous efforts to define the mechanisms whereby the spleen exerts a protective role in malaria have revealed that parasitized erythrocytes become trapped as they circulate through the splenic cords. Electron micrographic studies of the spleen of rhesus monkeys infected with *Plasmodium knowlesi* indicated that infected erythrocytes were unable to pass through the interendothelial spaces separating the cords and sinuses (9). On the basis of these observations, it was suggested that the relatively rigid intracellular parasite retarded the passage of the otherwise highly deformable erythrocyte. Independent measurements of the

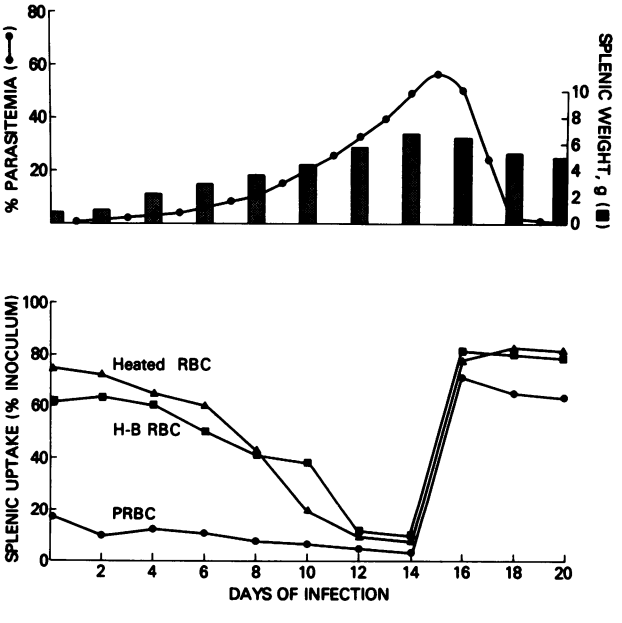

FIGURE 3 Splenic uptake of ^{51}Cr-labeled *P. berghei*-infected erythrocytes (PRBC) heat-damaged erythrocytes (heated RBC) and Heinz body-containing erythrocytes (H-B RBC) in rats on different days of infection. Mean spleen weight is shown in the upper panel by vertical bars. Mean parasitemia (percent infected RBC) on each day of infection is shown for comparison in the upper panel. The crisis period occurred between day 16 and 18 of infection. SEM <10% of mean for all parameters.

TABLE I

Hepatic Uptake of ^{51}Cr-labeled Erythrocytes during P. berghei Malaria in Rats

Stage of infection	Organ wt	Hepatic uptake of erythrocytes*		
		P. berghei-infected	Heat-damaged	Heinz body-containing
	g		%	
Noninfected	7.4±0.3‡	36.2±3.2	11.8±2.1	16.8±2.7
Precrisis§	8.5±0.7	30.2±4.1	26.1±4.4	26.2±3.9
Crisis‖	9.3±1.1	12.4±3.8	4.4±1.2	11.2±2.5

* Percentage of inoculum taken up by liver 24 h after inoculation (Mean±SE).
‡ Mean±SE.
§ Period of rising parasitemia.
‖ Period of falling parasitemia.

deformability of infected erythrocytes suggested that the host cell membrane itself might be poorly deformable as a consequence of the infection (4). These observations suggest that the altered rheologic properties of the infected erythrocytes might be the major determinant of their removal by the spleen. The present study, designed to test this hypothesis, has revealed that malaria infection indeed has strikingly similar effects on the clearance of parasitized and uninfected damaged (rigid) erythrocytes.

The alterations in splenic clearance function during malaria infection that we observed can be understood in relation to the role of splenomegaly and splenic microcirculation in the trapping process. It has been well established that a variety of damaged erythrocytes are trapped more readily by an enlarged spleen (10) and this has been confirmed specifically in the case of *P. berghei*-infected erythrocytes (2). Since splenomegaly characteristically develops in most acute malaria infections and specifically during *P. berghei* malaria in the rat (Fig. 3), one would have anticipated that damaged erythrocytes would be cleared at a supernormal rate during the infection. The observation that splenic clearance actually decreased progressively during the period of rising parasitemia (precrisis) (Fig. 3) thus appeared paradoxical. One explanation for this was the observation that relative cordal microcirculation was significantly reduced during the precrisis period (Table II). Since trapping of the damaged erythrocytes occurs largely within the cords, decrease in circulation through the open pathways and increase in circulation in the closed pathways would be expected to decrease the efficiency of splenic trapping of these cells (6). Confirmation of this interpretation was provided by the observation that at the time of crisis, circulation in the open pathways was restored toward normal and efficient trapping of the damaged erythrocytes once again occurred. The fact that trapping of the cells was actually supernormal during the crisis period could be explained by the presence of splenomegaly during this period, in conjunction with restoration of normal microcirculation.

The mechanisms responsible for the observed decrease in splenic cordal blood flow in the precrisis period are presently uncertain. One possibility is that large numbers of parasitized erythrocytes become sequestered in the cords during the period of rising parasitemia and thereby retard microcirculation in this re-

TABLE II

Effect of Malaria on the Distribution of Plastic Microspheres in Rat Spleen

Stage of infection	Number of rats	Parasitemia	Percent microspheres*			Total microspheres counted
			Sinuses		Cords	
		%				
Noninfected	6	0	1.7±0.3	$P < 0.0001$‡	98.3±0.3	5,271
Precrisis§	6	24–50	38.2±2.6		61.8±2.6	3,446
Crisis‖	6	0–10	10.6±1.9	$P < 0.0001$	89.4±1.9	3,485

* Mean±SEM.
‡ Comparison of means by Student's *t* test.
§ Period of rising parasitemia.
‖ Period of falling parasitemia.

gion. Indeed, we could observe in histological sections large numbers of parasitized erythrocytes in the splenic cords of animals during the precrisis period (unpublished observations). However, it is difficult to imagine how obstruction based solely on parasite sequestration would be alleviated at the time of crisis. A more likely explanation is that cordal obstruction results from the marked cellular hyperplasia that occurs in this region during malaria. In preliminary studies (unpublished observations) we noted that during the precrisis period marked hyperplasia of erythroid precursors occured in the region of the cords, undoubtedly in response to the progressive anemia. At the time of crisis, large numbers of reticulocytes were released into circulation (11) and the splenic erythroid islands were noted to have decreased substantially in size. Thus, the observed alterations in splenic blood flow may have resulted from transient obstruction of the cords by erythroid precursors.

Our earlier observations, which are confirmed here (Fig. 3), that parasitized erythrocytes are cleared at a supernormal rate during crisis, when parasitemia began to decrease, suggested a potential causal relationship between increased clearance and resolution of infection. Certain considerations suggest that increased splenic trapping is probably a necesary but not a sufficient basis for resolution of the infection. Although parasites were trapped in the spleen very early in infection, parasitemia continues to rise (Fig. 3), indicating that trapping is insufficient to curtail the infection. Furthermore, although the degree of trapping of infected erythrocytes could be greatly augmented by inducing splenomegaly nonspecifically by methylcellulose treatment, the rise in parasitemia in the treated animals was indistinguishable from that in untreated controls (unpublished data). In addition, it seems unlikely that the spleen could rapidly remove the huge number of plasmodia replicating within erythrocytes present at the time of peak parasitemia. Therefore we propose that during the precrisis period an additional splenic defense mechanism is initiated which, in concert with trapping, results in resolution of infection during the crisis period. One additional mechanism that has been proposed is the elaboration by cordal macrophages of soluble substances, which might retard the intracellular development of the parasite (12).

The demonstration that clearance of damaged uninfected erythrocytes undergo similar alterations during acute malaria as do *P. berghei* infected erythrocytes, the evidence that these alterations are related to changes in microcirculation and the suggestion that these changes may be causally related to resolution of the infection have important implications to understanding host defense in malaria.

ACKNOWLEDGMENTS

We thank Doctors L. Miller, G. Keusch, S. Wolff, and J. Jandl for helpful suggestions in preparation of the manuscript and Ms. C. Heim for excellent secretarial assistance.

REFERENCES

1. Wyler, D. J., C. N. Oster, and T. C. Quinn. 1979. The role of the spleen in malaria infections. *In* The Role of the Spleen in the Immunology of Parasitic Diseases. Tropical Disease Research Series No. 1. Schwabe & Co., Basel, Switzerland. 183–204.
2. Quinn, T. C., and D. J. Wyler. 1979. Intravascular clearance of parasitized erythrocytes in rodent malaria. *J. Clin. Invest.* **63**: 1187–1194.
3. Quinn, T. C., and D. J. Wyler. 1979. Mechanisms of action of hyperimmune serum in mediating protective immunity to rodent malaria (*Plasmodium berghei*). *J. Immunol.* **123**: 2245–2249.
4. Miller, L. H., S. Usami, and S. Chien. 1971. Alteration in the rheologic properties of *Plasmodium knowlesi* infected red cells. A possible mechanism for capillary obstruction. *J. Clin. Invest.* **50**: 1451–1455.
5. Weiss, L. and M. Tavassoli. 1970. Anatomical hazards to the passage of erythrocytes through the spleen. *Semin. Hematol.* **7**: 372–380.
6. Chen, L. T., and L. Weiss. 1973. The role of the sinus wall in the passage of erythrocytes through the spleen. *Blood.* **41**: 529–537.
7. Chen, L. T. 1978. Microcirculation of the spleen: an open or closed circulation? *Science (Wash. D. C.).* **201**: 157–159.
8. Rudolph, A. M. and M. A. Heymann. 1967. The circulation of the fetus *in utero*: methods for studying distributions of blood flow, cardiac output and organ blood flow. *Circ. Res.* **21**: 163–184.
9. Schnitzer, B., T. M. Sodeman, M. L. Mead, and P. G. Contacos. 1973. An ultrastructual study of the red pulp of the spleen in malaria. *Blood.* **41**: 207–218.
10. Jacob, H. S. 1972. Hypersplenism. *In* Hematology. W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, editors. Mc-Graw Hill Book, Co., New York. 511–520.
11. Quinn, T. C., and D. J. Wyler, 1980. Resolution of acute malaria (*Plasmodium berghei* in the rat): reversibility and spleen dependence. *Am. J. Trop. Med. Hyg.* **29**: 1–4.
12. Allison, A. C., and I. A. Clark, 1977. Specific and nonspecific immunity to hemoprotozoa. *Am. J. Trop. Med. Hyg.* **26**: 216–222.