TRAFFICKING OF Plasmodium chabaudi adami-INFECTED ERYTHROCYTES WITHIN THE MOUSE SPLEEN

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ABSTRACT Plasmodium chabaudi adami causes a nonlethal infection in mice. We found that crisis, the time of rapidly dropping parasitemia, was abrogated by splenectomy, indicating the role of spleen in parasite killing. The factors that mediate spleen-dependent immunity are not known. An earlier study in Plasmodium berghei-infected rats showed an association between increased clearance of heat-treated erythrocytes and the onset of crisis [Wyler, D. J., Quinn, T. C. & Chen, L.-T. (1982) J. Clin. Invest. 67, 1400-1404]. To determine the potential effects of different vascular beds in parasite killing, we studied the distribution of parasitized erythrocytes and bacteria in the spleens of P. chabaudi adami-infected mice during precrisis (a period of rising parasitemia) and during crisis. After intravenous injection, bacteria were localized predominantly in the marginal zone. In contrast, parasitized erythrocytes were found in the red pulp. We also found that during precrisis, a time of no immunity, the uptake of radiolabeled infected erythrocytes by the spleen was increased, not decreased. These data imply that no change occurs in the flow of parasitized erythrocytes through the spleen during the transition to an immune state (crisis). Our observations suggest that immune effector mechanisms, not circulatory changes, account for spleen-dependent parasite killing during a P. chabaudi adami infection in mice.

Plasmodium, as a result of its location within erythrocytes, evades the host recognition system in nucleated cells that presents antigen through major histocompatibility complex class I and class II pathways. The host, on its side, has evolved a highly sophisticated system in the spleen to kill intraerythrocytic parasites. One feature of the spleen that differs from other organs and may be responsible for parasite killing is the direct contact of blood with immune effectors in the extrascular beds. Blood flows in direct contact with immune effectors in two regions of the spleen: the marginal zone and the reticular meshwork of the red pulp. The marginal zone—the region between the white pulp and the red pulp—contains a vascular sinus (1). Blood and microorganisms within the blood can pass through slits in the endothelium of the marginal zone sinus. Marginal zone macrophages (MZMs), located on the red pulp side of this sinus, ingest bacteria and other particulate matter (2). The second potential site of contact between infected erythrocytes and effector cells is the red pulp, where arterioles open directly into the reticular meshwork through which blood percolates on its way to the collecting veins.

An acute, nonlethal plasmodial infection has two phases: a period of rapidly rising parasitemia (precrisis) followed by a period of falling parasitemia (crisis). Several studies have demonstrated the role of the spleen in the resolution of both primary and reinfection immunity (3–5). In addition, splenectomy during crisis caused a rapid rise in parasitemia in Plasmodium berghei-infected rats (6), indicating the role of the spleen in the effector phase of parasite killing. In this model, the clearance of intravenously injected, heat-treated erythrocytes by the spleen was reduced during precrisis, and injected microspheres were found in increased numbers within the venous sinuses of the spleen (7). With the onset of crisis, the spleen again trapped heat-treated erythrocytes, and injected beads were found in increased numbers in the reticular meshwork in the red pulp. These studies, however, did not measure the clearance of infected erythrocytes.

Ultrastructural studies on Plasmodium yoelii in the mouse have shown the formation of a new splenic cell type, the barrier cell, during precrisis that may channel blood through the red pulp from the arteriole to the vein, producing a closed circulation (8, 9). The barrier cells disappeared during crisis. Based on these studies, it was proposed that the fall in parasitemia (crisis) is associated with a switch from a closed to an open circulation in the spleen.

In the present study, the distribution and uptake of infected erythrocytes and bacteria in the spleen were determined during both precrisis and crisis in order to delineate potential sites for effector mechanisms. We chose murine malaria, Plasmodium chabaudi adami, which causes a nonvirulent infection that is controlled by an antibody-independent, spleen-dependent mechanism (4). mAbs against different populations of mononuclear phagocytes were used to identify splenic compartments, and Salmonella typhimurium was used to assess the functionality of the marginal zone. In addition, we monitored the flow of parasitized erythrocytes through the spleen during the precrisis and crisis periods. We found that bacteria were taken up by MZMs during both precrisis and crisis; infected erythrocytes were detected only in the red pulp.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from the National Institutes of Health (NIH) and from Charles River Breeding Laboratories and were housed at the NIH animal facility according to ref. 10. Mice were 8–12 weeks old at the time of study.

Splenectomy. BALB/c mice infected with P. chabaudi adami were spleenectomized during the crisis period when their parasitemia had decreased to between 5 and 0.5%. Mice were anesthetized for splenectomy with methoxyflurane (Pittman-Moore, Washington Crossing, NJ). To determine the effect of surgery independent of splenectomy, infected mice underwent sham surgery; that is, the spleen was exposed but not removed.

Bacteria. An aroA mutant, ampicillin-resistant strain of S. typhimurium (SLA 33–2) was grown and processed as described (11).

Parasite. P. chabaudi adami was maintained in mice by weekly intraperitoneal injections. For each experiment, mice were injected intravenously with 107 parasitized erythrocytes...
from an infected donor mouse. Parasitemia was estimated by counting Giemsa-stained slides of thin blood smears made from a drop of blood from the tail.

**Parasite Purification.** *P. chabaudi adami* infection progresses synchronously in mice. We determined that, with our infection protocol, mature parasites (late trophozoites and early schizonts) appeared in the peripheral blood between noon and 4 p.m. (data not shown). Erythrocytes infected with late trophozoites and early schizonts were purified from uninfected erythrocytes according to the method of McNally et al. (ref. 12 and J. Dalton, personal communication). Briefly, 1 ml of packed erythrocytes, which had been passed over a Sucrosecl R-500 column (Baxter Health Care, Deerfield, IL) to remove leukocytes, was layered over 12.17 ml of a 74% Percoll (density, 1.13 g/liter; Pharmacia Biotech) cushion and centrifuged at 5000 × g for 20 min at room temperature. The Percoll cushion was prepared by mixing 9 ml of Percoll, 2.17 ml of solution A (22.5 g of glucose per 100 ml of 10× RPMI 1640 medium), and 1 ml of a 1:10 dilution of solution A. The band at the top of the cushion, containing ~90% parasitized erythrocytes, was removed and washed twice with 50 ml of RPMI 1640.

**PKH Labeling of Parasitized Erythrocytes and *S. typhimurium.** Percoll-purified erythrocytes were washed twice with RPMI 1640 and once with 5% D-glucose (in distilled water) to remove residual RPMI 1640 and were resuspended in 5% D-glucose. The parasitized erythrocytes, at a concentration of 10^9/ml, were added to an equal volume of 5% D-glucose containing 10 μM PKH26 (Sigma). The cell suspension was mixed gently for 1 min. An equal volume of heat-inactivated fetal bovine serum was added to remove unincorporated dye. The pellet was resuspended in 5% glucose and an equal volume of fetal bovine serum to remove any remaining unbound dye. The cells were washed twice with RPMI 1640. *Salmonella* cells were stained under similar labeling conditions using 10^9 bacteria/ml and 10 μM PKH26.

**Radiolabeling of Parasitized Erythrocytes and *S. typhimurium.** Parasitized erythrocytes were resuspended in RPMI 1640 and labeled with 40 μCi of Na_2^51CrO_4 (1 Ci = 37 GBq; specific activity, 48.6 mCi/ml; DuPont/NEN) per 10^9 cells for 20 min at room temperature with constant shaking. Cells were washed with RPMI 1640, and the radioactivity in 10^9 cells was determined in a LKB universal γ counter (Turku, Finland). *S. typhimurium* were labeled under similar conditions using ~12 μCi of Na_2^51CrO_4 per 10^9 bacteria.

**Measurement of ^51Cr-Labeled Parasitized Erythrocytes or *S. typhimurium in Blood and Spleen.** 10^8 ^51Cr-labeled parasitized erythrocytes or 10^9 ^51Cr-labeled *S. typhimurium in 200 μl were injected intravenously into the tail vein of mice; 100 μl of blood and the spleen were collected from each mouse 30 min after injection, and the radioactivity was measured. Spleens were weighed before counting. Radioactivity in the blood or spleen was calculated as a percent of inoculum (cpm in whole spleen or 100 μl of blood/cpm injected) × 100.

**Distribution of PKH26-Labeled Parasitized Erythrocytes or *S. typhimurium in the Spleen.** Spleens from uninfected normal, precrisis, or crisis mice injected with either PKH26-labeled parasitized erythrocytes or *S. typhimurium were embedded in OCT compound (Miles Diagnostic, Elkhart, IN) and cooled in isopentane over liquid nitrogen and stored at ~70 °C until use. Seven-μm-thick frozen sections were cut on a cryostat (International Equipment), air-dried for 1 hr, and fixed with acetone for 20 min at room temperature prior to staining. Fixed sections were washed in PBS and incubated with 1% bovine serum albumin in PBS to block nonspecific reactivity. Sections were incubated with rat mAbs (described in Results). An irrelevant rat mAb, GL113, directed against β-galactosidase of *Escherichia coli*, was used as a control. Fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Boehringer Mannheim) was used to detect the localization of primary mAb. After washing, sections were mounted in Fluoromount (Southern Biotechnology Associates, Birmingham, AL) and observed by fluorescence microscopy.

Cryosections of spleen were visualized by fluorescence microscopy as described (13). When both PKH26- and FITC-stains were used in the same section, PKH26 and FITC images of the same microscope field were captured and converted into 24-bit true color images. The green and blue channels of the PKH26 image and the red and blue channels of the FITC image were eliminated. The resulting red PKH26 image and green FITC image were merged in the computer, saved as a composite color image, and printed on Kodak Ektachrome 100 PLUS Professional film with a Lasergraphics LFR film recorder (Lasergraphics, Irvine, CA).

**RESULTS**

*P. chabaudi adami* causes a nonlethal, self-limiting infection in BALB/c mice. The parasitemia rose to a peak of approximately 15% on day 7. There were no detectable parasitized erythrocytes on day 15 (Fig. 1). The period of rising parasitemia is referred to as precrisis, and falling parasitemia is referred to as crisis. We present data comparing the uptake and distribution of injected parasitized erythrocytes and bacteria in the spleen during precrisis and crisis.

In an earlier study using the *P. chabaudi adami* model, splenectomy before infection has been shown to delay the resolution of primary infection (4). To specifically determine whether killing of parasites during crisis is spleen-dependent, we splenectomized *P. chabaudi adami*-infected mice on day 10 after infection, when parasitemia was rapidly falling. Following splenectomy, the parasitemia rose again and remained elevated until later in the infection; the parasites were eventually cleared (Fig. 1). The parasitemia in sham-operated mice rose for 1 day and then was cleared 2 days after the time of clearance in control mice. These data demonstrate that spleen-dependent events are required to control infection, even during crisis.

**Topographic Organization of Mononuclear Phagocytic Subsets During the Course of Infection.** Marginal sinus and red pulp reticular meshwork are points of entry of blood elements toward the white and red pulp extracellular matrices (Fig. 2). We used mAbs to different mononuclear phagocytic...
Blood flows from the central arteriole into the marginal sinus, marginal zone, or red pulp, where it comes into contact with macrophages and other immune effector cells before flowing into the veins [composite from figures by G. Kraal (14), L. Weiss (9), and from the data of Schmidt et al. (15)].

populations as markers to delineate these compartments. Since the spleen is enlarged during malaria, the number of any particular cell per unit area in a section may be influenced by spleen size. The average weight of 27 precrisis spleens was 0.28 g (range, 0.2–0.4 g), and that of 21 crisis spleens was 1.2 g (range, 0.8–1.5 g). This compares to the average of 32 age-matched normal spleens of 0.105 g (range, 0.07–0.2).

In steady-state conditions, the marginal zone contains two major mononuclear phagocyte populations: marginal metallophilic macrophages (MMMs) located between the white pulp and the marginal sinus and MZMs located between the marginal sinus and the red pulp. Using mAb SER-4, which identifies MMMs (16), we observed that, compared with normal spleens, the zone of MMMs appeared broadened in precrisis and crisis spleens (Fig. 3). mAb ER-TR-9 identifies MZMs (17). In our study, ER-TR-9 reacted with normal spleens but failed to react with the spleens from mice during precrisis and crisis and, as a result, could not be used to determine the distribution of bacteria and parasitized erythrocytes in the infected spleens. The failure of ER-TR-9 to react to MZMs is consistent with the observations of Stevenson and Kraal (18) who suggested that it was a consequence of loss of marginal zone during infection. We suggest that the lack of reactivity could be because the ER-TR-9 marker may have been down-regulated through P. chabaudi adami-induced cytokines (e.g., interferon γ) or due to the complete synchronous renewal of MZMs. However, M1/70 positive cells were not seen in the marginal zone area, indicating that this last speculation is unlikely. mAb M1/70, which recognizes complement receptor 3 (19), was used as a marker for the red pulp. Determinants recognized by M1/70 are widely distributed on polymorphonuclear leukocytes; monocytes in the red pulp; and CD5+, B220+, and IgM+ B cells (19). We found that the number of M1/70-reactive cells in the red pulp decreased from normal to precrisis and further decreased during crisis (data not shown). Although we did not quantify the number of cells in the spleen during malaria, the decreased staining appeared to mirror the increase in the spleen size. We observed the presence of FA/11-reactive cells, a mAb that recognizes macroscalin (20) in macrophages, in the outer marginal zone (data not shown). This, and the presence of bacteria in this zone (Fig. 3 and described below) confirms that there were, indeed, functioning MZMs in the outer marginal zone.

Clearance and Distribution of Salmonella. Bacteria were used to measure the phagocytic function of the marginal zone of the spleen during infection. Presumably, bacteria pass through openings in the endothelium of the marginal zone sinus and are trapped and ingested by MZMs. 51Cr-labeled S. typhimurium were intravenously injected to determine their clearance from blood and uptake by spleen 30 min after injection. Counts were determined in 100 μl of blood and calculated as the percentage of total counts injected. Percent of recovered counts in precrisis and crisis blood were similar to the counts in normal mice [normal, 0.54 ± 0.02; precrisis, 0.41 ± 0.08; crisis, 0.45 ± 0.03 (mean ± SEM)]. Tukey multiple comparison test (21) showed that none of the three comparisons for blood was significant. When percentage of these

Fig. 2. Schematic representation of the mouse spleen showing the marginal zone and red pulp areas. Blood flows from the central arteriole into the marginal sinus, marginal zone, or red pulp, where it comes into contact with macrophages and other immune effector cells before flowing into the veins [composite from figures by G. Kraal (14), L. Weiss (9), and from the data of Schmidt et al. (15)].

Marginal Zone Macrophages (●) Marginal Metallophilic Macrophages (●)

Marginal Zone Macrophages (●) Marginal Metallophilic Macrophages (●)

Normal

Normal

Pre-Crisis

Crisis

Salmonella

Salmonella

P. chabaudi

P. chabaudi

Fig. 3. Spleen sections of uninfected normal mice or P. chabaudi adami-infected mice during precrisis or crisis. Mice were injected with either PKH26 (red)-labeled Salmonella (upper) or P. chabaudi parasitized erythrocytes (lower). Sections were stained with antibodies against different macrophage populations and visualized with an FITC-labeled secondary antibody (green). An overlap of PKH-labeled cells with the FITC-stained cells is seen as yellow. Sections from normal spleens were stained with ER-TR-9, an antibody against the MZMs. Sections from normal and infected spleens stained with SER-4, a mAb against MMMs. Bar represents 100 μm.
Counts recovered in 100 μl was calculated for total blood volume of a normal mouse (approximately 2000 μl in a 25-g mouse), then around 10.8% of the injected counts would be present in the total blood volume. This indicates that the majority of the bacteria had been cleared from blood by 30 min after injection and that clearance of bacteria did not change during infection.

The percentage of injected counts (mean ± SEM) found in the spleen during precrisis (5.8% ± 0.47) increased over normal (3.57% ± 0.21); the crisis spleen had the highest uptake (8.43% ± 0.54). Each of the three comparisons (normal versus precrisis, normal versus crisis, and precrisis versus crisis) was significant at the P < 0.05 level, using Tukey multiple comparison test (21). It appeared that the function of the spleen in relation to the clearing of bacteria was not compromised during precrisis.

To study the distribution of bacteria in the spleen, the bacteria were surface-labeled with PKH26, a red fluorescent lipophilic dye that integrates into the membrane. The splenic compartments where PKH26-labeled bacteria localized was ascertained using the mAbs described above, and reactivity was visualized with a FITC-conjugated secondary antibody (green fluorescence). When the PKH26-labeled bacteria co-localized with the mAb-reactive cells and the two images were merged, a secondary yellow color occurred because of the overlapping of primary red and green colors. In the normal spleen, PKH26-labeled bacteria co-localized with ER-TR-9-labeled MZMs (Fig. 3). Most of the injected bacteria were found between the SER-4-positive MMMs and M1/70-positive macrophages that are found in the red pulp. Because of the lack of reactivity of ER-TR-9 during infection, we were unable to use this direct marker for the MZMs of infected spleens. As the clearance of 51Cr-labeled Salmonella through the spleen during precrisis was greater than in the normal spleen and the uptake of PKH26-labeled bacteria in the marginal zone appeared normal (Fig. 3), the function of the marginal zone—at least in its ability to clear bacteria—was normal throughout the acute malarial infection.

The Fate of P. chabaudi adami-Infected Erythrocytes. To determine the clearance of parasitized erythrocytes from blood and their uptake in the spleen, 51Cr-labeled parasitized erythrocytes were injected intravenously, and blood and spleen were collected 30 min later. Counts recovered from 100 μl of blood when converted to total blood volume approximated the total radioactivity injected. Counts in the blood in precrisis and crisis were not significantly different from the counts in normal animals; that is, about 100% of the counts were present in the blood.

Counts from parasitized erythrocytes in any organ are those distributed within the blood volume of that organ plus those retained or trapped during their transit through the organ. The blood volume of the normal spleen is around 180 μl/g of tissue (22); therefore, in a normal mouse whose spleen weighs approximately 0.1 g, the blood in the spleen would account for about 1% of the total blood volume. The spleen of normal mice had between 3.0 and 7.4% of the injected counts (Table 1), indicating some retardation of infected erythrocytes in the spleen. There was a statistically significant increase in the counts during precrisis (P = 0.02; Table 1). The crisis spleen had counts similar to those of the normal spleen (P > 0.20; Table 1). Thus, there was no evidence of decreased numbers of parasitized erythrocytes in the spleen during precrisis and no evidence of increased splenic uptake of parasitized erythrocytes during crisis.

The distribution of parasitized erythrocytes within the spleen was determined by observation of PKH26-labeled infected erythrocytes 30 min after injection. In contrast to bacteria—most of which were distributed within the marginal zone—the parasitized erythrocytes were found primarily within the red pulp in normal, precrisis, and crisis spleens (Fig. 3). The parasitized erythrocytes were mostly localized among M1/70-positive cells that are present in the red pulp but not found in the marginal zone (data not shown). A space separated the infected erythrocytes from the MMMs, indicating that they were not in the region of the MZMs. The PKH26-labeled infected erythrocytes did not superimpose on MZMs in the normal spleen (Fig. 3).

**DISCUSSION**

The acute course of *P. chabaudi adami* infection can be divided into two phases: a time of no or minimal immunity (rising parasitemia or precrisis) and a time of immunity (falling parasitemia or crisis). Our data show that splenectomy abrogates the immunity of crisis. The different immune mechanisms through which the spleen exerts its antiparasitic functions remain ill defined. It is likely to be related to the complex structure of the spleen as well as to the peculiarity of its vascular beds and blood flow. Two possible effector sites are the marginal zone and the red pulp, where macrophages and T cells can act as antiparasitic immune effectors. One approach to address the relative contribution of the effector sites was to monitor the early distribution of parasitized erythrocytes using phenotypic and functional markers of the outer marginal zone and red pulp. We show that parasitized erythrocytes are found in the red pulp. This is different from bacteria and other

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Table 1. Percent of injected 51Cr-labeled *P. chabaudi adami*-infected erythrocytes recovered in 100 μl of blood and whole spleen of normal mice and pre-crisis and crisis mice infected with *P. chabaudi adami*.
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foreign particulate matter, which are trapped in the marginal zone (ref. 2 and present study). Furthermore, infected erythrocytes in normal mice and in mice undergoing crisis are seldom seen in the marginal zone, where bacteria are cleared in both normal and Plasmodium-infected mice (Fig. 3). Therefore, the MZMs that are responsible for the clearance of bacteria such as S. typhimurium do not contribute to the control of the proliferation of Plasmodium through spleen-dependent mechanisms.

The second important observation was the absence of a difference between the numbers of infected erythrocytes in the red pulp between crisis spleens and normal spleens. Furthermore, the numbers of infected erythrocytes in the red pulp during precrisis was increased, not decreased. This conclusion derives from studies on the quantitative distribution in the spleen and the qualitative localization in the red pulp (Table 1, Fig. 3).

Quinn and Wyler (6), from their studies of P. berghei in the rat, showed that crisis was associated with a sudden rise in clearance of heat-treated erythrocytes and Heinz body-containing erythrocytes (7). They suggested that circulation through the spleen converts from an open circulation of the normal, uninfected animal to a closed circulation in precrisis. Crisis may be initiated by a reopening of the circulation in the red pulp. Weiss et al. (8), studying P. yoelli in the mouse, proposed an ultrastructural basis for this shift in circulation during precrisis. During this period, the reticular cells in the red pulp become highly activated, and their processes form a tightly knit meshwork that channels blood directly from the artery into the vein. Thus, a blood-spleen barrier is formed, causing limited access of blood into the filtration beds. These barrier cells disappeared during crisis, and normal blood circulation was restored.

Two differences exist between mouse spleen and rat or human spleen. The mouse spleen has no arteriovenous shunts, and the spleen is nonsinusoidal (24, 25). Do these differences invalidate the mouse model for the study of events in the spleen as they relate to human malaria? The spleen is critical in all malarials in all animals studied. Its function has rarely been studied precisely during crisis, but—in both P. chabaudi adami—mouse (Fig. 1) and P. berghei—rat models (7)—splenectomy during crisis abrogated immunity in that parasitemia again rose. The fact that the mouse can control the infection through splenic mechanisms is not surprising, since the survival of mice in nature requires the ability to handle intraerythrocytic infections such as Babesia spp., Plasmodium spp. and bacterial infections of the erythrocyte. Are there multiple spleen-dependent mechanisms of immunity again malaria that are redundant? Alternatively, both use cellular mechanisms in the red pulp that kill infected erythrocytes. The rat and human spleen may have an additional layer of complexity that involves arteriovenous shunting that affects exposure to these killing mechanisms.

We have shown that parasitized erythrocytes are not trapped in the red pulp extravascular spaces during crisis. In addition, the parasitized erythrocytes avoid a fully functional marginal zone. Our findings raise the possibility that some alteration in the spleen, other than dramatic changes in microcirculation, is critical for the killing of parasites. One such mechanism may be a change in effector cells such as macrophages in the red pulp during crisis.

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