Lymphology 16 (1983) 121–127

Splenic Functions in Malaria

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Summary

The spleen traps parasitized erythrocytes within its unique architecture, and this trapping function is modified during plasmodium infestation. Splenectomy dramatically exacerbates the course of experimental malarias probably through elimination of activated cordal macrophages responsible for killing intraerythrocytic plasmodia.

An essay on malaria in a monograph on the spleen might seem out of place, were it not for the fact that malaria is probably the infectious disease *par excellence* in which the spleen plays a critical (and perhaps also unique) role in host defense. Unfortunately, no careful documentation of malaria in groups of splenectomized patients has been published to define the clinical aspects, but tropical anecdotes attest to the increased risk of overwhelming malaria in asplenic individuals. In contrast, a substantial published experience with infections due to *Plasmodium* species in splenectomized experimental animals clearly indicates that no manipulation of the host is as consistently deleterious to antimalarial defense as splenectomy. Precisely how the spleen plays such an important function in this intraerythrocytic infection is uncertain, and efforts to enhance our understanding have been frustrated by limitations of technology for studying spleen physiology.

I will review here some findings from studies on the course of malaria in splenectomized animals and report results of recent investigations on the interaction of parasitized erythrocytes and the spleen. The concepts which emerge are that the spleen traps parasitized erythrocytes by virtue of its unique architecture, that this trapping function is modified during the course of infection, that the demise of intraerythrocytic plasmodia may depend on activated cordal macrophages, and that the spleen exerts a direct influence on parasite virulence by an as yet undefined mechanism. These conclusions regarding malaria reemphasize the multiplicity of splenic functions, the importance of the splenic microcirculation in some of these functions, and at the same time also introduce a new concept of the spleen as an organ modulating microbial virulence. With regard to splenic function, malaria must properly be viewed as both a hematologic disorder and an infectious disease.

Plasmodia invade and replicate within erythrocytes and when mature, rupture out of the host cell as the merozoite form which invades other erythrocytes. Depending upon the species, for each merozoite that enters, roughly 8–24 merozoites ultimately emerge from each red blood cell (RBC) following the intracellular process of development (schizogony). Schizogony may require 24–72 hours to be completed, the exact time depending on the parasite species. Morbidity and mortality in both human and experimental malarias are directly related to parasite density (percent infected RBC), so that an appropriate index of host defense is a restriction on the level of parasitemia attained or attrition of parasites as revealed by a declining parasitemia. Resistance to infection and host defense may be expressed at different times during malaria. Since *Plasmodia* species infect a relatively narrow range of hosts, some natural resistance is present at

the outset (1). This natural resistance depends in part on erythrocyte factors such as the presence of plasma membrane surface determinants including specific parasite receptors (2) and the age and hemoglobin composition (3) of the red cells. The spleen apparently also contributes to natural resistance, since splenectomy in absolutely or relatively resistant experimental hosts may render them fully susceptible to specific *Plasmodia* species. Several such examples exist for *Plasmodia* of fowl, rodents, and primates (4). Furthermore, where the course of malaria is highly virulent in the intact natural host (e.g. *P. falciparum* in man) but benign in an intact experimental host, splenectomy may convert the experimental malaria to a virulent form. These effects of splenectomy on virulence are largely restricted to the erythrocytic stage of infection since susceptibility to sporozoite-induced (i.e. mosquito-borne) infection is unaffected (5). How the spleen performs a sentinel role in natural resistance has not been elucidated but it seems likely that a filtering function (see below) is more important than protective "natural" antibody since natural resistance is not transferable with serum.

These experimental findings may have little relevance to clinical malaria inasmuch as there is no evidence that splenectomized humans are at an increased risk of acquiring infection with *Plasmodia* species of other primates. On the other hand, the early reported experiences with human infection with another intraerythrocytic protozoan parasite, *Babesia,* clearly indicate enhanced susceptibility of splenectomized patients (6). These individuals were infected with *Babesia* species which frequently infect cattle, virtually all patients had been splenectomized, and some experienced a fulminant course; in contrast, infection in intact individuals was rarely recognized. The more recent experience in the northeastern United States indicates that infection from another species, *B. microti,* which primarily infects deer and rodents, is by no means restricted to but may nonetheless be more severe in splenectomized individuals (7).

Once malaria is established in a susceptible intact host, the spleen may be essential for resolution of acute infection. A striking example of this phenomenon is *P. berghei* infection in the rat, its natural host. In this form of malaria, as in certain other rodent models, acute infection is characterized by a steady rise in parasitemia to a peak of about 40–60% infected RBC by day 14–18. Then, suddenly and dramatically over a 48–72 hours period, parasitemia drops precipitously to undetectable levels, and hosts become resistant to rechallenge. This sudden resolution, called "crisis" by malariologists, is also characterized by the presence of abnormally stunted intraerythrocytic parasites ("crisis forms"). Splenectomy at any time prior to crisis results in a fulminant course, and splenectomy during the crisis period reverses declining parasitemia (8). Indeed, within a few hours of splenectomy, crisis forms (which may have constituted as much as 60% of the infected RBC) can no longer be found in circulating blood. From these observations, Dr. *Thomas Quinn* and I concluded that in this malaria model, resolution of the acute infection was absolutely spleen-dependent and most likely involved a splenic mechanism of reversible inhibition of intracellular parasite development, giving rise to crisis forms. Because of the predictable and dramatic events in this model, we have employed it extensively in our efforts to elucidate mechanisms of splenic defense (see below).

One might reasonably ask at this point whether the spleen's important role in resolution of the acute infection depends upon the intact organ or arises from the performance of a specific subpopulation of splenic cells. We, therefore, resorted to mouse malaria models to determine whether reconstitution of malarial defense in splenectomized animals was possible by intraperitoneal transplantation of autologous spleen pieces or dissociated spleen cell suspensions (9). In addition, we used congenitally asplenic mice (Dh/+) and their intact littermates to determine whether during ontogeny in asplenic mice, putative "antimalarial cells" destined for the spleen might migrate to an alternative site and there be fully functional. Because we did not discern im-

portant and consistent differences between the course of malaria in congenitally asplenic mice and their splenectomized littermates, or infection in splenectomized and splenectomized-reconstituted animals, it is concluded that an intact splenic architecture not just immunocompetent cells was necessary.

Returning to the *P. berghei*-rat model, we next sought the mechanism whereby the spleen exerted such a dramatic role in resolving the acute infection. To do so, we labelled infected erythrocytes from donor rats with ^{51}Cr and analyzed red blood cell disappearance from the circulation of recipient rats with different malarial experiences (10). Following tail-vein injection of labeled cells, we sampled blood from the retroorbital plexus at frequent intervals and determined with a gamma counter the radioactivity present in a standard aliquot. In this way, we could calculate the percentage of the inoculum remaining in circulation at different times after infusion. We observed that in intact rats that had never experienced malaria, previously infected erythrocytes were cleared much more rapidly ($t_{1/2}$ = 9.3 ± 0.6 hours) than uninfected erythrocytes ($t_{1/2}$ = 16.1 ± 1.2 days). Attrition of infected cells occurred in a biphasic pattern. During the first hour, these cells were rapidly removed to the spleen (as determined by counting tissue samples of spleen in a gamma counter and comparing splenic radioactivity with that in liver, lung, kidney, gut, and bone marrow). Thereafter, a more gradual attrition took place, which we believe represented primarily erythrocyte rupture due to parasite maturation. The rapid clearance phase during the first hour was entirely abolished in splenectomized rats, confirming that it was indeed spleen-dependent.

When we performed these studies on rats that had recovered from malaria and were resistant to reinfection, we found that there was as much as a three-fold greater disappearance of labelled cells from the circulation during the first hour as compared to that seen in non-immune rats. On the other hand, as was true in non-immune rats, splenectomy in immune rats entirely abolished early parasite clearance. As we subsequently learned in our serum transfer studies (11), the enhanced clearance in immune rats was not antibody-dependent. Rather, we could relate it to the presence of splenomegaly in these animals, a condition that characteristically accompanies plasmodial infection. Indeed, we determined that the rate of clearance in the first hour correlated directly with spleen size. In fact, splenomegaly induced by methylcellulose injection into rats was just as effective in accelerating clearance of infected erythrocytes as was prior malaria (*Quinn, T.C.*, and *D.J. Wyler;* unpublished observations).

Putting together our observations, we postulated that clearance involved rheological interactions of the infected RBC with the spleen rather than opsonization (10). Since infected cells were less deformable than uninfected RBCs (12), they became trapped as they coursed through interendothelial slits between splenic cords and sinuses. Whereas deformable cells squeezed through these narrow spaces, relatively rigid cells were impeded and thus filtered out from the circulation. Electron micrographic studies of the spleen of infected animals provided non-quantitative support for this hypothesis (13). In addition, evidence that enlarged spleens more efficiently removed rigid erythrocytes (14) was compatible with our observations with infected RBCs in immune rats (see above).

To test the hypothesis further, we performed clearance studies in groups of rats at different stages of infection with *P. berghei* (15). Some had been infected on the previous day, others were experiencing a peak parasitemia just prior to the onset of crisis, and still others were undergoing crisis. We examined in different rats the attrition of not only infected RBCs but also of uninfected RBCs which were made less deformable by heating (50 °C for 20 min) or phenylhydrazine treatment (Heinz-body-containing). The following observations were made: 1) during the period of rising parasitemia, clearance of ^{51}Cr-tagged infected RBCs was markedly reduced and resembled that seen in uninfected splenectomized rats, 2) immediately with the onset of crisis, the clearance suddenly increased to a supernormal rate (i.e. much faster than in uninfected intact rats),

and 3) this pattern observed for infected cells was reproduced with heated or Heinz-body-containing uninfected erythrocytes! The supernormal clearance during crisis was accounted for by the presence of splenomegaly. Thus, we had strong circumstantial evidence (on the basis of analogy with rigid uninfected cells) that clearance of infected erythrocytes was most likely rheologically determined. Furthermore, the dramatic increase in clearance with the onset of resolution of the acute infection suggested that there might be a causal relationship between splenic trapping of infected cells and host defense.

We reasoned that if trapping depended upon the poorly deformable infected cells being caught during passage through interendothelial spaces between cords and sinuses, perhaps splenic microcirculation was altered during infection. That is, if infected cells were diverted primarily to a closed pathway of circulation, they would not be trapped in the cords and could re-enter the peripheral circulation. Accordingly, we joined forces with our anatomist collaborator, Dr. *Li-tsun Chen,* to study splenic microcirculation during malaria infection (15) using a technique he previously described (16). We injected carbonized plastic microspheres into uninfected rats and ones experiencing a rising parasitemia (pre-crisis) or undergoing resolution of acute *P. berghei* infection (crisis). These microspheres had a nominal diameter of 3–4 μm permitting them to enter the cords but not traverse the interendothelial slits, which are estimated to be approximately 0.5–2.5 μm wide (17). Since the distribution of such particles has been repeatedly shown in other systems to be a function of microcirculatory blood flow (18), it was possible to estimate the *relative* flow through the open and closed pathways by quantitating the location of the microspheres within the spleen (16). Within seconds after the particles were injected intravenously into anesthetized rats, blood flow was stopped instantaneously by guillotine transection at the level of the diaphragm. Histological sections of the spleen were then examined and microspheres enumerated in the cords and sinuses. As anticipated, whereas in uninfected rats splenic microcirculation was predominantly through the open pathway (98.3 ± 0.3%; mean ± SEM) and closed pathway circulation was minimal (1.7 ± 0.3%), in infected rats (pre-crisis) the open circulation was markedly reduced (61.8 ± 2.6%) and the closed pathway relatively increased (38.2 ± 2.6%). This finding supported the contention that decreased splenic clearance in infected (pre-crisis) rats was due to diversion of infected cells away from the cords where they could otherwise be trapped. With the onset of crisis, normal cordal blood flow was restored.

We concluded, therefore, that in this malaria model, rising parasitemia was associated with "functional asplenia" in the sense that infected erythrocytes were excluded from the cords and thus not trapped because they were unable to traverse the interendothelial slits between cords and sinuses. On the other hand, during this period the spleen enlarged so that when cordal flow was restored during crisis (by a totally obscure mechanism), trapping occurred at a supernormal rate thereby aiding resolution of the acute infection. There were only two problems with the theory. When we estimated the number of parasites that had to be removed at the time of peak parasitemia, we could not reconcile this huge amount with the clearance capacity of the spleen. The other issue was how to explain the curious "crisis forms", i.e. infected erythrocytes circulating at the time of crisis that contain "stunted" parasites. The following notion based on these and related studies was, therefore, postulated. During infection, several T cell-dependent (23) immune responses are initiated in the spleen, which result in macrophage accumulation (20) and activation with potential parasiticidal consequences. Since these activated macrophages are primarily located in the cords where close cell-cell interactions with infected RBCs are possible, reduction of cordal blood flow would prevent expression of these parasiticidal mechanisms. At crisis, when cordal flow is restored, not only are more of the infected cells trapped but they also make contact with activated macrophages. These macrophages

secrete soluble products, perhaps oxygen radicals or tumor necrosis factor (21), which in turn are toxic to the parasite (crisis form). Thus far this hypothesis has not been tested directly.

In addition to its role in resolution of acute malaria, the spleen also is important for resistance to reinfection in many immune hosts. Animals can acquire the ability to resist infection entirely (e.g. *P. berghei* in the rat) or to convert a potentially lethal infection into a brief benign course (e.g. *P. knowlesi* in the rhesus monkey) either by virtue of prior infection or vaccination. If these animals are splenectomized prior to challenge, however, all or a substantial portion of the acquired resistance may be lost (22). Since in intact humans and experimental animals, protection is transferable with immune globulin (23, 24) and specifically IgG (25) antibody and the spleen must act in concert in host defense, the most obvious candidate among potential mechanisms is opsonization of parasitized erythrocytes by antibody and subsequent immune clearance by the spleen. Despite successfully transferring malarial protection to naive rats with hyperimmune serum, however, we were unable to obtain evidence that *P. berghei*-infected erythrocytes were more readily cleared by the spleen in recipients of serum hyperimmune compared with normal rats (11). On the other hand, we have obtained evidence suggesting that hyperimmune serum transfer blocked the ability of merozoites to invade erythrocytes and also may have opsonized them for removal by the spleen (11). Moreover, preliminary studies of clearance of *P. knowlesi*-infected autologous RBCs in rhesus monkeys suggest that in contrast to *P. berghei*-infected erythrocytes in rats, these cells might indeed be cleared through opsonization (*Wyler* and *Quinn*, unpublished observations). A major determinant of whether the spleen can clear parasites by immune (antibody-mediated) mechanisms, therefore, probably lies in the expression of the appropriate antigens on the surface of the infected erythrocyte or merozoite. As a corollary, failure of antigen expression (sequestration) may be an important evasion mechanism for the parasite. Thus, in *P. berghei* in the rat perhaps only the extracellular form of the parasite (merozoite) expresses such antigens, whereas in *P. knowlesi*, merozoites *and* infected erythrocytes express surface antigens. If this is the case, then we could presume that the same types of factors that regulate clearance of erythrocytes in autoimmune hemolytic anemia (26) and of pneumococci (see Role of the Spleen in Pneumocemia), such as antibody isotype, density, and complement activation, are operative. Clearly, this consideration in malaria deserves close attention and investigation.

Some malarias are characterized by marked chronicity, such as *P. malariae* in man. In contrast to relapsing malarias (e.g. *P. vivax*), in which recurrence is due to emergence of latent exoerythrocytic forms from the liver, intraerythrocytic *P. malariae* can persist in the circulation for years (up to 53) (27) at subclinical and even undetectable levels. How this parasite evades host defense is a fascinating mystery. In studies conducted in collaboration with Drs. *Leon Schmidt* and *Louis Miller* (28), we obtained evidence that the chronicity of such malarias might also be spleen-dependent. We carefully analyzed the course of infection of *P. inui* in rhesus monkeys, a model closely resembling *P. malariae* in man. We found that infected monkeys with intact spleens developed recurrent waves of relatively low-grade parasitemia (recrudescences) for over 13 years! On the other hand, monkeys that were splenectomized two to three months following infection experienced an immediate rise in parasitemia to extremely high levels, which, however, they were able to resolve only to have a few more recurrent high peak parasitemias. Interestingly, these recrudescences invariably ceased within a year after splenectomy, and blood from these monkeys failed to induce infection in intact or splenectomized naive recipients, indicating that the donors had totally cleared their infection. Although splenectomy prior to infection was associated with high mortality, the few surviving monkeys progressed to self-cure in less than a year. Thus, sequestration of parasites in the spleen, thereby evading host defenses, is not an explanation for our observations. Although we pro-

posed that the spleen might be exerting both a protective and immunosuppressive role, recent findings (see below) raise the alternative possibility that the spleen actually can modify parasite virulence. Therefore, our original explanation (32) is probably wrong, and in *P. inui* at least, the spleen might be affecting a fundamental biological property of the parasite which permits its protracted survival.

Schmidt (as reported by *Garnham* (29)) observed several years ago that *P. cynomolgi* (a *P. vivax*-like monkey malaria) lost its virulence for intact rhesus monkeys if it was passed initially into splenectomized animals. More recently, *Barnwell* and his colleagus (30) found a similar effect with *P. knowlesi,* a species of primate malaria which causes lethal infections in non-immune intact rhesus monkeys. They investigated the course of *P. knowlesi* infection in rhesus monkeys as well as the expression of serologically-identifiable antigens on the surface of the circulating schizont-infected erythrocytes. These antigens (referred to as schizont-infected cell agglutination (SICA) antigens) are detected by the ability of antisera to agglutinate the infected cells in vitro. With each recurrent wave of parasitemia, new SICA antigens are expressed on the cell surface, to which the host then produces antibody, which in turn promotes the appearance of yet other serologically-distinct antigenic variants.

This phenomenon of antigenic variation is thought an important mechanism for parasite evasion of host defense (31). When *P. knowlesi* infected erythrocytes were passed in splenectomized rhesus monkeys, the SICA antigens no longer were expressed on the surface of the cells. Furthermore, when clones of these SICA-negative infected cells were then inoculated into naive intact monkeys, the resulting infected cells which appeared in the circulation also were SICA-antigen negative. Remarkably, the course of infection with these SICA-negative variants was benign (peak parasitemia of 1–6% and spontaneous resolution) compared to a uniformly lethal course generally experienced with *P. knowlesi.* These findings indicate that the spleen somhow alters the expression of parasite antigens on the surface of infected erythrocytes and that the expression of these antigens is correlated with virulence. Neither how the spleen exerts this influence nor the relationship of the SICA-antigens to virulence has yet been elucidated. The spleen nonetheless appears to exert a dual role in malaria by promoting parasite virulence while at the same time also serving as the major organ of defense.

This brief review of the spleen in malaria has highlighted the major points of knowledge regarding the critical role of this organ in one of mankind's most important infectious disease. We have considered the highly reproducible, dramatic, deleterious effects of splenectomy on the course of experimental malarias, examined some possible ways in which the spleen might exert a protective role in defense during acute infection and in resistance to reinfection, and explored recent evidence for an intriguing interplay between the spleen and parasite virulence. Considering that *Plasmodia* have likely been pathogens for a variety of terrestrial animals over a long period of evolution (32), it may not be too presumptious to suggest that the evolution of splenic function may have occurred under a selective pressure from malaria itself! Even if this notion is overly speculative, the study of malaria serves nonetheless to call attention to the remarkable variety of host-parasite interactions which occur in the spleen and therein holds potential fruit for the labors of biologists, physiologists, hematologists and immunologists alike.

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