Chronic, Patent *Plasmodium berghei* Malaria in Splenectomized Mice

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It was shown that splenectomized mice could develop a certain resistance to *Plasmodium berghei* but usually no real immunity, since the infection became chronic, often with high parasitemias. A patent infection lasting at least 2 weeks was necessary for the development of this degree of protection. Prolonged suppression to subpatent levels (sulfonamide treatment), rather than radical cure (chloroquine), after 2 weeks of patency yielded a higher proportion of mice resistant to superinfection. An increasing proportion of B10LP, but not C57BL/Rij or BALB/c, mice cleared their chronic infection spontaneously in time. Chronic patent infections were accompanied by anemia, elevated serum glutamate oxaloacetate transaminase levels, indicating liver pathology, and decreased immune reactivity, but the magnitude to these pathological changes was limited compared with changes in primary, lethal infections in intact controls. Parasitemia and pathology did not always develop synchronously.

The spleen is an important organ for defense against blood-borne pathogens. In many malaria models splenectomy has an adverse effect on the host's defense against the infection: hosts with innate resistance become susceptible, or transient infections become lethal, and immunity either may not be expressed or may be lost (16), although at least temporary beneficial effects were also observed (6, 11). The spleen is considered essential for resolution of an acute infection or at least for the induction phase of immunity (2, 12, 16) to both the avirulent and the virulent murine malaria parasites. Avirulent parasites produced lethal, primary infections in spleenless animals, whereas vaccinated (2), or congenitally asplenic, mice as well as splenectomized mice (12) showed normal resistance when rechallenged after a drug-cured infection.

There is a basic contradiction in these findings: on the one hand, the spleen appears to be essential for induction but not for maintenance of immunity; on the other hand, it was found that splenectomized as well as congenitally asplenic mice could develop normal resistance. To further explore the role of the spleen in virulent malaria, immune induction was attempted in splenectomized mice.

MATERIALS AND METHODS

Parasite strain. The parasite *Plasmodium berghei*, strain K173, was maintained by intraperitoneal subinoculation of 10^5 parasitized erythrocytes (PE) obtained from infected donor mice into clean mice of the corresponding strain and sex at weekly intervals.

Parasitemia was determined from thin smears made from tail blood, and smears were stained with May-Grünwald-Giemsa solutions. In these studies mice were considered negative when no parasites were observed in at least six microscope fields of a thin smear.

Mice. Mice used for passage of the parasite and experiments were 6 to 8 weeks old and were kept in plastic cages on standard food (RMH Hope Farms) and water ad libitum. Outbred Swiss mice and inbred C3H/StZ, BALB/c, and C57BL/Rij mice were obtained from colonies of the animal facilities of the University of Nijmegen. Inbred B10LP mice were obtained from TNO, Zeist, The Netherlands.

Chemotherapy. Chemotherapy was administered by addition of sulfonamide (30 mg of sulfadiazine per liter) to the drinking water. This treatment suppressed patent infections quickly to subpatent levels, but did not lead to radical cure in most of the animals in a short time (7). For radical cure, chloroquine treatment was given, consisting of five intraperitoneal injections of 0.8 mg of chloroquine free base per mouse on consecutive days, supplemented with 100 mg of chloroquine per liter of drinking water for 1 week.

Splenectomy. Splenectomy was performed 2 weeks before infection. After a lateral incision the splenic pedicles were ligated, the spleen was extirpated, the body wall was sutured, and the skin was closed with wound clamps. The whole procedure was carried out under ether anesthesia.

Serological test. Serum glutamate oxaloacetate transaminase (SGOT) activity was measured as a marker for liver pathology. Approximately 10 μl of blood was withdrawn each time from the retro-orbital plexus under ether anesthesia. The SGOT level was measured in 50 μl of plasma, using a commercially available UV test (Biochemica Tests, Boehringer,

Mannheim Corp., Mannheim, West Germany) adapted to this volume.

Hematocrit values. Hematocrit values were determined by standard procedures, using tail blood.

Hemagglutinating antibody responsiveness. The hemagglutinating antibody responsiveness to rabbit erythrocytes was determined in intact controls, uninfected and infected splenectomized mice, and chronically infected mice. Freshly drawn leukocyte-free rabbit erythrocytes were used as antigen. Each mouse received 0.2 ml of a 10% suspension of rabbit erythrocytes intraperitoneally, and serum was collected for analysis 7 days later. Antibody was determined by routine procedures (10), using 2-mercaptoethanol for differentiation of immunoglobulin M (IgM) and IgG antibodies. Antibody titer was expressed as the final dilution step of serial twofold dilutions giving agglutination. The geometric mean value of results from groups of five mice was determined.

RESULTS

Immunization of splenectomized mice. The standard immunization procedure developed in our laboratory has been described previously (4). Briefly, it consists of chemotherapeutically suppressing the development of a primary infection (10^7 PE intraperitoneally) by administration of sulfadiazine (30 mg of sulfadiazine per liter of drinking water) 2 days postinfection and continued for 31 days, followed by a challenge (10^5 PE) 2 days later. When applied to groups of 20 splenectomized C3H/StZ and C57BL/Rij mice, this regimen did not yield a single immune mouse either 14 days or 2 months after splenectomy. Only 2 of 40 splenectomized Swiss mice developed immunity.

Intact mice develop solid immunity if the patent primary infection is cured chemothera-

peutically (7). This method was also applied to splenectomized mice. Groups of 20 mice were splenectomized and infected (10^5 PE) 14 days later. In separate groups parasitemia was suppressed and controlled by chemotherapy (30 mg of sulfadiazine per liter of drinking water for 26 days), starting after increasing periods of patent infection. All mice were challenged (10^5 PE) 2 days after termination of chemotherapy. The results of experiments with splenectomized Swiss, B10LP, C57BL/Rij, and BALB/c mice are given in Fig. 1.

When the period of patent infection was increased to 14 days or more (before the sulfonamide was added to the drinking water), the proportion of mice surviving challenge infection later increased considerably, although results varied with the mouse strain used. Since the mean survival time of splenectomized Swiss and C3H/StZ mice is <14 days (6), the survival of mice of these strains was low by this method. None of the mice developed solid immunity; all became patent after challenge. Since the proportion of surviving mice decreased with time (Fig. 3), the percentages given in Fig. 1 were arbitrarily determined 4 weeks after challenge, when a more or less stable level was observed among B10LP mice.

Type of chemotherapy. To determine the effect of the presence of living parasites during the recovery period after clinical drug cure, several chemotherapeutic procedures were tested. Groups of 20 mice were splenectomized and infected (10^5 PE) 14 days later. Chemotherapy was started either 14 or 21 days after infection and continued for 33 days. Chemotherapy was either sulfadiazine treatment (30 mg/liter of
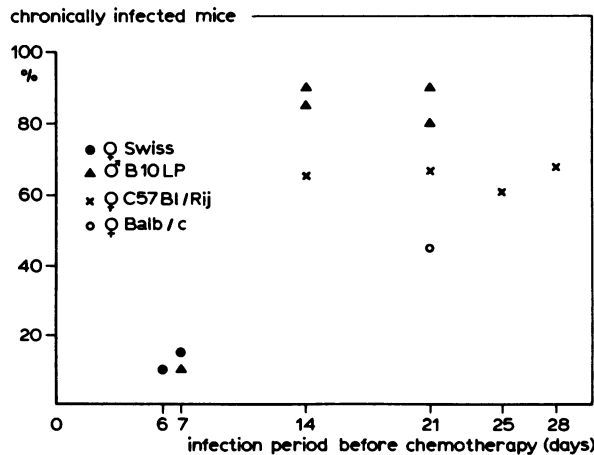

FIG. 1. Effect of infection period on development of chronic infections in splenectomized mice of different strains. Splenectomized mice were infected, and after increasing periods separate groups were cured and kept under chemotherapy for 26 days, followed by a challenge 2 days later. Data indicate the proportion of mice surviving challenge, though exhibiting chronic, patent infection.

drinking water for 33 days) or radical cure by chloroquine treatment (see Materials and Methods). Mice were challenged (10^5 PE) 35 days after chemotherapy began (Table 1).

A high proportion of chronically infected survivors was obtained when sulfadiazine therapy was started 14 or 21 days after infection. After chloroquine treatment the proportion of survivors was lower, particularly in B10LP mice, but it increased with increasing period of patent infection before therapy.

The sulfonamide regimens used in these experiments are known to suppress an infection to subclinical levels (7), whereas the chloroquine treatment effects radical cure in intact animals (unpublished data). These results suggest that the persistence of living parasites plays an important role in the development of protection against lethal disease in splenectomized mice.

Therapy regimen. To determine how long antigenic stimulation by subpatent numbers of parasites is necessary for the development of optimal protection, six groups of 20 male B10LP mice were splenectomized and infected (10^5 PE) 2 weeks later, and surviving mice were clinically cured by sulfadiazine treatment 3 weeks after infection. Patent infection was suppressed after 3 days, but spontaneous recrudescence was prevented by continuing suppressive therapy. Therapy was stopped 12 ($n = 19$ and 20), 19 ($n = 19$), 26 ($n = 20$), and 33 ($n = 15$ and 20) days later, followed by challenge (10^5 PE) 2 days thereafter (Fig. 2). Increasing subpatent periods yielded increasing numbers of surviving mice.

Characteristics of a chronic infection. Parasitemias were repeatedly determined in chronically infected mice. Groups of male C57BL/Rij ($n = 40$), female BALB/c ($n = 20$), and male B10LP
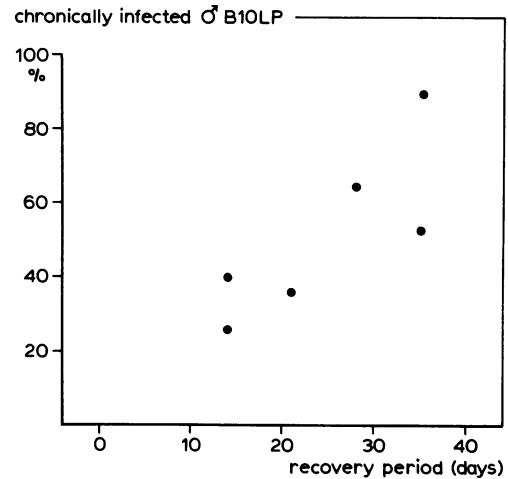

FIG. 2. Effect of therapy regimen on development of chronic infection in splenectomized mice. Splenectomized mice were infected, and chemotherapy was administered 3 weeks later. In separate groups, chemotherapy was stopped after increasing periods and followed by challenge. Data indicate the proportion of mice surviving challenge, though exhibiting chronic, patent infection.

($n = 40$) mice were splenectomized and infected (10^5 PE) 14 days later. The day of infection is day 0 of the experiment (Fig. 3). Sulfadiazine treatment (30 mg/liter of drinking water) was started 21 days after infection and continued for a period of 33 days, followed by a challenge (10^5 PE) 2 days thereafter. Mortality was registered daily and expressed as cumulative mortality (percentage) of the number of challenged mice (C57BL/Rij, $n = 33$; BALB/c, $n = 20$; B10LP, $n = 38$). Parasitemia was determined and expressed as the geometric mean value of all positive mice ($P > 1\%$). The proportion of blood-negative mice was expressed in relation to the number of challenged mice (see above) (Fig. 3).

All mice developed patent infection with high parasitemias usually for long periods, many of them throughout life. The course of a chronic infection varied in different strains. Mortality was low in chronic B10LP mice compared with BALB/c and C57BL/Rij mice. A comparatively high number of B10LP mice (35%) were able to clear their chronic infections spontaneously in time (Fig. 3). In separate experiments (not shown) such mice were reinfected (10^5 PE) and appeared to clear the challenge infection in a much shorter time (2 to 3 weeks) than at the beginning of the experiment. A considerable proportion (45%) of B10LP mice remained chronic throughout the experiment, and 20% died (Fig. 3). Chronic BALB/c mice were also

TABLE 1. Effect of drug used for chemotherapy on immunization of splenectomized infected mice[a]

Mouse strain	Chemotherapy		Chronic %
	Drug	Started on day:	
Male B10LP	Sulfonamide	14	90
Male B10LP	Sulfonamide	21	80
Female C57BL/Rij	Sulfonamide	21	85
Male B10LP	Chloroquine	14	5
Male B10LP	Chloroquine	21	30
Male B10LP	Chloroquine	21	35
Female C57BL/Rij	Chloroquine	21	65

[a] Splenectomized mice were infected, and separate groups were cured by chemotherapy after a period of 14 or 21 days. Mice were challenged 35 days after chemotherapy began, and the proportion of surviving, though chronic, patent mice was determined.
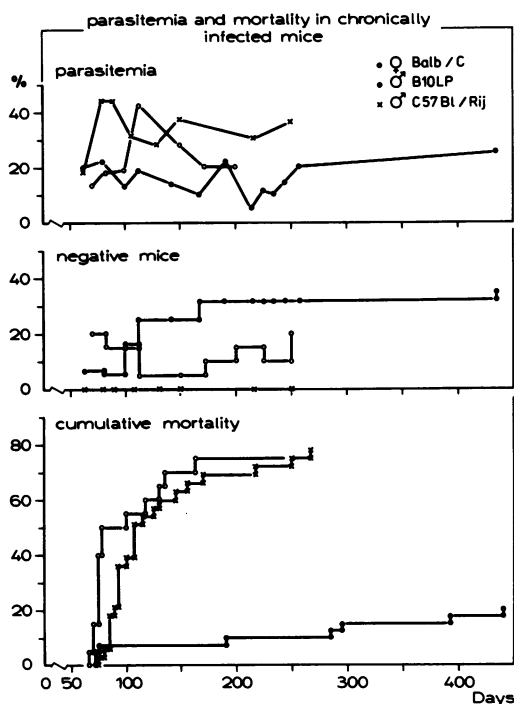
FIG. 3. Course of infection in splenectomized mice with chronic infection. Data indicate mean parasitemia in patent mice, proportion of mice that spontaneously resolved their infection, and cumulative mortality in groups of chronic BALB/c, B10LP, and C57BL/Rij mice.

able to suppress a patent infection to subpatent levels, but, in contrast to B10LP mice, which remained blood negative, they suffered frequent spontaneous recrudescences. None of the C57BL/Rij mice was able to suppress the infection to subpatent levels. Parasitemias dropped to <10% occasionally. Parasitemias in mice of all strains fluctuated considerably, often exceeding 50%, which, however, did not kill them.

Parasites and serum from chronic mice. Experiments were carried out to determine whether blood from chronic blood mice contained specific factors related to chronicity. The course of infection in normal intact mice was the same, whether the infectious inoculum was prepared from infected blood of a chronic or an intact animal with a primary lethal infection.

Large numbers of parasites (10^9 per animal) collected from chronic mice could not break an established immunity in intact mice, not even when given four times on consecutive days. Parasites were rapidly cleared to subpatent levels. The same numbers of parasites collected from intact infected donors did not kill or markedly affect parasitemias when injected into chronic mice.

Sera of chronic mice did not change the course of infection when injected (0.5 ml per mouse intraperitoneally) together with an infectious inoculum (10^5 PE per mouse) into intact control mice.

Anemia and SGOT levels in chronic mice. Anemia and liver pathology, as indicated by SGOT activity, are severe in the course of a primary infection (6). In chronic mice morbidity is apparently reduced, since mice survive despite parasitemias as high or higher than those observed in lethal primary infections in intact controls. Parasitemia, hematocrit, and SGOT activity were determined in 34 chronic mice (16 male B10LP, 10 male C57BL/Rij, and 8 female C57BL/Rij mice) longitudinally. All mice had an established chronic infection for various periods of time when the experiments were started, and the moment of the first sampling was designated day 0. To prevent interference due to sampling, not all determinations were performed on each animal.

Representative examples of the relation between parasitemia and hematocrit are given in Fig. 4. Strongly fluctuating parasitemias and even temporary subpatency were observed. In some cases changes in hematocrit followed changes in parasitemia, but a direct correlation was repeatedly absent. Some mice died during the course of the experiment, which may have been caused by problems related to repeated sampling (e.g., stress, superinfection).

Comparable results were obtained for the absence of correlation between parasitemia and SGOT activity (Fig. 5). The SGOT activities accompanying chronic infections in C57BL/Rij mice were usually higher (roughly twofold) than those in B10LP mice.

Immune responsiveness in splenectomized, infected, and chronic mice. The effect of a chronic infection on the ability to mount an immune response against heterologous (rabbit) erythrocytes was determined in chronically infected C57BL/Rij and B10LP mice and compared with responses of intact controls, splenectomized noninfected animals, and splenectomized mice on several days of a primary infection (10^5 PE) (Table 2). The antibody response was markedly suppressed in splenectomized mice, and a further reduction was observed in the presence of malaria. It should be noted that the primary antibody response in splenectomized, primary infected, or chronic mice was not completely suppressed.

Early after infection of splenectomized mice (5 days postinfection) an enhanced antibody response was found in both strains. The IgG antibody to rabbit erythrocytes was completely suppressed by splenectomy in nonmalarious C57BL/Rij mice, whereas IgG production in the
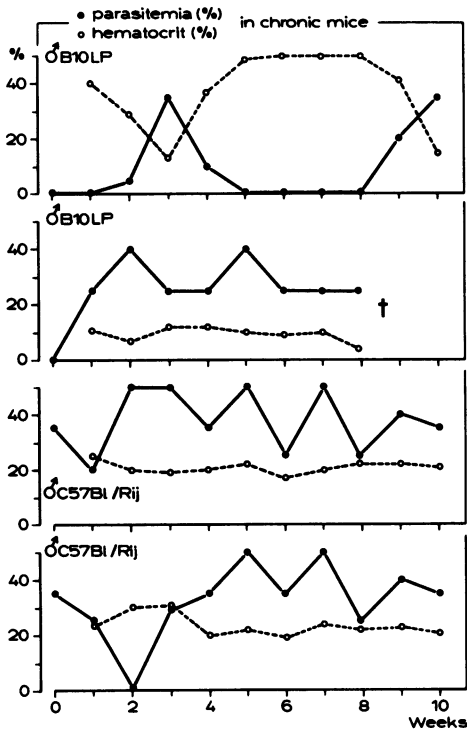
FIG. 4. Relation between parasitemia and anemia, as determined by changes in hematocrit values in splenectomized mice with chronic infection. Data represent longitudinal observations in individual B10LP and C57BL/Rij mice.

course of malaria infection was prevented in splenectomized and absent in chronic B10LP mice.

Chronic infections apparently suppressed heterologous immune reactivity. They prevented IgG antibody production, but not IgM responses.

DISCUSSION

Usually, the spleen is considered to play an essential role in development of immunity (16). The experiments described in this paper show that splenectomized mice cannot be immunized by a chemotherapeutically subpatent infection, a method successful in intact mice (7). This result supports the importance of the spleen as an essential site for immune induction against parasites, as observed by others (2, 12). However, splenectomized mice were able to develop a certain degree of protection after a patent infection which was terminated by chemotherapy (Fig. 1 and 2; Table 1). Although all mice treated in this way developed chronic infection after reinoculation, some were eventually able to effect a spontaneous cure. Parasitemias were fluc-

tuating, sometimes reaching levels as high or higher than those seen during (lethal) primary infection. The mice were protected against superinfection.

It was shown that prolonged stimulation with large quantities of antigen is required to reach this degree of protection. Patent, untreated infection lasting 2 weeks or longer (Fig. 1) followed by subpatent persistence of living parasites under chemotherapy for 4 to 5 weeks (Fig. 2) was required for optimal results. More mice were protected when patent infection was suppressed by sulfadiazine treatment, allowing persistence of parasites, rather than chloroquine, which radically cured. Persistent parasites might function as an additional antigenic signal during recovery, which is in line with the importance of living parasites observed during immune induction in intact animals (7).

The time span for induction of protection in splenectomized mice is comparable to the immunization period needed in intact mice (7). In humans, too, considerable time elapses between the first contact with antigen and established antimalarial immunity. Although the recovery period in infected splenectomized mice included
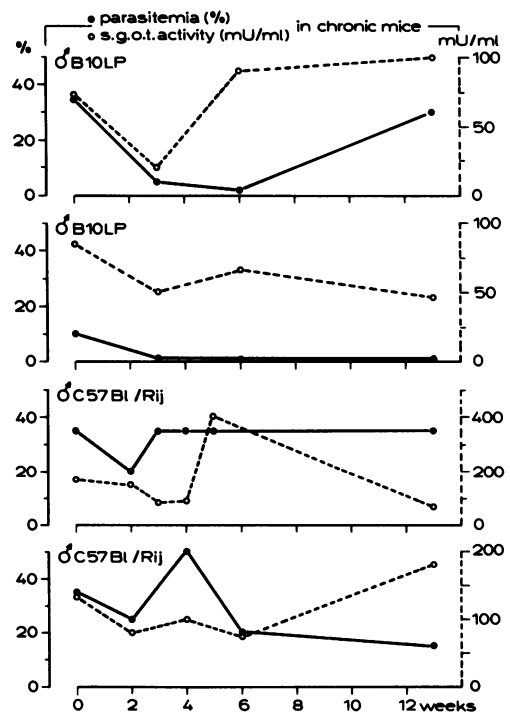

FIG. 5. Relation between parasitemia and liver pathology, as detected by elevation of SGOT activity, in splenectomized mice with chronic infection. Data represent longitudinal observations in individual B10LP and C57BL/Rij mice.

TABLE 2. Agglutinating antibody response in splenectomized, infected, and chronic mice[a]

Mice	Antibody response (titer)[b]			
	Female C57BL/Rij		Male B10LP	
	IgG + IgM	IgG	IgG + IgM	IgG
Intact controls	6.2 ± 0.3	4.2 ± 0.8	4.8 ± 1.4	3.4 ± 2.0
Splenectomized	2.8 ± 0.4	0	1.8 ± 0.6	0.4 ± 0.5
Splenectomized plus infected (days p.i.)[c]				
5	3.2 ± 0.4	0	3.4 ± 0.6	1.0 ± 0.7
7	2.4 ± 0.5	0		
9	2.6 ± 0.5	0	2.4 ± 0.5	0
12	2.0 ± 0.7	0	1.4 ± 0.7	0
Chronic	2.6 ± 0.5	0	1.8 ± 0.6	0

[a] Total (IgG + IgM) and mercaptoethanol-insensitive (IgG) antibody response against rabbit erythrocytes was determined in groups of five mice. The geometric mean value of each group was used to determine the fractional response in splenectomized, splenectomized and infected, and chronic mice compared with controls.

[b] Expressed as the final dilution step (mean ± standard deviation) exhibiting agglutination.

[c] p.i., Postinfection.

the recovery period of the immune system, this may have been compensated for by information already processed during acute infection.

Chronic infection, with clinical symptoms, in splenectomized mice is different from that in intact animals (14) and persistence of parasites in immune mice, which are usually not accompanied by clinical disease. The protection achieved is of a different quality: the splenectomized mice are able to live with sometimes high parasitemias, but are capable of eliminating large numbers of superinoculated parasites. Whereas liver pathology, anemia, and reduced immune responsiveness vis-à-vis a heterologous antigen are present, their extent is not always correlated to the parasitemias, and they are less severe than in primary infection. Chronic, patent infection with clinical symptoms over a long time (Fig. 3 to 5) implies a dissociation between parasite proliferation and lethal pathological effects which is never observed in primary infections, after immunization (7), in relation to fading of immunity (3), or in immune mice after splenectomy. In chronic patent infections the lethal factors either are not produced or are neutralized in some manner, although the lack of any effect of serum from chronic mice on a primary infection might argue against the latter.

Spontaneous cure (B10LP, BALB/c), absence of recrudescence (B10LP; Fig. 3), and rapid clearance of challenge infections in mice that spontaneously cured their chronic infection suggest that improved immune reactions could eventually develop. Unlike some B10LP and BALB/c mice, splenectomized C57BL/Rij mice with a chronic patent infection were unable to suppress their infection spontaneously. Previous experiments showed that intact C57BL/Rij mice could not be immunized by either a controlled subpatent infection or a chemotherapeutically suppressed patent infection (5). These results suggest a defect in the effector part of the immune system of C57BL/Rij mice.

Splenectomy alone reduced, but did not block completely, the immune reactivity (Table 2). Some genetic differences were observed in this respect. Production of immunoglobulin was blocked completely in uninfected splenectomized C57BL/Rij mice, but not in other strains. Thus, either some immune reactions can occur not only in the spleen, but also in other organs (e.g., lymph nodes), or compensatory immune reactions may be established to some extent in other organs after splenectomy. Immunodepression with regard to heterologous antigens was also less severe during infection. Whereas the immune response to rabbit erythrocytes was completely absent in intact mice 7 days postinfection (13), splenectomized mice still exhibited 30% of the normal response 12 days after infection (Table 2). The induction of the IgG response, however, appears to be blocked in the course of infection, and this remains so after the mice become chronic (Table 2). Better immune reactivity in splenectomized, infected mice, in comparison to intact infected mice, supports previous work (6) that had indicated that splenectomy eliminates certain (immuno-)pathological responses. As a consequence, morbidity was reduced during infection and mortality was delayed (6), although strain idiosyncrasies were observed. More protected mice were obtained after longer patent infection before suppression by chemotherapy (Fig. 1), which may indicate that elimination of (immuno-)pathological responses by splenectomy allowed triggering of

immune responses that gave rise to the development of protective reactions during the subsequent recovery period, protecting against lethal disease during chronic patency after reinfection. Similar observations were made in intact infected animals (7). It is possible that certain immune reactive sites not normally exposed to the antigen (e.g., lymph nodes) eventually become available during prolonged patent infection (15). A considerable increase in size and accumulation of pigment in mesenteral and paratracheal lymph nodes in chronic animals (unpublished data) supports this view.

Fluctuating parasitemia in chronic mice might be caused by antigenic variation (1), but in the absence of IgG the variant specific antibody would have to be IgM. Even large amounts of serum from chronic mice did not affect primary infections, although this does not necessarily exclude the presence of variant specific antibodies. The course of infection and the survival period of mice infected with parasitized erythrocytes from either chronic patent or preimmune mice were the same as in mice infected with an inoculum from a primary infection. Moreover, parasites from chronic mice were not able to evade the immune system of intact immune animals. Conversely, superinfection of chronic mice with parasites from a primarily infected intact mouse did not affect morbidity, mortality, or parasitemia significantly. These results suggest that chronicity depends predominantly on qualities of the host rather than of the parasite.

Our results are somewhat comparable to those of Grun and Weidanz (9). They demonstrated that B-cell-deficient mice developed long-lasting, low-grade infections after challenge in the absence of antibody production. The similarity to splenectomized chronic mice that do not produce IgG (Table 2) suggests that blocking of parasite proliferation, but not protection against lethal complications, depends on IgG production.

Chronic patency was not observed in mice splenectomized and challenged after immunization. This suggests that the spleen is an important organ for the induction of immune reactions operating in the effector part of immunity. Once developed, immunity appears eventually to become independent of the spleen, at least in some of the mice (2, 5, 6, 12).

In summary, in splenectomized mice the development of protection against the lethal effects of a malaria infection and the eventual development of protection, and even immunity, require prolonged exposure to large numbers of parasites and possibly immune induction at other sites.

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