

***Plasmodium berghei*: suppression of antibody response to sporozoite stage by acute blood stage infection**

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SUMMARY

Mice infected with the erythrocytic stages of *Plasmodium berghei* show an impaired host response to immunization with irradiated sporozoites of the same malarial parasite. The stage-specific anti-sporozoite response was measured by indirect immunofluorescence upon adsorption of the sera with parasitized red blood cells. *P. berghei*-infected mice, immunized with irradiated sporozoites on the fourth day of a blood-induced malaria infection, developed a normal anti-sporozoite antibody response. However, this antibody response was more short-lived compared with the antibody response in normal mice immunized with a similar dose of irradiated sporozoites. The immune response was severely depressed when the animals were immunized on day 7 or later after malaria infection. None of the sporozoite-immunized animals, including those which responded to the first immunization, developed a secondary antibody response on reinoculation with irradiated sporozoites. A fully established anti-sporozoite immune response, obtained after multiple immunizations with irradiated sporozoites and which resulted in stage-specific protection of the animals, was not affected by a superimposed blood stage malaria infection. The titres of the anti-sporozoite antibodies in these animals were unaltered, in spite of their high parasitaemias. Reduction of the malaria parasitaemia by chloroquine treatment abolished the immunosuppressive effects of the disease.

These observations are discussed in relation to anti-sporozoite immunity and immunosuppression in man in malaria endemic areas.

INTRODUCTION

McGregor & Barr (1962) first reported that African children infected with malaria respond poorly to inoculation with tetanus toxoid. Since then, many other studies have shown that plasmodial infections, in man and experimental animals, suppress the immune response to a variety of antigens (reviewed by Wedderburn, 1974; Greenwood, 1974; McBride, Micklem & Ure, 1977; Williamson & Greenwood, 1978). However, it is believed that malaria-induced immunosuppression is a selective rather than a generalized process (Greenwood, Playfair & Torrigiani, 1971; Barker, 1971).

The basic question of whether malaria infection impairs only the immune responses to foreign, unrelated antigens, or whether it also affects host resistance to certain invasive stages of plasmodia, and modulates the course of this disease, has not yet been answered. It would appear that malaria does not depress the immune response to the erythrocytic stages of plasmodia significantly, since antibodies to these stages are produced during the infection. However, plasmodia have very complex life cycles and each stage has antigenic characteristics of its own (Cohen, McGregor & Carrington, 1961; Vanderberg

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et al., 1972; Vanderberg, 1973; Danforth, Orjih & Nussenzweig, 1978). Therefore, the immune response to each stage may be affected differently during the course of infection.

Immunological parameters to measure the host response to plasmodia, and to evaluate the possible effects of immunosuppression, are only available for certain stages of the parasite. Among these, the response to sporozoites has been well characterized. Irradiated sporozoites induce stage-specific immune responses in both man and experimental animals (reviewed by Nussenzweig, 1977; Nardin & Nussenzweig, 1978). Analysis of the host response to sporozoite immunization during ongoing malaria infection would provide a suitable experimental system to detect the possible effects of the disease on the immune response to a specific stage of the malaria parasite.

In this report, we show that an acute infection with the erythrocytic stages of *P. berghei* renders mice immunologically unresponsive to inoculations with irradiated sporozoites of the same plasmodial strain.

MATERIALS AND METHODS

Animals. Adult female A/J mice (Jackson Memorial Laboratories, Bar Harbor, Maine), 8–12 weeks of age, were used throughout the studies, except when concentrated parasitized erythrocytes were obtained from infected blood of other rodents (rats and hamsters).

Malaria parasites. The erythrocytic stages and irradiated sporozoites of *Plasmodium berghei*, NK strain, were used to induce malaria infection and for immunization, respectively. Both stages were also used as antigens for serological tests. The laboratory maintenance of the NK strain of *P. berghei*, its cyclical transmission and the characteristics of this infection in mice have been described by Vanderberg, Nussenzweig & Most (1968).

Malaria infection. Mice were infected intravenously (i.v.) with 1×10^4 parasitized A/J mouse erythrocytes. This infection resulted in a very low parasitaemia (0.1%) 4 days later, but rose to about 10% within 10 days. The parasitaemia usually continued to rise until the animals died.

Concentration of parasitized erythrocytes for adsorption. Rodents (mice, rats and hamsters) infected with *P. berghei* were the donors of heavily parasitized blood. They were anaesthetized with ether and bled from the axillary vessels. The blood was collected in glass centrifuge tubes containing heparinized phosphate-buffered saline (PBS), pH 7.5, to prevent clotting. After centrifugation of the blood at 1300 g for 10 min, two distinct layers of cells were observed in the pellet. The top layer, which was brown in colour, was made up of about 85 parasitized erythrocytes (PE), as determined in Giemsa-stained smears. The bottom layer contained mostly uninfected red blood cells and was discarded after careful collection of the PE layer. The concentrated PE were then washed four times with PBS to remove the donor's serum components. The packed PE were then distributed in aliquots of 0.1 ml into small plastic tubes and centrifuged again at 8733 g (microfuge) for 5 min to remove excess fluid prior to their use for adsorption.

Adsorption of antibody form antibodies with PE. Antisera diluted 1 : 16 with PBS were incubated with an equal volume of concentrated PE for at least 30 min at room temperature. It was then centrifuged at 8733 g for 5 min. This procedure was repeated at least five times with the resulting supernatant.

Sporozoite antigens. (a) *For immunization.* Salivary glands of *P. berghei*-infected mosquitoes (*Anopheles stephensi*) were obtained by dissection 18 days after the mosquitoes' blood meal on malaria-infected hamsters. The sporozoites contained in the glands were harvested as described by Cochrane *et al.* (1976) in tissue culture Medium 199 (M 199) which contained 10% normal A/J mouse serum. The sporozoites were γ -irradiated at 15 K rads prior to being used for immunization. All *in vitro* handling of the organisms was done on ice.

(b) *For IFA tests.* The sporozoites were harvested as described above, but in M 199 without serum. To remove mosquito debris, the sporozoites were purified on a DEAE-cellulose column (Moser *et al.*, 1978). The parasites were then concentrated by centrifugation at 8733 g for 5 min. They were resuspended in PBS at a concentration of 8×10^5 sporozoites/ml. Drops (0.015 ml) of the suspension were air-dried on multi-well glass slides and acetone-fixed for 1 min. The slides were then stored in the freezer at -20°C until used.

Schedule to establish sporozoite-induced protection. Mice were immunized weekly for 5 weeks by an i.v. inoculation of 1×10^4 irradiated sporozoites in M 199 containing 10% serum. During the week after the last inoculation the animals were challenged with 1×10^4 infectious sporozoites. No blood infections developed from this challenge, since these animals had developed solid protection (sterile immunity).

Immunofluorescence test for detection of anti-sporozoite antibodies. Sporozoite antigen slides stored at -20°C were defrosted at room temperature, immersed in PBS for 5 min and air-dried again. The sera to be tested were diluted with PBS in serial two-fold dilutions, starting at 1 : 16. The antigen wells were covered with the dilutions and the slides incubated in a wet chamber for 30 min at 37°C . The slides were then exhaustively rinsed with PBS.

Fluorescein-conjugated polyvalent rabbit anti-mouse immunoglobulin purchased from Behring Diagnostics (New Jersey) was used for indirect immunofluorescent antibody (IFA) staining. The conjugate was titrated and diluted appropriately with PBS which contained 5% (v/v) of a saturated solution of Evan's blue dye. The antigen slides were then reacted with the conjugate for 30 min at 37°C and the washing process was repeated after the incubation. Finally, the slides were air-dried

and mounted with coverslips in phosphate-buffered glycerol. The reactions were examined under a Zeiss universal microscope with epi-illumination. The reactions were scored positive when sporozoites showed yellow-green fluorescence. Negative reactions did not show fluorescing sporozoites. The antibody content of the test sera was recorded in titres, reflecting the highest serum dilution which gave a positive reaction. The lowest serum dilution assayed and considered positive was 1 : 16. At lower dilutions sera from normal A/J mice sometimes give false positive IFA reactions with air-dried and acetone-fixed sporozoites.

Immunofluorescence test for anti-erythrocytic stage antibodies. This test was performed with washed, air-dried and acetone-fixed PE slides. The same procedure described above for the detection of anti-sporozoite antibodies was also applied to these titrations.

Sporozoite immunization of malaria-infected, chloroquine-treated mice. Mice were infected with 1×10^4 PE and on the ninth and tenth days of the infection the animals were treated intraperitoneally (i.p.) with non-curative doses of chloroquine (0.15 mg/day). The next day (day 11 following infection) they were inoculated with 1×10^5 irradiated sporozoites. Three days later, the surviving animals received another chloroquine treatment, followed by treatments on days 19, 20 and 23 of the infection. On day 27, the animals were reinoculated with irradiated sporozoites. This treatment schedule was designed merely to control, but not to cure the malaria infection. Uninfected mice (controls) were similarly treated with chloroquine and immunized with irradiated sporozoites. Sera from all the animals were assayed for anti-sporozoite antibodies 7 days after the first sporozoite inoculation and 4 days after the second inoculation.

RESULTS

Differentiation between stage-specific anti-sporozoite antibodies and those directed to common plasmodial antigens

Mice inoculated with blood stages of *P. berghei* produce antibodies which react with the blood stage and with air-dried acetone-fixed sporozoites in the IFA test. We differentiated these cross-reacting antibodies from stage-specific anti-sporozoite antibodies by repeated adsorption of the sera with concentrated PE. As illustrated in Table 1, this procedure removed antibodies directed against the blood stages of malaria parasites from the immune sera, but did not alter the titre of anti-sporozoite antibodies. Thus, if the serum from an experimental animal retained a positive IFA test with sporozoites after adsorption with PE, and had no reaction with blood forms, we considered this reaction to be sporozoite-specific.

Time course of the anti-sporozoite antibody response

The sera of mice inoculated with a single dose of 7×10^4 irradiated sporozoites were tested every 2 days for levels of IFA. The results are illustrated in Fig. 1. Two months after the single injection of irradiated sporozoites, the IFA in these animals were still detectable at relatively high titres (1 : 64).

Effect of malaria infection on the primary immune response to sporozoites

Mice were infected i.v. with 1×10^4 PE on day 0 (experimental animals). On days 4, 7, 10, 13 or 16 after the inoculation, groups of these animals were immunized i.v. with 7×10^4 irradiated sporozoites.

TABLE 1. Stage specificity of anti-malaria antibodies

Immune serum	IFA titres			
	Before adsorption with PE		After adsorption with PE	
	Sp Ag	PE Ag	Sp Ag	PE Ag
Single inoculation with irradiated sporozoites	256	< 16	256	< 16
Blood stage infection only	64	128	< 16	< 16
Blood stage infection and inoculation with irradiated sporozoites	16	256	16	< 16
Inoculations with sporozoites followed by patent infection	256	256	256	< 16

Stage specificity of malarial antibodies in the sera of animals inoculated with either irradiated sporozoites and/or erythrocytes parasitized by *Plasmodium berghei*. All titrations were done by indirect immunofluorescence using air-dried sporozoites (Sp) and parasitized erythrocytes (PE) as antigens before and after adsorption of the antisera with parasitized erythrocytes, as described in the Materials and Methods section.

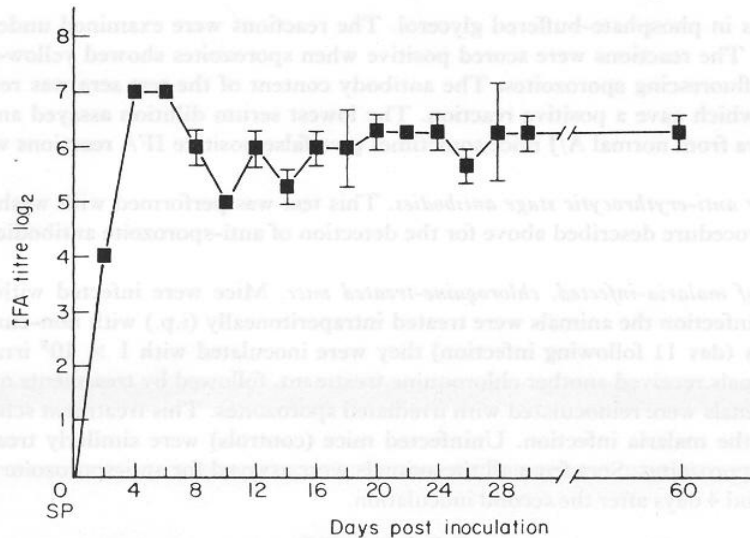


FIG. 1. Time course and level of anti-sporozoite antibodies in normal A/J mice inoculated with 7×10^4 γ -irradiated *P. berghei* sporozoites (SP) in 10% mouse serum. Antibody levels are expressed as mean titre \pm s.e. for groups of three mice.

Normal mice were similarly immunized and used as controls. All the animals were bled from the retro-orbital plexus 6 days after the immunization and the sera assayed individually for primary IFA anti-sporozoite response. The results of this experiment (Fig. 2) indicated that experimental animals immunized on day 4 of the malaria infection responded normally, when compared with their controls. In contrast, almost all of the mice immunized on day 7 or later during the ongoing malaria infection produced no detectable anti-sporozoite antibodies.

Effect of malaria infection on the secondary antibody response to sporozoites

Animals immunized with 7×10^4 irradiated sporozoites 4, 7, or 10 days after being injected with PE, and their respective controls, were reinoculated with the same dose of irradiated sporozoites. When they were examined 6 days later for anti-sporozoite antibodies, it was found that (1) none of the animals

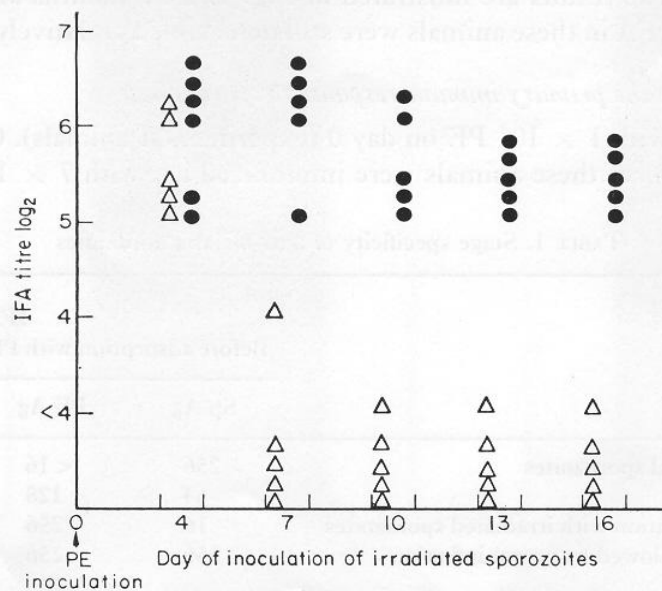


FIG. 2. Effect of a continuing *P. berghei* infection on the development of a primary antibody response to immunization with irradiated sporozoites of *P. berghei*. Mice were injected with parasitized erythrocytes on day 0. Groups of four to six of these malaria-infected animals (Δ) and equal numbers of uninfected controls (\bullet) were immunized with 7×10^4 irradiated sporozoites on day 4, 7, 10, 13, or 16. Sera for IFA tests were collected 6 days after the sporozoite inoculation. Each symbol represents the IFA titre of one mouse.

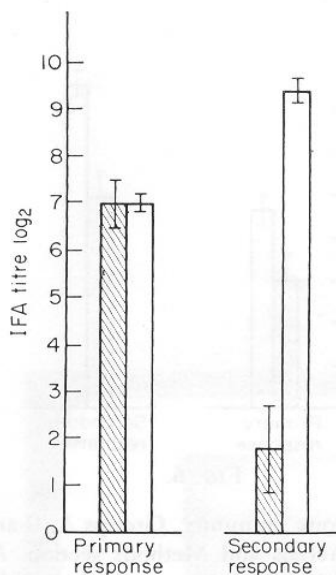


FIG. 3.

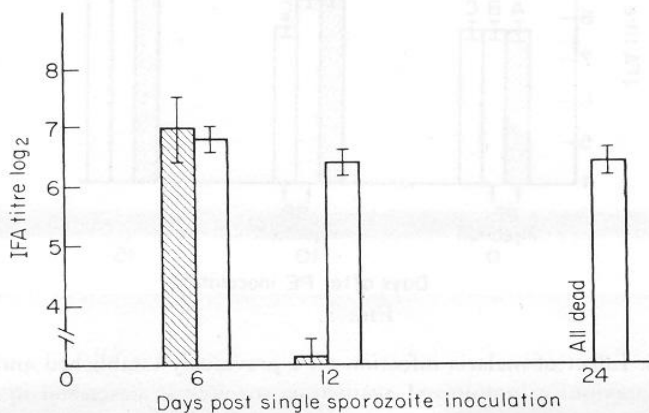


FIG. 4.

FIG. 3. Primary and secondary anti-sporozoite antibody responses in mice primed with irradiated sporozoites during the early phase of malaria infection. Experimental animals (▨) infected with PE on day 0 were inoculated with single dose of 7×10^4 irradiated sporozoites 4 days after the PE injection. Six days later, the animals received a second inoculation of the same dose of irradiated sporozoites. Uninfected controls (□) were similarly immunized with irradiated sporozoites.

FIG. 4. Decline of anti-sporozoite antibodies in mice immunized during the early phase of malaria infection. Animals injected 4 days earlier with PE (▨) and their uninfected controls (□) were inoculated with 7×10^4 irradiated sporozoites on day 0. They were then examined for IFA against sporozoite antigens on days 6, 12, and (where possible) 24. Results are given for each group of five mice as mean titre \pm s.e. All the malaria-infected mice died before day 24.

which failed to respond to the primary immunization produced detectable anti-sporozoite antibodies on revaccination; (2) those animals which responded to the first immunization, were either negative after the second injection of sporozoites or frequently had IFA titres lower than those reached during their primary response; and (3) animals which were immunized during the very early phase of the malaria infection (day 4) gave normal antibody responses to the first inoculation with irradiated sporozoites, but failed to respond to the reinoculation of sporozoites (Fig. 3). In fact, most of the experimental animals had no detectable antibodies when tested for their secondary response. As expected, all uninfected controls showed an increase in antibody titre on reinoculation with sporozoites (secondary response).

Decline of anti-sporozoite antibodies in mice immunized during ongoing malarial infection

Since mice immunized with sporozoites on day 4 of their malaria infection responded normally to the single inoculation but became negative upon sporozoite reinoculation, we examined the persistence of the primary anti-sporozoite antibody response in an ongoing malarial infection. A group of mice inoculated with 7×10^4 irradiated sporozoites on day 4 of malaria infection was examined for anti-sporozoite antibodies on days 6 and 12 after the single immunization. The levels of sporozoite-specific antibodies in these animals infected with malaria were compared with those found in uninfected controls which had been immunized with a similar dose of irradiated sporozoites. As shown in Fig. 4, it was found that in most of the malaria-infected animals, anti-sporozoite antibodies declined within 12 days after the single inoculation with irradiated sporozoites. In contrast, uninfected controls retained their antibody response to the sporozoites much longer than 24 days (see also Fig. 1).

An established anti-sporozoite immunity is not abrogated by subsequent malaria infection

We investigated the effect of malaria on anti-sporozoite immunity established prior to a patent infec-

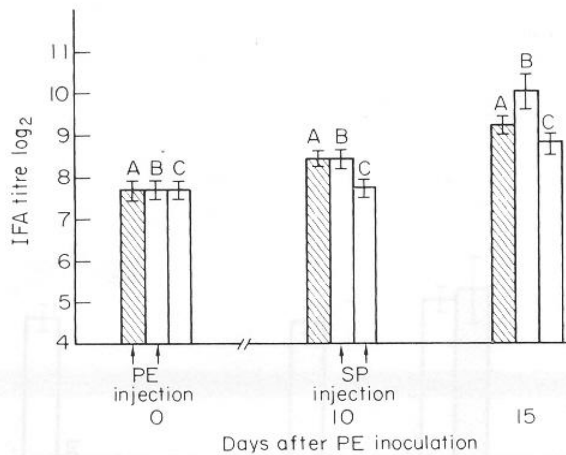


FIG. 5.

FIG. 5. Effect of malaria infection on a previously established anti-sporozoite immunity. Groups A, B and C were previously immunized against sporozoites as described in the Materials and Methods section. Anti-sporozoite antibody titres were determined on day 0 before groups A and B were injected with 1×10^4 PE, on day 10, when groups B and C were inoculated with 7×10^4 irradiated sporozoites, and on day 15. The anti-sporozoite antibody titres given here for groups A and B on days 10 and 15 were obtained after adsorption of the immune sera with PE. Results are expressed as mean titre \pm s.e. for groups of five mice.

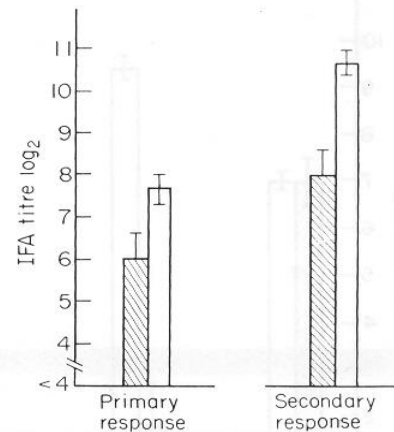


FIG. 6.

FIG. 6. Primary and secondary anti-sporozoite antibody responses in chloroquine-controlled *P. berghei* infection. Malaria-infected animals (▨) and uninfected controls (□) were treated with non-curative doses of chloroquine as described in the Materials and Methods section. During the period of controlled parasitaemia, the animals were inoculated twice with 1×10^5 irradiated sporozoites. The titres of the primary antibody responses were determined 7 days after the first immunizing dose of irradiated sporozoites and the secondary responses were determined 4 days after the second dose. Results are given for each group of three mice as mean titre \pm s.e.

tion. Fifteen mice which had been repeatedly immunized with irradiated sporozoites and were protected against challenge were divided into three equal groups (A, B and C). Groups A and B were injected with 1×10^4 PE; Group C was not infected. The levels of anti-sporozoite antibodies in all the animals were determined before the PE injection. Ten days after the PE injection, when the parasitaemia reached 5%, all the animals were re-examined for anti-sporozoite IFA. On the same day mice in groups B and C were reinoculated with 7×10^4 irradiated sporozoites. Animals in group A received no sporozoite booster. All three groups were retested for anti-sporozoite antibodies on day 15 after PE inoculation. As shown in Fig 5, there was no adverse effect of the malaria infection on the established anti-sporozoite antibody titres, nor on the ability of the infected animals to recognize the antigens to which they had been previously exposed. However, there was a non-specific rise in anti-sporozoite antibody titres in the malarious animals before the sporozoite booster on day 10. Both the infected and non-infected animals showed anamnestic antibody responses after the reinoculation.

Anti-sporozoite antibody response in drug-controlled malaria infection

We looked next at whether a reduction of parasitaemia in infected animals would reduce the severity of immunosuppression. Starting from day 9 of the malaria infection, a group of mice was treated with non-curative doses of chloroquine (see the Materials and Methods section). The parasitaemia was approximately 10% when the treatment was started and after two doses of chloroquine it fell to 0.1%. The drug treatment schedule enabled us to maintain the infection at a subpatent level for 21 days during which time the animals and the uninfected controls were immunized with two doses of irradiated sporozoites. When the chloroquine treatment was terminated, all the infected animals developed high parasitaemia once again, indicating that the parasites were not totally absent during the immunization. As illustrated in Fig. 6, the infected animals showed primary as well as secondary antibody responses to the sporozoite inoculation.

DISCUSSION

We have demonstrated that malaria-induced suppression of the host immune response markedly lowers the antibody levels to certain stages of the life cycle of the causative plasmodium. In the host-parasite system studied, patent infection resulted in the abolition of the antibody response to sporozoites in the great majority of the animals from day 7 onwards, and persisted until their death. Since the impairment was maximal on day 7 (Fig. 3), when the parasitaemia of the animals was only about 2%, a high parasitaemia may not be essential for the malaria-induced suppression of the immune response. However, the fact that a normal primary antibody response was observed in animals immunized on day 4 of malaria infection, when the parasitaemia was still barely detectable, suggests that a certain level of infection must be reached before the immunosuppression becomes effective.

Severe immunosuppression induced by *P. berghei* has been described in response to non-plasmodial antigens. Sengers, Jerusalem & Doesburg (1971) reported that mice immunized with rabbit red blood cells on day 7 or later of malaria infection were unable to produce detectable agglutinating antibodies against the erythrocytes. Poels & van Niekerk (1977) infected BALB/c mice with 1×10^5 PE and immunized them on either day 7 or 14 with sheep red blood cells (SRBC). Both groups of animals failed to produce antibodies against the heterologous erythrocytes. Loose & Di Luzio (1976) found that the impairment of the immune response to SRBC was noticeable at the cellular level, resulting in a decrease of splenic plaque-forming cells 2 days after the malaria infection, and that on day 4, the humoral response was also affected.

The focus of these investigations of malaria-induced immunosuppression has generally been on its mechanism and the effects on the host resistance in its broadest sense. However, the most important effect of this immunosuppression is on the host-parasite relationship itself. Suppression of host resistance to the development of the plasmodial life cycle would result in a very effective mechanism of evasion by the parasite of the host's defence. Other manifestations of malaria-induced immunosuppression might be side effects of this central mechanism of parasite preservation.

The mechanisms of malaria-induced immunosuppression have not been fully defined. It is thought that in malarious animals the induction phase of the immune response is defective. Some authors have attributed this to macrophage dysfunction (Loose, Cook & Di Luzio, 1972; Warren & Weidanz, 1976; Tanabe *et al.*, 1977).

In our studies, even a fully established primary immune response, induced early during a malaria infection, was still found to be subject to the constraints imposed by the suppressive activities of the disease. The primary antibody response declined faster in the malaria-infected animals than it did in uninfected controls (Fig. 4), and the infected animals did not respond to a second injection of antigen despite the apparently successful priming (Fig. 3). The duration of the primary response and the capacity to mount a secondary response were particularly affected. Therefore, it is likely that other additional mechanisms which impair the synthesis and/or secretion of antibodies are also in operation during malaria infection. Another factor which could account for the suppression of the secondary immune response is the depletion of B and T lymphocytes which is known to occur in malaria-infected hosts (Krettli & Nussenzweig, 1974; Gravely, Hamburger & Kreier, 1976; Wyler, 1976). This might affect, in particular, immune-competent lymphocytes which had been sensitized to antigens, thus lowering the production of specific antibodies.

An important finding in the experimental system was that once the anti-sporozoite immunity had been solidly established and the animals had become protected against challenge, a subsequent malaria infection failed to reduce the antibody response to the sporozoite antigen.

The results presented here may be of particular relevance to studies aimed at the development of a sporozoite vaccine for malaria. On the basis of the findings of the rodent-*P. berghei* model, we suggest that suppression of the immune response by the blood stages of these parasites may interfere with, or even prevent, the establishment of anti-sporozoite immunity in nature. However, reduction of the malaria parasitaemia by chloroquine treatment seems to abolish the immunosuppressive effects of the disease (Fig. 6). Therefore, if the immunosuppression to sporozoite antigens occurs in nature, the use

of sporozoite immune prophylaxis in malaria endemic areas would require the application of anti-malarial drugs to prevent patent malaria infection and its immunosuppressive consequences.

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