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Development and decline of antiplasmodial indirect fluorescent antibodies in mice infected with *Plasmodium berghei* (NK65) and treated with drugs

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Malaria parasites in mice present a simplified rodent model for the immunological study of malaria. Experiments have been performed to determine the pattern and persistence of malaria antibody as detected by the indirect fluorescent antibody (IFA) test utilizing specific antimouse IgM and IgG conjugates. The antibody levels in mice inoculated with Plasmodium berghei *and treated with antimalarial drugs were traced after complete elimination of the parasites from the host. Within 1–2 weeks after inoculation, both specific IgM and IgG reached peak levels, which thereafter declined rapidly. The results suggest that a high IFA titre may be taken as an indication of recent parasitaemia when the parasites are absent from the host. The protective role of the specific immunoglobulin was not found in the cured animals at the time when the animals showed a high IFA titer. It seems that the detected IFA may not reflect protective immunity against reinfection with malaria parasites.*

Spleen and parasite rates are two major quantitative measurements in malariometric surveys for eradication programmes. Splenomegaly is usually the result of repeated malaria infections but may also occur in other tropical diseases such as kala-azar, schistosomiasis, epidemic relapsing fever, and some other communicable diseases, or after recent mass vaccinations against smallpox. For this reason, a malaria survey based on spleen examinations alone is unsatisfactory for the promotion of a malaria eradication programme. The parasite rate, obtained by counting the plasmodia in blood films under a microscope, has been employed for estimating more precisely certain aspects of malaria epidemics, particularly in the advanced phase of a malaria eradication programme, e.g., at the beginning of the consolidation phase (14). However, in many malaria programmes, positive slides have not infrequently been missed during microscopic examinations. Raghavan (15), for instance, reported that if there were 18 parasites in a total of 1 000 microscope fields in a thick smear and if only 100 fields were examined, there was a statistical probability of 15% that the slide would be declared negative.

The malaria immunofluorescent antibody (IFA) test, introduced by Tobie & Coatney (16) and by Voller & Bray (23), has been especially appreciated for the detection of asymptomatic infections (i.e. without detectable parasitaemia) in individuals (18). Although the usefulness of this test for large-scale surveys has been confirmed by many authors (5, 6, 10, 12, 19, 23), its employment over traditional microscope examinations can be justified only when the parasitaemia is below the microscopic threshold (1) because of the difficulties in interpreting the results of serologic surveys. However, a clarification of the following points should lead to reasonable interpretations of the results. *(1)* Is it possible to detect a recent malaria infection by the IFA method? *(2)* How long do the IFA antibody titres last, and what is the pattern of their development and decline in individuals and in mass populations after an exposure to malaria infection? *(3)* Can measured values of the IFA titre reflect protective immunity against reinfection with malaria parasites?

The present study was carried out on a simplified rodent model to clarify these three points in the IFA

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test. The development and decline of IgM and IgG levels, measured by the indirect fluorescent antibody technique, were traced in mice infected with *P. berghei* (NK65) after complete elimination of the parasite by drug treatment. The protective role of the specific immunoglobulin was examined in the cured animals by reinoculating parasites at the time when the animals showed a high antibody titre.

MATERIALS AND METHODS

Strain of Plasmodium and of the animals employed

The NK65 strain of *P. berghei* (21) and 19–22-g female DDY strain white mice were used throughout the experiment. The parasites were donated by Professor M. Yoeli in 1969, since when they have been maintained by blood transfers in mice with occasional freezing at −70°C (21).

Doses of the drugs used

Previous work by one of the authors showed that with doses of 20 mg per kg of body weight per day of sulfamonomethoxine (liquid form) (22), of chloroquine phosphate (liquid form), and of pyrimethamine (suspended in 10% CMC medium by ultrasonic treatment) infected mice could be cured in 4 days (Suzuki, unpublished report to WHO, 1972). The doses of each of these three drugs, which were administered to different groups of mice in the present experiments, are shown in Table 1.

Preliminary test for the immunosuppressive action of the drugs

Five mice for each drug were inoculated intraperitoneally with 1×10^7 sheep red blood cells (SRBC) on day 0 (D0). On day 3 (D3) and daily thereafter for 3 days (D4–D6), the specified doses of each drug were administered subcutaneously each day to 3 separate groups of mice. Peripheral blood was drawn by cutting the tail vein of each mouse on days 3, 6, and 9. The anti-SRBC serum titres were compared with those in control mice (i.e. untreated with drugs) injected with SRBC. As shown in Table 1, there were no significant differences in the anti-SRBC antibody response between the treated and control groups.

Procedure of the experiment

Thirty-three mice in the 3 test groups were inoculated intraperitoneally with 1×10^7 parasitized red blood cells and 24 mice in the 4 control groups were inoculated with normal mouse red blood cells on day 0. Then, as described above, the drugs were administered for 4 successive days (D3–D6). On days 6, 13, 27, 41, 55, 69, 84, 97, 111, 139, and 342, a small

Table 1. The immunosuppressive effect of antimalarial drugs on mice

Antimalarial drug	Doses of drugs (mg/kg/day)	No. of mice	Haemolysin titre [a] days since SRBC inoculation		
			3	6	9
sulfamonomethoxine	200	5	24	1248	1008
	100	4	24	1124	374
	20	5	24	3016	648
chloroquine	50	4	42	900	521
	25	5	19	1248	648
	5	5	72	3016	521
pyrimethamine	50	4	24	900	900
	25	3	33	648	300
	5	5	46	648	174
control	—	7	46	2172	723

[a] The haemolysin titre was measured by the microtitration system. It is the reciprocal number of the serum dilution that gives approximately 50% haemolysis with the addition of complement.

sample of blood was taken into a sterilized capillary tube by cutting the tail vein of each mouse; the tube was sealed and centrifuged at 2 500 rev/min within 12 hours. The resulting sera were taken into other sterilized capillary tubes, sealed, and kept at 4°C until needed.

Estimation of antibody levels by the immunofluorescence technique

On the 2nd day after inoculation of the mice, some heavily infected blood was taken in order to prepare blood films for malaria antigens. The blood smears on the glass slides were dried in a current of air, dipped in acetone for 1 minute, and rapidly dried again. The prepared antigen films were wrapped in tissue paper and stored at −70°C in a freezer until required.

All the sera taken on the same day in each test were diluted with 0.15M phosphate buffered saline. The end-point of the dilution that showed a significant fluorescence was taken as the titre of the serum. The tests were carried out with the aid of a standard antiplasmodial serum, which showed 1 : 256 in every titration, as a control.

Highly specific rabbit anti-mouse IgM and anti-mouse IgG sera for the present study were prepared by Dr Nariuchi using his own method (13).

RESULTS

The time course of the development of fluorescent antibodies in untreated infected mice

Both specific IgM and IgG levels were traced in 6 mice inoculated with 1×10^7 parasitized red blood cells. The number of survivors on each day were as follows: D0 (day of inoculation), 6/6; D1, 6/6; D2, 6/6; D3, 6/6; D4, 6/6; D5, 6/6; D6, 6/6; D7, 5/6; D8, 5/6; D9, 4/6; D10, 4/6; D11, 2/6; D12, 2/6; D13, 1/6; and D14, 0/6. Both classes of the specific immunoglobulin reached maximum titres by the 7th to the 9th day after inoculation, a time when the animals became moribund; thereafter, the Ig levels declined rapidly (Fig. 1).

The development and decline of fluorescent antibodies in infected mice after chemotherapy

The results are shown in Fig. 2. The curve of IgM levels was traced only in mice treated with sulfamonomethoxine; the IgG levels were followed up in the

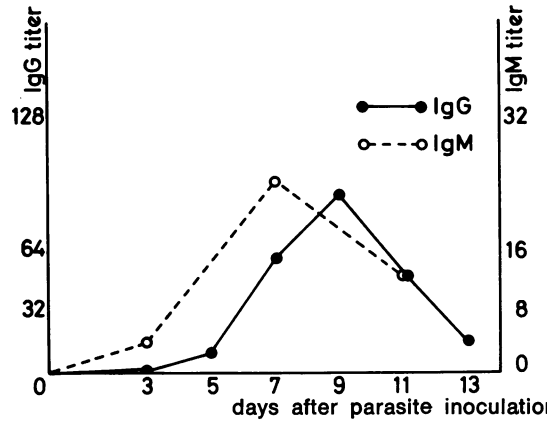

Fig. 1. Malaria antibody response as measured by the IFA test in mice infected with *P. berghei* (NK65)

groups treated with chloroquine, pyrimethamine, and sulfamonomethoxine. Each dot on the curves in Fig. 1 and 2 represents the mean of the logarithm of IFA titres from the mice in one group on each day. The IgM curve showed its highest titre as early as the 6th day after inoculation, which was when the last dose of drug was given. After the completion of drug treatment, the IgM level in the cured mice declined rapidly and was undetectable on the 27th day after inoculation. The IgG titres, on the other hand, continued to rise even after the disappearance of parasitaemia, reached a maximum on the 13th day after inoculation, and then declined gradually although IgG was still detectable 342 days after inoculation.

No significant difference was found in the pattern of IgG production curves between the groups of mice treated with the 3 different drugs.

The relationship between the fluorescent antibodies and protective immunity

Whether the developed antibodies could protect against reinfection when the infected and cured mice were reinoculated with parasites was examined in another experiment. On the 18th day after the initial inoculation with parasites followed by treatment with sulfamonomethoxine as described above, challenge inoculations of 1×10^3, 10^4, 10^6, and 10^7 parasites were performed on 4 test groups of 3 mice each. Four control groups had the same number of uninfected mice of the same age. The control mice were injected with non-parasitized mouse blood and

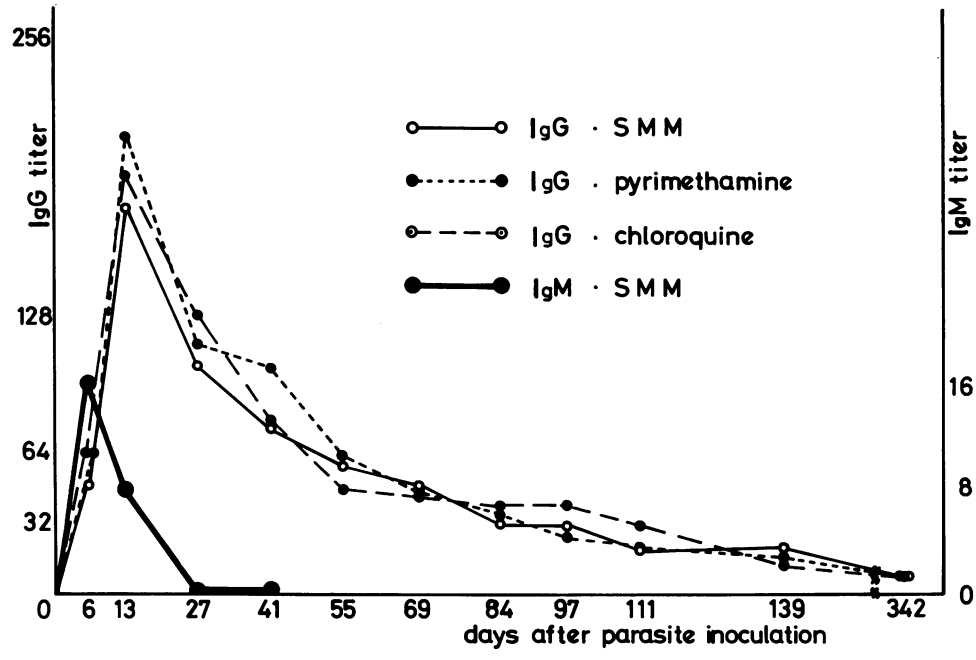

Fig. 2. The course of development of malaria indirect fluorescent antibodies in mice after the treatment of *P. berghei* (NK65) infection with sulfamonomethoxine (SMM), pyrimethamine, and chloroquine.

treated with drugs concurrently with the test group. The periods of survival of the mice in the test and control groups were compared. As shown in Table 2, the immunized mice did not show any resistance against the effects of reinoculation with parasites.

DISCUSSION

It is generally agreed that although malaria indirect fluorescent antibodies persist for a long time when the infection is not completely cured, the radical cure of malaria infection may lead to a rapid decline in antibody levels (18). However, there is disagreement concerning the development and decline of the serum titres in patients. Collins et al.(4) reported that syphilitic patients who had been inoculated with *P. malariae* followed by drug treatment frequently showed a positive fluorescent antibody (IFA) response even 15–24 years after the last known patent parasitaemia. Their earlier reports (2, 3) indicated that the antibody titres in some of the volunteers, inoculated with *P. malariae* or *P. falciparum* and subsequently treated with drugs, persisted at high levels for several hundred days after the first onset of the fever. On the other hand, Wilson et al. (20) showed that the majority of patients infected with falciparum and vivax malaria reversed to a negative IFA response within 6 months to a year of radical cure. Lupascu et al. (11) demonstrated the rapid decline of IFA titres in patients with *P. malariae* infection treated with an antimalarial drug.

Table 2. Protective immunity in mice against challenge inoculation following radical cure of *P. berghei* infection

Parasites inoculated	Death rate of mice	Mean survival days (range)
1×10^7	immunized 3/3	8.7 (6–13)
	control 3/3	6.7 (6– 8)
1×10^6	immunized 3/3	9.3 (7–13)
	control 3/3	8.7 (7–10)
1×10^4	immunized 3/3	12.7 (8–20)
	control 3/3	14.7 (11–20)
1×10^3	immunized 3/3	14.7 (14–16)
	control 3/3	20.0 (20)

Because of the difficulties in interpreting the differences in the reported time courses of IFA titres, further discussions on clinical cases offer little hope of establishing a fundamental basis for field surveys. Studies on simple animal models are therefore now required.

Although there are considerable differences between rodent and human malarias, the *P. berghei*–mouse system should be a useful model for investigating the basic features of malaria seroepidemiology.

The present study may be compared with that by Cox & Turner (9) employing *P. berghei yoelii*. In their experiment, infected mice showed a low peak of parasitaemia on the 12th day, after which the parasites disappeared spontaneously by the 15th day of inoculation; the levels of malaria IgM and IgG increased in association with the infection and remained high even in the absence of parasitaemia. In the present study, both the IgM and IgG levels in *P. berghei*-infected mice declined rapidly after reaching their peaks. The findings are similar to the antibody production curve in the *P. berghei*-infected rat, as shown by Voller (17). Again, there are differences in the results according to the models employed. It will be necessary therefore to exclude the parasite completely from the infected host to simplify the factors being studied.

Suzuki (unpublished report to WHO, 1972) looked for parasites in mice that had been infected and cured in the same manner as in the present experiment, and showed that after the treatment with drugs the parasite was absent from the brain, lungs, heart, spleen, liver, thymus, retroperitoneal lymphnodes, kidneys, pancreas, intestine, bone marrow, and adipose tissue of the host. Subsequent recrudescences were not observed in the treated mice. Thus, the treated animals in the present study could be considered to be free of malaria parasites. In these animals, both specific IgM and IgG declined rapidly after reaching their peaks. These results are in contrast to those obtained by Cox et al. (8) in *P. vinckei*-infected mice which had been freed from parasites by a single treatment with chloroquine (7); the curves of specific IgM and IgG levels in these mice were maintained at a high plateau for longer than 50 days. One difference between Cox's and the present experiments concerns the duration and level of parasitaemia in the mice before chemotherapy. In the present experiment, the parasites were suppressed by 4 days of drug treatment, which started on the 3rd day; by the 6th day of inoculation no parasites were to be found in the peripheral blood. Cox applied chemotherapy on the 5th day of inoculation when the parasitaemia was already nearly 70% and found a slight recrudescence between the 10th and 20th days of inoculation. These facts suggest that if an infected animal is radically cured shortly after the onset of parasite multiplication but before this can reach a high degree of parasitaemia, then the antibody levels will decline rapidly; but when parasite multiplication and the resulting parasitaemia persist for some time after chemotherapy, then the antibody level is maintained at a high plateau.

Cox (7) also investigated the extent of protective immunity in *P. vinckei*-infected mice that had been cured by a single dose of chloroquine. His results showed two alternatives: if the infected mice were cured when the parasitaemia was low, then primary inoculation conferred no protection to the host against a reinoculation, but if the infected mice were cured in the same manner at 70% parasitaemia, then the following challenge inoculation was not lethal and the treated animals were found to be sterile (i.e. free of parasites) when their tissues were inoculated into healthy mice. In the present study, no protective immunity was demonstrable in any of the infected mice after chemotherapy.

From all these observations, it is reasonable to conclude that in treated, parasite-free mice, the IFA levels which reach a high titre and remain elevated for a long time do confer protective immunity, whereas those antibody levels which decline rapidly after reaching a peak do not. In the latter, the high IFA titres may be an indication of recent parasitaemia. It seems that a critical threshold of parasite multiplication determines the pattern of the IFA curve after chemotherapy. Recent work by one of the authors (S. W.) has shown that sterile protective immunity was conferred on mice by repeated inoculations followed by drug treatment in the same manner as described above. In this experiment, the curve of IFA titres was found to remain at a high plateau. This finding partly supports our present speculations.

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RÉSUMÉ

APPARITION ET DÉCLIN DES ANTICORPS ANTIPLASMODIQUES DÉCELÉS PAR IMMUNOFLUORESCENCE INDIRECTE CHEZ DES SOURIS INFECTÉES PAR *PLASMODIUM BERGHEI* (NK65) ET TRAITÉES PAR DES MÉDICAMENTS

L'évolution des anticorps spécifiques IgM et IgG a été étudiée par la technique de l'immunofluorescence indirecte chez des souris infectées par la souche NK65 de *Plasmodium berghei* et traitées par chimiothérapie.

L'examen du sang, pratiqué au 6e jour suivant l'inoculation, n'a fait découvrir aucun plasmodium chez les souris inoculées par 10^7 parasites et traitées par la sulfomonométhoxine, la chloroquine ou la pyriméthamine les 3e, 4e, 5e et 6e jours à la dose de 20 mg/kg/jour. Les titres d'IgM ont présenté un clocher au 6e jour puis ont décliné rapidement pour devenir négatifs 27 jours après l'inoculation. Les titres d'IgG, d'autre part, maximaux au 13e jour, se sont abaissés dans les 5 à 6 semaines suivantes pour s'établir à un niveau faible, mais étaient encore décelables 342 jours après l'inoculation.

On a recherché le rôle protecteur des immunoglobulines contre une réinfection par le même parasite chez les animaux traités. Des inoculations d'épreuve de 10^3, 10^4, 10^6 et 10^7 parasites ont été pratiquées lorsque les titres d'anticorps étaient les plus élevés. On n'a constaté aucune différence entre souris traitées et souris témoins en ce qui concerne la résistance à une seconde infection. Il semble donc que chez les rongeurs étudiés des titres élevés d'anticorps fluorescents, s'ils sont l'indice d'une parasitémie récente, ne confèrent aucune immunité protectrice à l'hôte.

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