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Crossreactivity with sporozoites, exoerythrocytic forms and blood schizonts of *Plasmodium berghei* in indirect fluorescent antibody tests with sera of rats immunized with sporozoites or infected blood

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SUMMARY

IFA studies are reported using plasmodial antigens from three different stages of the life cycle of *Plasmodium berghei*: sporozoites (SP); exoerythrocytic schizonts in rat liver (EEF); and parasitized rat erythrocytes (SCH = schizonts). Two series of specific sera were applied: sera from adult rats with a blood-induced infection (series A) and sera from rats immunized against sporozoites by mosquito bites and protected against parasitaemia by chloroquine (series B). In series A antibody titres with all three antigens were seen, but those with SCH were generally the highest. Superinfection with parasitized rat blood did not change the titre. In series B sera, collected from rats after a single exposure to infected mosquitoes, showed only titres with SP from day 3 onwards, but after a second exposure titres to all three antigens developed. Crossreactivity with the heterologous antigens in series B was clearly less than in series A. Anti-*P. berghei* sporozoite antibodies did not crossreact with *P. vivax* sporozoites.

Rats of series A were resistant to a challenge of parasitized blood and could also inhibit the development of sporozoites. Rats of series B were protected against a challenge of sporozoites but not of infected blood. The results are discussed.

INTRODUCTION

Information on the immunogenicity of plasmodial sporozoites and exoerythrocytic forms is limited. The erythrocytic stages of the plasmodial cycle have been studied much more intensively than the other stages. Only a few methods were occasionally used to detect antibodies with sporozoites as antigen: agglutination tests (Mulligan, Russel & Mohan, 1940); circum-sporozoite precipitation tests based on development of a precipitate on the sporozoite (Vanderberg, Nussenzweig & Most, 1969); and a direct fluorescent antibody test with serum samples from chickens immunized by red blood cells infected with *P. gallinaceum* (Sodeman & Jeffery, 1964).

Exoerythrocytic forms (EEF) in the liver of infected mammals can hardly be detected. Therefore, critical studies on this stage of the malaria life cycle are difficult to perform and almost absent (Brown, 1976). Nevertheless Krotoski, Collins & Jumper (1973) succeeded to demonstrate anti-EEF antibodies by the IFA technique in sera of rhesus monkeys infected by *P. cynomolgi*. Vanderberg (1973) suggested that antigenic differences may exist between sporozoites and exoerythrocytic parasites.

The question concerning immunological properties of different plasmodial stages is not new. Boyd & Kitchen (1936) suggested that sporozoites might be antigenically different from trophozoites and that the immunity of the host might be directed mainly against trophozoites rather than against the sporozoites. Unfortunately the immune mechanisms operating against stages other than the blood stages are still not well understood. Only a few scientists have succeeded in immunizing with sporozoites

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(reviewed by Beaudoin *et al.*, 1976), and discussions on the basic biology of sporozoites and EEF are still based more on hypothesis than on facts (Coatney, 1976).

The present study reports the details of experiments in which rats were immunized against sporozoites or against erythrocytic schizonts of *P. berghei*. The titres of antibodies against sporozoites and schizonts as well as against exoerythrocytic forms in the liver are presented. The immunized rats were also challenged with blood parasites or sporozoites, and the results indicate a partial correlation between the stage-specific protection obtained by such immunization and the antibody level. Although we used the antibody titre as a marker of the immune response, we did not exclude a role for cellular immunity.

MATERIALS AND METHODS

Sporozoite source. (a) Laboratory-bred *Anopheles stephensi* or *A. atroparvus* were infected with *P. berghei* (Anka strain). The latter was kept in Swiss mice by weekly blood passage alternating once a month with cyclical transmission through mosquitoes. The *P. berghei*-infected mosquitoes were kept at 20–21°C under appropriate conditions for sporozoite development (Vanderberg & Yoeli, 1966). Mosquitoes with at least a 50% infection rate served as sporozoite source between the 15th and the 18th day after the infective blood meal.

(b) Laboratory-bred *A. atroparvus* were fed on a *P. vivax*-infected volunteer. The 22-year-old volunteer was accidentally infected during a trip in Indonesia and came back to the Netherlands with fever. Examination of blood films revealed *P. vivax*-infected erythrocytes and gametocytes. The man was treated and cured, after allowing about 100 mosquitoes to feed, all of which became infected. The mosquitoes were kept at 21°C and served as sporozoite source on the 17th day after the infective blood meal.

Preparation of sporozoite antigen from infected mosquitoes. (a) For *P. berghei* sporozoite antigen preparation we used a modification by Beaudoin & Strome (personal communication) of the method introduced by Krettli, Chen & Nussenzweig (1973). 600 *A. stephensi* female mosquitoes with about 70% infection rate were used. The mosquitoes were triturated with 4 ml tissue-culture medium 199 (M 199) in a glass tissue grinder. This and all other preparations were done at 4°C. The homogenate was centrifuged at 49 ***g*** for 5 min in siliconized tubes and the supernatant was separated. The pellet was resuspended in 4 ml of M 199 and the suspension was again ground and centrifuged. The supernatant was added to the former one and centrifuged together at 17,000 ***g*** for 20 min. The pellet was resuspended in 2 ml of M 199 and applied to a two-layered gradient composed as follows: a bottom layer containing 3 ml Urografin® 60% (Schering Ag Berlin/Bergkamen, Germany), 1 ml heat-inactivated normal rat serum and 3 ml M 199; a top layer containing 2 ml Urografin® 60%, 1 ml heat-inactivated normal rat serum and 4 ml M 199. The gradient was centrifuged at 17,000 ***g*** for 30 min. A visible white band at the interphase of the gradient contained the sprozoites. This band was collected, washed by centrifugation at 17,000 ***g*** for 20 min, once with 30 ml of M 199 and once with PBS pH 7·2. The pellet was resuspended in 5 ml PBS, centrifuged at 43 ***g*** for 5 min to get rid of some insect tissue contaminating the band of sporozoites. After this final centrifugation the supernatant contained $1{\cdot}2\times10^6$ sporozoites/ml. About 2 μl of this product was put on each of ten places on glass slides, divided by spraying fluorocarbon in a grid pattern (Fluoro Glide Chemoplast Inc., Wayne, New Jersey). 500 slides can be obtained from such a batch of mosquitoes. This antigen, coded SP (sporozoite antigen), served for indirect fluorescent antibody (IFA) tests.

(b) Thirty *A. atroparvus* infected with *P. berghei* or *P. vivax* were dissected. The salivary glands were separated and homogenized in 0·4 ml of PBS pH 7·2. 5μl drops of these homogenates were applied to glass slides for IFA tests comparing antibodies to *P. berghei* and *P. vivax* sporozoites.

Preparation of antigen from infected blood. Four splenectomized Wistar rats were infected with *P. berghei*. The rats were killed 6 days later when parasitaemias were between 12·2–16·3% and the schizont rate was relatively high. The blood (collected with sodium citrate 3·8% as anticoagulant) was centrifuged at 681 ***g*** for 5 min. Plasma was discarded, and the erythrocytes washed four times with PBS pH 7·2. 2 ml of packed cells were diluted 1:20 in PBS. The solution containing $2{\cdot}8\times10^8$ erythrocytes/ml was divided into 2 μl drops for the IFA tests. This antigen was coded SCH.

Processing of liver sections for counting exoerythrocytic forms and preparation of the sections for IFA tests. Rats were injected i.v. with different numbers of *P. berghei* sporozoites. The density of exoerythrocytic forms (EEF) was used as a measure for numbers of sporozoites that escaped natural and acquired immunity. Livers were removed 45 hr after inoculation, since the maturation time of EEF of the Anka strain of *P. berghei* is about 48 hr (Killick-Kendrick, 1974). They were fixed in modified Carnoy's solution (10% glacial acetic acid, 30% chloroform and 60% ethanol) for 5 hr with a change of the fixative after 1 hr. The fixative was then replaced by ethanol 96%, which was also changed after 8 hr. Pieces of liver were passed through acetone, methylbenzoate and toluene into a mixture of equal parts of paraffin and paraplast. 5μm sections were cut with a microtome, mounted on slides with white gelatine (Merck) and after drying stained according to the Giemsa-colophonium method (Shute & Maryon, 1966).

The number of EEF per cm^2 of liver tissue was estimated by microscopic observation and calculation of the area of nine different sections from different sites of the median lobe of the liver (Verhave's method, 1975). Livers of rats with high densities of EEF (60 EEF/cm^2) were sectioned at 3 μm. The fluorescent antibody titre with EEF antigen was estimated by a modification of the method of Krotoski *et al.* (1973), using as antigen dry liver sections placed on glass slides.

Immunization procedure. (a) Rats were injected i.v. with sporozoites. In some experiments the animals were exposed to the bites of infected mosquitoes. All rats which received sporozoites were protected against parasitaemias by addition of chloroquine to the drinking water (100 mg Nivaquine/1) for the whole period of the experiments.

(b) Rats were injected i.p. with erythrocytes parasitized with *P. berghei*. The inoculum was obtained from the stock of *P. berghei* which was kept in mice and mosquitoes, after a passage through young rats before each experiment. Samples of blood were collected in heparinized capillaries from the tail vein of experimental and control rats at different days after the infection. The sera were separated and stored at −20°C until use.

Indirect fluorescent antibody (IFA) tests. Sporozoite antigen (SP) obtained by gradient separation or by homogenization of salivary glands, infected blood from rats (SCH), and exoerythrocytic schizonts (EEF) in sections of rat livers served as antigens.

IFA tests were performed on sera of experimental and control rats. The sera were examined with rabbit anti-rat globulin (Institut Pasteur). This conjugate was diluted 1:80 for EEF and SP, and 1:160 for SCH after titration according to Johnson & Holborow (1973).

Each serum sample was tested at least twice and all experiments were repeated twice. The results are expressed as the mean titre index (MTI) ± s.d. MTI is the arithmetic mean of the individual titre indices. The latter are obtained by dividing the reciprocal values by 10 and calculating the logarithm to the basis of 2; for instance a titre 1:320 corresponds with a titre index of 5 (= $1:10\times 2^5$). A titre < 1:20 corresponds with a titre index of 0.

RESULTS

1. *IFA titres to sporozoites (SP), exoerythrocytic forms (EEF) and erythrocytic schizonts (SCH) in rats immunized with either the erythrocytic stage or sporozoites of P. berghei*

1.1. *The effect of infection induced by erythrocytic schizonts on IFA titres.* Table 1 gives the results of an

TABLE 1. MTI* values ± s.d. in sera of three rats, examined by IFA tests with sporozoites (SP), exoerythrocytic forms (EEF) and erythrocytic schizonts (SCH) as the antigen. Serum samples were collected during the course of blood-induced infection with *P. berghei*

	Days after inoculation						
	3	6	9	12	18	27	38
Parasitaemia (%)	0·43 ± 0 01	3·45 ± 0·52	2·66 ± 1·02	0·80 ± 0·17	0·01 ± 0·00	0·00	0·00
SP	0·3 ± 0·6	2·3 ± 1·1	6·0 ± 1·3	6·3 ± 0·6	3·6 ± 0·8	5·2 ± 1·0	5·3 ± 1·5
EEF	0·2 ± 0·3	1·0 ± 0·0	2·2 ± 1·6	3·8 ± 1·2	2·7 ± 0·3	3·2 ± 0·8	3·7 ± 0·6
SCH	3·0 ± 0·0	4·5 ± 0·5	7·1 ± 0·5	8·0 ± 0·9	5·6 ± 1·0	5·5 ± 1·0	4·3 ± 0·6

* MTI is the arithmetic mean of the individual titre indices. The latter are obtained by dividing the reciprocal titres by 10 and calculating the logarithms to the basis of 2; for instance a titre of 1:320 corresponds with a titre index of 5 (= $1:10\times 2^5$). A titre < 1:20 corresponds with a titre index of 0 (= $1:10\times 2^0$). The titre indices of non-infected control rats to SP, EEF and SCH did not exceed 1, 0 and 2 respectively.

experiment in which 13-week-old rats were infected with 2×10^7 parasitized erythrocytes. The parasite number reached a peak 7 days after inoculation, when about 4% of the erythrocytes were infected with *P. berghei*. The infection became latent some 11 days later. IFA titres to SP and SCH rose steadily until around the 9th day when a plateau was reached. The titre to SCH was generally higher, however, than the titre to SP. Both tended to be higher than the titre to EEF. The titre of control rats to SP, EEF, and SCH did not exceed 1:20, 1:10 and 1:40 respectively. The influence of superinfection with *P. berghei* on IFA titres can be seen in Table 2. Eight rats, 11 weeks old, inoculated with *P. berghei*, reached a peak parasitaemia of 3·7–5·1% at 4–6 days after inoculation and recovered about 17 days later. 36 days after the first inoculation four rats were super-infected with 10^8 RBC parasitized with *P. berghei*. The superinfection did not elevate the titres. The inter-relationship of antibody titres with the three antigens as found also in the foregoing experiment was as follows: titres with SCH > SP > EEF.

TABLE 2. MTI* values ± s.d. in sera of eight convalescent rats, examined by IFA tests with sporozoites (SP), exoerythrocytic forms (EEF) and erythrocytic schizonts (SCH) as the antigen. Serum samples were collected after infection with *P. berghei* had been reduced to latency and after superinfection

	Days after first inoculation			
	Convalescent			Superinfected
	25	35	42	42†
SP	3·7 ± 0·6	5·3 ± 0·9	4·5 ± 0·7	5·6 ± 1·6
EEF	3·4 ± 0·5	4·6 ± 0·8	3·8 ± 0·5	4·3 ± 0·5
SCH	5·8 ± 0·9	7·6 ± 0·9	7·5 ± 0·6	7·5 ± 0·6

* For MTI see legend to Table 1. The titre indices of non-infected control rats to SP, EEF and SCH did not exceed 1, 0 and 2 respectively.

† Four rats superinfected with red blood cells parasitized with *P. berghei*, 36 days after first inoculation.

1.2. *IFA titres in rats immunized with sporozoites.* The following results were obtained: all rats immunized against sporozoites by the bites of infected mosquitoes developed high antibody titres to SP and low titres to SCH and EEF. The cross-immunity between antibodies to sporozoites and to the other stages of malaria was obviously lower than in rats infected by parasitized blood.

Three rats, 10 weeks old, were each bitten by about thirty infected mosquitoes, 14 days after the infective blood meal. The rats were protected against parasitaemia by chloroquine. Control rats were bitten by non-infected mosquitoes and treated also with chloroquine. Anti-sporozoite antibodies were visible already after 3 days and reached maximum titres within 1 week (see Table 3). In the next experiment, five rats, 11 weeks old, were bitten three times at day 0, 10 and 25. The antibody response is shown in the graph. After one bite the most striking IFA response was to the sporozoite antigen. The second bite by infected mosquitoes caused elevation in titres to all antigens, but the third bite did not cause any further increase. Unlike the single bite, whereafter no significant titres to EEF and SCH could be observed, the second and the third bite provoked a considerable response of antibodies to

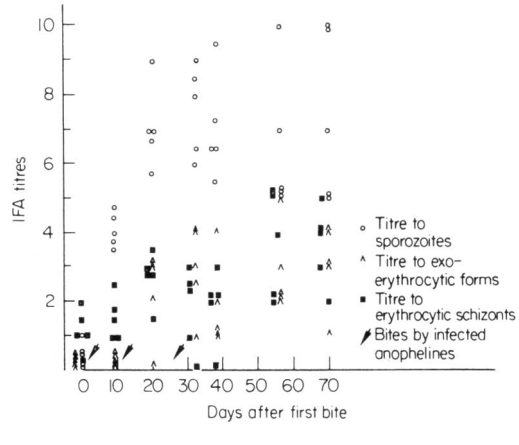

FIG. 1. IFA titres to different stages of *P. berghei* malaria in rats after 1 (day 0), 2 (day 10) and 3 (day 25) bites by infected mosquitoes. Each figure represents one rat. The results are expressed on a $\log_2$ scale where the initial dilution is 1:10. For more details see legend to Table 1. The titres of control rats bitten by non-infected mosquitoes and also treated with chloroquine did not exceed 1, 0 and 2 to SP, EEF and SCH respectively.

TABLE 3. MTI* values ±s.d. in sera of three rats immunized against sporozoites by mosquito bites, protected by chloroquine from parasitaemia and examined by IFA tests with sporozoites (SP), exoerythrocytic forms (EEF) and erythrocytic schizonts (SCH) as the antigen

	Days after immunization					
	3	6	9	12	15	22
SP	2·7±1·6	5·1±1·5	4·7±1·8	5·2±1·2	5·0±0·0	4·7±1·5
EEF	0·0	0·0	0·0	0·0	0·2±0·3	0·3±0·6
SCH	1·0±1·0	1·3±1·1	1·3±1·1	0·3±0·6	0·0	0·0

* For MTI see legend to Table 1. The titre indices of non-immunized control rats to SP, EEF and SCH did not exceed 1, 0 and 2 respectively.

these antigens. Control rats bitten by non-infected mosquitoes could not develop a significant response to any of these antigens.

1.3. *Comparison between IFA titres to P. berghei and P. vivax sporozoites.* Rats were immunized three times against *P. berghei* sporozoites by mosquito bites. The sera with high titres to *P. berghei* sporozoites (but also showing low titres to *P. berghei* EEF and SCH) were examined for crossreactivity with *P. vivax* sporozoites. The results are summarized in Table 4. The antibodies to the rodent *P. berghei* sporozoites did not crossreact with sporozoites of human type; no titre $\geqslant 1:40$ could be found against the sporozoites of *P. vivax*.

TABLE 4. Comparison of MTI* values in IFA tests with *P. berghei* and *P. vivax* sporozoites. Serum samples were collected from three rats, immunized against *P. berghei* by mosquito bites and protected by chloroquine against parasitaemia. Control rats bitten by non-infected mosquitoes were also treated with chloroquine

	P. berghei	*P. vivax*
Negative controls	0·2±0·4	0·0
Positive controls	6·3±0·6	0·5±0·9

* For MTI see legend to Table 1.

2. *The influence of immunization with erythrocytic schizonts or sporozoites on the development of those stages*

2.1. *The influence of trophozoite-induced parasitaemia upon challenge with sporozoites.* Adult, 14-week-old, and young, 6-week-old, rats were inoculated i.p. with 5×10^7 RBC parasitized by *P. berghei*. Peak parasitaemias of less than 4% and up to 50% were monitored for the adult and the young rats, respectively. Control animals and infected animals received an i.v. inoculation of about 10^5 sporozoites at different stages of the parasitaemia. 45 hr later, the livers were removed for the estimation of the density of EEF. In all experiments, densities of EEF in rats with a present or past parasitaemia were lower than in controls. Even in heavily infected rats, 50·2% parasitized RBC could prevent the development of the liver stages. Considerable reduction of developing EEF was especially seen during the rising phase of the infection. At that time no EEF could be detected at all in thirteen out of eighteen young rats (72%) and in seventeen out of twenty-five rats (68%). Table 5 summarizes the results of representative experiments estimating the influence of parasitaemia on the EEF density.

These results indicate that blood stages can be involved in the neutralization of infective sporozoites or can interfere with their development to EEF in liver parenchymal cells.

TABLE 5. The influence of trophozoite-induced parasitaemia upon the development of EEF

Days after inoculation	Age of rats (weeks)	Mean per cent parasitaemia of experimental group	* Ratio of EEF density controls/experimental group
5	6	12·88	1·42/0·00 = ∞†
	14	0·01	3·71/0·00 = ∞
11	6	3·82	6·39/1·74 = 3·7
	14	0·08	8·30/0·85 = 7·1

* EEF density is expressed as number of exoerythrocytic forms (EEF) in cm^2 liver tissue.
† The EEF density in control groups differs in separate experiments, according to the day of sporozoite harvest after the blood meal of the mosquitoes, the number of injected sporozoites, the viability of the sporozoites and the age of the rats. Each group consisted of six to eight rats.

2.2. *The influence of trophozoite-induced immunity upon a challenge with parasitized blood.* All rats that overcame an infection initiated by the injection of parasitized blood were fully resistant against the challenge of parasitized blood.

2.3. *The influence of immunization with sporozoites upon the development of parasitaemia following the challenge with parasitized blood.* Four rats, 11 weeks old, were immunized against sporozoites by one exposure to infected mosquitoes. The animals were protected against parasitaemia during the course of immunization by chloroquine. 2 weeks after the bite chloroquine treatment was stopped, and 2 weeks later the rats were challenged by parasitized blood. Another group of five rats also 11 weeks old were immunized three times by exposure to infected mosquitoes at 10-day intervals. These animals were similarly protected against parasitaemia by chloroquine. The drug administration was interrupted 2 months after the last bite, and 2 weeks later the rats were challenged with parasitized blood. Blood smears, taken every other day until challenge, did not reveal parasites. All the rats immunized once and even three times against sporozoites were susceptible to the challenge of the parasitized blood. The parasitaemias in the experimental group were similar to those of control rats exposed to non-infected mosquitoes and treated either with chloroquine or not at all.

2.4. *The influence of immunization with sporozoites upon the development of the parasites following a challenge with sporozoites.* Eight rats were immunized against *P. berghei* sporozoites by the bites of thirty infected mosquitoes. The rats were protected against parasitaemia by chloroquine. Six control rats were bitten by non-infected mosquitoes and treated also with chloroquine. 2 weeks after the mosquito bites chloroquine was removed, and another 2 weeks later all the rats were challenged by infective bites. In spite of marked differences in titres against sporozoites even on the day of challenge, parasitaemias in the control and experimental groups were the same, except for a delay in the onset of parasitaemia in the experimental group. The mean titre indices against sporozoites in the control and experimental groups were 0·2 and 6·8, respectively, while the incubation periods were 2·8 and 4·5 days respectively.

Attempts were made to immunize animals via repeated bites of infected mosquitoes. Seven rats (E1 and E3) were each exposed to fifty female *P. berghei*-infected anophelines, three times at intervals of 1 week. Three other rats (E2) were bitten once. 10 days later the latter group and three rats of the former group (E1) were challenged i.v. (7×10^5 sporozoites per rat) together with animals that had been bitten previously by non-infected mosquitoes (C1). The four remaining animals (E3) were challenged after 11 months (2×10^5 sporozoites per rat). Group C2 animals similarly exposed to non-infected anophelines served as controls for the latter group. Results of both challenges are given in Table 6.

TABLE 6. The influence of repeated immunization against sporozoites upon the development of EEF

10 days after last bite			11 months after last bite	
3 exposures (E1)	1 exposure (E2)	Control (C1)	3 exposures (E3)	Control (C2)
0·0*	0·8	12·8	2·3	6·7
0·0	1·0	15·0	2·6	13·4
0·0	2·1	51·4	3·2	20·3
Mean			3·4	
±s.d. 0·0	1·3±0·7	26·4±21·7	2·9±0·5	13·5±6·8

* EEF density expressed as number of exoerythrocytic forms (EEF) in cm^2 liver tissue. Each data point represents one rat.

In none of the three-times-exposed animals (E1) were EEF detected, and even group E2 differed markedly from group C1. The mean EEF number of group E2 was 5% of that in group C1. After about 1 year the interfering effect was still present, as judged by the difference between the results of groups E3 and C2; the mean number of EEF in group E3 was 21% of that in control group C2. The results suggest that increasing the number of immunizing bites causes not only a rise in titre against sporozoites (as shown in the graph) but also in protection.

DISCUSSION

Three plasmodial antigens from three different stages of *P. berghei* parasites were used throughout these experiments; sporozoites (SP); exoerythrocytic schizonts in rat liver (EEF); and schizonts in rat erythrocytes (SCH). The nature of the indirect fluorescent antibody (IFA) test allows the use of particulate antigen suspensions, even if these contain contaminants. SP antigen-containing sporozoites, also some bacteria and insect tissue, liver slices which contained about 60 EEF/cm^2 and blood with a parasite density of 12–16% were used. The objects fluorescing after application of immune sera could always be definitely identified as the particular stages of the parasite.

Rats immunized against sporozoites by the bites of infected mosquitoes and protected against parasitaemia by chloroquine developed high antibody titres to SP as early as 3 days after the first mosquito bites. Maximum titres of about 1:320 were reached within a week. The titres to SCH and EEF were lower than those to SP, and generally below 1:40. The anti-sporozoite antibodies were detectable much earlier in the IFA test than with the circum-sporozoite precipitation (CSP) test which becomes positive only after 3 weeks (Nussenzweig *et al.*, 1972). However, the CSP tests were performed on sera from animals injected with sporozoites by syringe; therefore, a direct comparison of the experiments is difficult. Subsequent exposure of the rats to the bites of infected mosquitoes enhanced anti-sporozoite antibodies but a third bite did not cause a further increase in titre. Unlike the single bite, that did not induce significant titres to EEF and SCH, the second and the third exposures provoked considerable antibody responses to these antigens. Control rats bitten by non-infected mosquitoes did not develop significant responses to any of these antigens. Spitalny, Rivera-Ortiz & Nussenzweig (1976) also found that both CSP and sporozoite-neutralization activity (SNA) titres rose considerably after one booster and reached a plateau after four boosts at levels of 1:40 and 1:80 in mice.

In blood-induced malaria of rats, titres of antibodies against SCH were already detectable 3 days after the inoculation of parasitized blood. Unlike the experiment with the natural bites (where parasitaemia was prevented by chloroquine) the rats with blood-induced infections showed high titres to all three antigens but the homologous reaction predominated. Superinfection with parasitized blood did not change titres.

This is in agreement with Ambroise-Thomas' observation (1969) that superinfection with *P. berghei*-infected blood did not change IFA titres to schizont antigen. The most likely explanation of the difference between the antibody production that follows sporozoite- or blood-induced infection and their reaction with different developmental stages of the parasite is as follows: the antigenic stimulus given by one dose of sporozoites is probably less intense than that of blood parasites with their repetitive multiplication cycle of 48 hr, causing a continuing administration of increasing quantities of antigen. Under these circumstances it can be expected that the antibody response towards sporozoites is more stage-specific than that of a blood-induced infection. Another dose of sporozoites will cause not only an increase in the anti-sporozoite antibody titre but also a gradual increase in the content of high-affinity antibodies with their greater potential for crossreactivity with similar antigenic determinants on EEF and SCH antigens. Other possible explanations for the enhancement of antibody titres to EEF and SCH antigen following repeated inoculation with sporozoites could be: (1) antigenic stimulus from developing EEF in the liver. These are less likely to exist since leakage out of parasitized liver cells would probably have provoked a cellular reaction, which is not the case (Vanderberg, 1973). (2) Stimuli from liver merozoites on their way to an (abortive) infection of erythrocytes. Although we did not find parasites in blood smears of rats immunized with sporozoites and treated with chloroquine, one can not exclude the possibility of a subpatent blood infection.

The influence of immunization with sporozoites or erythrocytic stages upon a challenge, either with sporozoites or with infected erythrocytes, was examined by estimating the density of EEF in the liver or by monitoring the developing parasitaemias. Rats which were immunized up to three times with sporozoites and challenged with infected blood could not prevent a normal development of parasitaemias. The same result was obtained in mice by Nussenzweig *et al.* (1969). Rats, immunized with sporozoites only once and challenged by bites of infected mosquitoes showed only a delay of the onset of parasitaemia by about 2 days. Increasing the number of immunizing bites caused a marked potentiation of the protection against the sporozoite-induced infection. Rats immunized by mosquito bites developed high antibody titres to sporozoites after one exposure, nevertheless the protection was obtained only after repeated exposures. This result can be explained by either a change in the quality and quantity of antibodies or by a longer induction period required for other immune effector mechanisms.

Rats with present or past parasitaemias were inoculated with sporozoites and 45 hr later the livers were removed for the estimation of the density of EEF. In all experiments densities of EEF in rats which suffered parasitaemia were lower than in controls [confirming and extending preceding work (Verhave, 1975)]. This protection-like effect could be due to antibodies reacting with the inoculated sporozoites, to non-specific activation of mononuclear phagocytes by the parasitaemias (Zuckerman, Spira & Ron, 1973), or to a concomitant change in the liver parenchymal cells, causing a loss in their capacity to function as host cells for EEF. Rats which overcame the infection initiated by the injection of parasitized blood were fully resistant to a homologous challenge. This is a well-known phenomenon that was also observed in our experiments. The overall results appear to suggest the induction of stage-specific immune responses. A role for circulating antibodies in the effector mechanisms is likely, but other phenomena such as the activation of mononuclear phagocytes as specific immune effects may be involved. With regard to the antibody response, high titres to SCH coincide with protection against reinfection with parasitized blood, and high titres to SP with varying degrees of protection against an infection with sporozoites. However, high titres to EEF and SCH in serum of rats three times exposed to sporozoites did not coincide with protection to a blood-borne infection. Also, high titres to SP in sporozoite-immunized rats do not necessarily mean that the animals are solidly protected against sporozoite-induced infection. We suppose that next to stage-specific antigenic determinants in the developmental stages of the parasite there also similar-antigenic determinants; the former would relate to stage-specific immunity, the latter to the are development of serological crossreactivity. This would explain why no clear correlation was found between high antibody titres of any serological test and protection. Moreover, since protection against sporozoite or blood-induced malaria is probably the result of concurrent or co-operating humoral and cellular mechanisms, all *in vitro* tests supposed to indicate a state of *in vivo* immunity should probably include both humoral and cellular reactions.

Further studies on the antigenicity of these parasites should also include the species-specific antigenic determinants that are clearly present, as indicated by the results of serological tests with *P. vivax* and *P. berghei* sporozoites: *P. vivax* sporozoites do not crossreact with *P. berghei* sporozoites. Though Nussenzweig and colleagues initially found crossreactivity among sporozoites of rodent plasmodia (1969), they could not demonstrate serological crossreactivity between sporozoites from different primate plasmodia (Nussenzweig *et al.*, 1973). Future sero-epidemiological work aiming at insight into immune reactions following life-long exposure to bites of infected mosquitoes seems feasible, therefore, only with sporozoite antigens from the specific *Plasmodium* transmitted in the endemic areas under study.

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