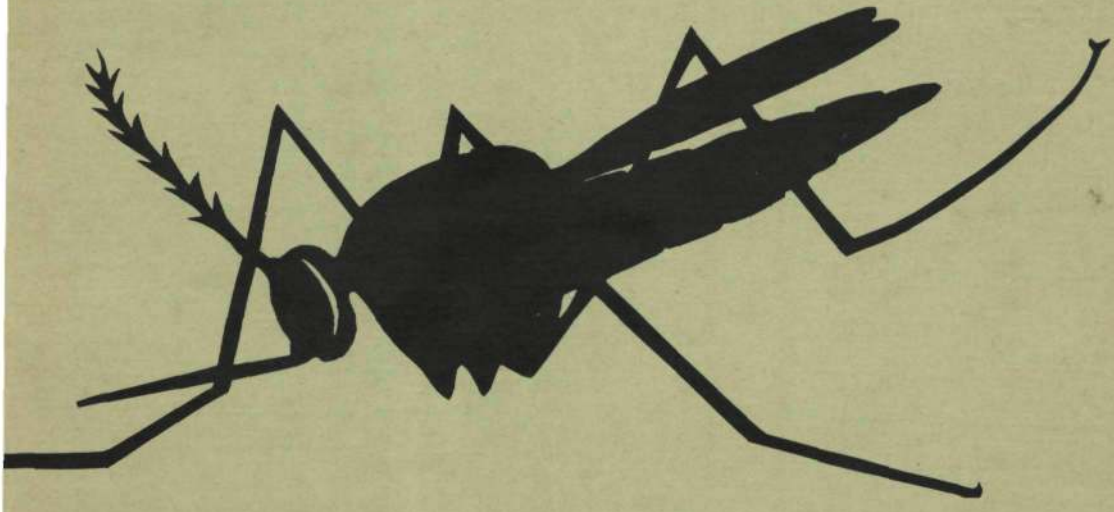
1981

IMMUNIZATION WITH SPOROZOITES

AN EXPERIMENTAL STUDY OF PLASMODIUM BERGHEI MALARIA

J. P. VERHAVE



IMMUNIZATION WITH SPOROZOITES

AN EXPERIMENTAL STUDY OF PLASMODIUM BERGHEI MALARIA

druk: Krips Repro B.V. Meppel

PROMOTOR

DR. J. H. E. Th. M E U W I S S E N

IMMUNIZATION WITH SPOROZOITES

AN EXPERIMENTAL STUDY OF PLASMODIUM BERGHEI MALARIA

PROEFSCHRIFT

ter verkrijging van de graad van Doctor in de Geneeskunde
aan de Katholieke Universiteit te Nijmegen
op gezag van de Rector Magnificus
Prof. Mr. F. J. M. Duynstee
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag 2 oktober 1975
des namiddags te 4 uur.

door

JAN PETER VERHAVE
geboren te Bussum

Het practisch werk voor dit proefschrift werd verricht van 1972 - 1975 op het Instituut voor Medische Parasitologie, onder leiding van Dr. J.H.E.Th. Meuwissen, lector aan de Faculteit der Geneeskunde van de Katholieke Universiteit te Nijmegen.

GENERAL INTRODUCTION

Malaria is still an important disease of the tropics. Despite the considerable achievements of malaria eradication in the past, still nearly 500 million people are unprotected. Logistical, sociological, financial and technical problems interfere with the interruption of malaria transmission. Multidisciplinary research devoted to the bio-medical aspects of the vector-parasite-host interrelationship is required to bring closer the goal of global malaria eradication.

Some of these aspects can be elucidated by the study of malaria parasites in animal models, and it is often practicable to apply and extrapolate knowledge from such laboratory systems to the problem of human malaria. Even then, understanding of human malaria remains difficult, since four species of malaria parasites occur in man, and the course of the disease varies with each species.

Roughly, the life cycle of these parasites, belonging to the genus *Plasmodium*, is as follows: different species infect various vertebrate hosts, including reptiles, birds and mammals. They all need an insect host for part of their development. Mammalian plasmodia require mosquitoes of the genus *Anopheles* as vectors. While infected mosquitoes bite, parasite stages called sporozoites, are inoculated into the blood. Within one hour infective sporozoites disappear from the circulation, and in susceptible mammals they invade parenchymal cells of the liver. After intracellular replication as exo-erythrocytic forms, newly formed merozoites are released into the blood, where they parasitize and multiply inside erythrocytes. Schizogony during the erythrocytic cycle initiates the known pathological effects such as

fever, anaemia and splenomegaly. Depending on the host-parasite system, a more or a less efficient immune response occurs after prolonged contact with these parasites.

Various approaches exist for interrupting the life cycle of the parasite in its mosquito- and human host. Two main ways of interference are the control of the vector, and the use of antimalarial drugs.

However, in large areas of the world even a combination of these methods cannot achieve eradication. It is doubtful if the extermination of parasites with such complex ways of life is attainable. In practice it appears impossible, due to the resilient nature of the eco-system: many species of the mosquito vector have become resistant to promising insecticides, and plasmodia have developed resistance to otherwise highly effective drugs.

These aspects have contributed to the general trend of a halt in the progress of malaria eradication programs over the past few years.

Greater insight into the immune responses of vertebrate hosts appears to be a prerequisite for breaking this impasse, and may facilitate the production of more advanced and ecologically more acceptable control methods, such as vaccines. Knowledge of additional conditions for the production of vaccines is still very incomplete. Neither the cultivation of parasites, nor their biochemical analysis have been succesfully performed, nor do we know enough about the effective antigens. Hence the prospects for vaccination of man against malaria are still beyond our practical possibilities.

Nevertheless, attempts to vaccinate laboratory animals and humans with non-virulent, attenuated parasites have been made. Passages *in vitro* or through insusceptible hosts have been tried. Killed parasites, or fractions thereof have been used, but protection against virulent parasites has only been attained in exceptional cases. Recently, however, immunization with *in vitro* grown merozoites in adjuvant turned out to be an effective way of inducing protection in monkeys.

Also the use of irradiation for attenuation of the parasites has appeared to be a promising approach. In a number of studies erythrocytic stages have been attenuated by irradiation, since it is these that are directly responsible for the pathogenic effects of a normal infection. Again, consistent and reliable protection of animals against infection with virulent parasites appeared to be a difficult problem.

Other stages of the parasite have also been used for attenuation. Evidence has been presented that irradiated sporozoites can be used as a vaccine.

During the past eight years Nussenzweig and her co-workers of the New York University Medical School, have made considerable progress in the demonstration and understanding of innate and acquired responses to single and repeated inoculations of mammalian plasmodial sporozoites. It was demonstrated that irradiated sporozoites elicit stage specific antibodies and that after repeated contact with these sporozoites, the hosts are completely protected against the development of parasitaemias following the administration of normal sporozoites.

At present important questions concerning the role of antibodies, and lymphoid and phagocytic cells in the protective immune response are still incompletely solved.

The main objective of the present study has been the collection of more data on the sporozoite- host interrelationship. The aim was to study the effect of repeated contact with normal sporozoites of a rodent *Plasmodium* in rats. Attention was paid to the decrease of susceptibility to infective sporozoites, after previous exposures to malaria parasites.

A method for the assessment of sporozoite infectivity was applied, which gives more direct information than the techniques in common use.

The study of the anti-sporozoite immune mechanisms is not very advanced yet, and results are still preliminary. Insight into the hosts responses to normal sporozoites may be of value for future progress in the production and application of sporozoite vaccines, and for better comprehension of the epidemiology of malaria.

CHAPTER I

THE PRE-ERYTHROCYTIC DEVELOPMENT OF A MALARIA INFECTION

1.1 The infection with sporozoites

The strain of *Plasmodium (Vinckeia) berghei* Vincke & Lips 1948 used this study, was isolated in Zaïre, in a forest gallery near Lumumbashi in 1965 by Vincke and Bafort. In 1968 some properties of this, so called Anka-strain, were published (1).

The local insect host is *Anopheles dureni millecampsi* and the mammalian host is the ticket-rat, *Grammomys surdaster*, also referred to as *Thamnomys surdaster*.

Sporogony is obtained in laboratory-bred *A. stephensi*, when maintained at 18-21° C (2). A variety of rodents are susceptible to blood-induced infections, whereas white rats, hamsters, and, to a lesser extent, mice are susceptible to infection with sporozoites (3).

Aspects of the pre-erythrocytic phase of *P. berghei* are reviewed here, in order to facilitate the appreciation of the results presented in this study. Stages of the life cycle of the malaria parasite can be found in a variety of habitats: the mosquito, the liver and the blood of vertebrate hosts. That part of the life cycle which starts with the inoculation of sporozoites into the vertebrate host and ends with the infection of erythrocytes, is described here as pre-erythrocytic development. Exo-erythrocytic forms (EEF) are those stages in the pre-erythrocytic development that parasitize liver parenchymal cells. The sporozoites in particular lead a variegated life. They are generated in the oocyst on the outer wall of the mosquito stomach and released into the haemocoel. From there, they move or are transported through the haemolymph to the salivary glands. They penetrate the secretory cells and enter the secretory mass, where they are

stored (4) and then have a mean length of about 12 µm (5).
The sporozoites are released with the saliva of the mosquito. They are mobile, and this activity may attribute to the penetration of salivary glands in the mosquito and of liver parenchymal cells in the vertebrate host (6). Vanderberg recently established that sporozoites glide with their apical ends (=conoid) trailing when moving (7).
The sporozoites are deposited intracutaneously or directly in the circulation by the bite of the mosquito. Nothing is known of tissue reactions to sporozoites which stick in the subcutaneous tissue. It has been demonstrated by Beckman (8, 9), however, that the skin of an incompatible host (mouse) is a very effective barrier to sporozoites (*P. gallinaceum*).
Those sporozoites that enter the circulation either by the bite of a mosquito, or by artificial intravenous inoculation are exposed to phagocytic cells lining the bloodvessels. 1-2 hours after inoculation sporozoites can no longer be demonstrated in the circulation, as concluded from subinoculations of blood of infected volunteers (10).
These short circulation times have also been found by Nussenzweig and coworkers in an experimental system with A/J mice and *P. berghei*. Most of the sporozoites appeared to be cleared from the circulation within one hour (11). Since the blood is recirculating, they could pass the liver several times, and on one of these occasions they may succeed in penetration of the parenchymal cells. The processes of migration towards the liver, and of subsequent recognition, attachment and penetration have never been described. The sporozoites that do not succeed in penetration are likely to be phagocytosed. An *in vivo* demonstration of such a process has never been given. Presumably the Kupffer cells of the liver and perhaps the spleen macrophages, are the main cells involved.
When sporozoites have finally succeeded in penetrating parenchymal cells, they will start to develop into exo-erythrocytic forms (EEF). Maturation of this stage takes about 48 hours in *P. berghei* (4, 12), and at the end of this period the schizont has a diameter of about 40 µm. Estimations of the number of merozoites, produced in the liver of white rats, range from 4000 - 8000 (4, 13). In *Grammomys* these numbers may be twice, and in mice half as large (13).
The schizogony inside liver parenchymal cells is followed by the liberation of formed merozoites, when the parasitized host cells are destroyed. Immediately after this rupture the site is infiltrated by leucocytes. These

cells, which are mainly polymorphonuclear granulocytes and macrophages, phagocytose liberated merozoites. It is a wellknown fact that a considerable number of these merozoites are prevented from penetrating into erythrocytes (14). This should not be ignored in estimations of the number of erythrocyte-penetrating merozoites that originate from one sporozoite.

1.2 Biological aspects of sporozoites

Recently Vanderberg (6) clearly demonstrated that very few sporozoites from oocyts are infective (i.e. cause a parasitaemia in mice). Sporozoites acquire infectivity when migrating via the haemocoel to reach the salivary glands. Maximal infectivity was observed in sporozoites that were stored for some days in the glands. Comparing immature stages from oocysts and mature stages from salivary glands, a 10.000-fold increase in infectivity was found. This increase appears to be time-dependent rather than site dependent. When sporozoites remain in mosquitoes for longer times they tend to loose their infectivity again. Infectivity of sporozoites also rapidly decreases when they are kept *in vitro*.

Chilling the medium is favourable for the retention of infectivity, and the choice of the medium may also be important. Vanderberg demonstrated that serum albumin strongly preserves the infectious capacity of sporozoites, and that motility was also considerably increased (7). This correlates with the fact that blood is a natural habitat for sporozoites.

In Vanderberg's studies *infectivity* was defined as the proportion of mice developing a parasitaemia, whereas the percentage of sporozoites which develop into mature exo-erythrocytic forms was defined as *viability*.

A review of the literature on sporozoites reveals a confusing use of the terms viability and infectivity. Sometimes sporozoites are considered viable when they are motile, or capable of developing into EEF, or inducing a parasitaemia; on the other hand sporozoites are called infective, when they invade liver parenchymal cells, with or without causing an erythrocytic infection.

An *in vitro* test for the detection of their capacity to multiply is not available and *in vivo* estimates can be obtained only retrospectively. Therefore definitions should be strict, and accepted generally. Moreover they must reflect modern insights into the described properties and phenomena.

With regard to the above mentioned definitions, it should be stated that a

fig. 1. Schematic presentation of the development of Plasmodium in the mammalian host, with emphasis on the various possibilities of its interruption during the pre-erythrocytic stage.

parasitaemia is not necessarily a reflection of the properties of a sporozoite, and by consequence it is only an indirect measure of the infectivity. On the other hand, when viability is defined as the capacity to develop into EEF, one must assume that sporozoites from the same source, failing to develop into mature EEF in an unsusceptible host, should be considered non-viable. Also immature sporozoites should generally be considered non-viable by this definition. It is hard to avoid the impression that this is a non-functional approach.
The following definitions are proposed and used throughout this study.

Viable are those sporozoites that are apparently healthy, intact and motile, and supposedly capable of multiplication.
Pending the development of methods for *in vitro* cultivation of the exo-erythrocytic stage in cell lines, assessment of the viability is unattainable.

Infective are those viable sporozoites that are able to develop into exo-erythrocytic forms within (liver parenchymal) cells of a distinct host.

In fig 1 some of the possibilities are depicted.

Using these definitions, viability is considered a property of the organism, whereas infectivity is subject to a given host-parasite inter-relationship. Sporozoites in the salivary glands of a mosquito are thought to represent a variety of maturation stages (6), but also degenerating, non-viable sporozoites might occur (6). Moreover, when an inoculum is transferred to a susceptible host via a mosquito bite or intravenous inoculation, apparently only a small and variable fraction of sporozoites actually develops into EEF (16).
This means that, depending on the host's susceptibility, many viable but non-infective sporozoites are lost. The possible consequences of this are considered in sections 5.3 and 5.6.

1.3 Immune responses to sporozoites

1.3.1 *Early attempts at immunization with sporozoites*

The question concerning antigenic properties of sporozoites is not new. In 1936 Boyd and Kitchen (cited in 17) had wondered if the acquired homologous immunity to *P. vivax* was equally effective against sporozoites and trophozoites. They suggested that the sporozoite might be antigeni-

cally different from the trophozoite, and that the immunity of the host might be directed mainly against the trophozoite rather than against the sporozoite. A more systematic study on the immunogenicity of sporozoites was initiated some years later by Mulligan, Russell and Mohan (18). They demonstrated that salivary gland sporozoites of *P. gallinaceum*, *P. malariae* and *P. falciparum* readily agglutinate in sera obtained during the chronic stages of homologous parasitaemias of sporozoite-induced infections. Considerably weaker agglutination was found in sera obtained during accute stages of homologous parasitaemias of such infections and in sera from animals with heterologous parasitaemias. This agglutination was claimed to be a specific reaction though normal sera also agglutinated the sporozoites at low titre. Sera of trophozoite induced infections also agglutinated sporozoites at low titre. Apparently the reaction was not completely stage specific. They also found that, when sporozoites of *P. gallinaceum* were attenuated in ultraviolet light and repeatedly inoculated, sera of these chickens showed sporozoite agglutinating properties (19).

Interestingly, parasite densities induced with non attenuated sporozoites were lower in such birds, and parasitaemias were less frequently lethal than trophozoite-induced parasitaemias. Fowls immunized with mixtures of many dried sporozoite containing thoraces invariably developed parasitaemias after being bitten by two infected mosquitoes, but percentages and mortality rates were lower than in controls.

The immunized animals were not protected against infections with trophozoites. It was concluded that sporozoites elicit a clear specific immune response (10).

Much later, however, Garnham and Bray (21) found that sporozoites of *P. cynomolgi* developed normally as EEF in monkeys, previously immunized by a sporozoite-induced infection. Numbers of EEF in these animals were reported to be comparable with those in normal monkeys.

Garnham (22) further stated that *P. cynomolgi* sporozoites, incubated in immune sera, were capable of developing into normal EEF and of inducing a parasitaemia. A third finding concerned the preservation of infectivity of sporozoites exposed *in vivo* to the blood of a sporozoite-immunized monkey and subinoculated into a non-immune monkey.

These arguments led the author to state that sporozoites provoke no immune response, either because they are present in the circulation for too short

a time, or because the number of phagocytosed sporozoites is too small for providing a sufficient quantity of antigen to stimulate the production of antibodies. Further he failed to boost the immune response with formalinized sporozoites in adjuvant, in a monkey that had previously been infected with sporozoites of *P. c. bastianellii*.
After challenge, EEF of normal morphology were found in comparable numbers to those found in a control monkey. Sporozoites were found to agglutinate in serum of this immunized monkey.
Garnham concludes that living sporozoites in a susceptible host are apparently non-antigenic, presumably because their antigenic components remain masked in liver parenchymal cells. He considered the non-viable sporozoites that are not able to penetrate liver parenchymal cells as the only possibility for anti-sporozoite responses. In our discussion on epidemiological consequences of antisporozoite immunity we shall come back to these conditions (see section 5.6).

At the same time Richards (23) repeated the experiments of Mulligan's group with sporozoites of *P. gallinaceum*. He confirmed that three inoculations of dead or U.V. attenuated *P. gallinaceum* sporozoites into young chickens induced high antibody titres in the sporozoite agglutination test. When challenged with viable sporozoites the animals survived subsequent low grade parasitaemias. U.V. and formalin treated sporozoites and dried sporozoites were highly immunogenic. Freeze-thawed sporozoites also induced partial protection *in vivo*, and agglutination *in vitro* to a lesser degree. Since then the *P. gallinaceum*-chicken system has been abandoned in further studies on the anti-sporozoite immune response. Only recently was some work published on immunization with formalin killed merozoites from in vitro cultivated EEF of *P. fallax* of the turkey. A stage specific immune response was demonstrated which conveyed no protection against erythrocytic stages (24, 25).

The promising approach of Richards has been applied in the mouse-*P. berghei* model by Nussenzweig. Together with her colleagues she greatly contributed to our knowledge of cyclical transmission of *P. berghei* in the laboratory (26, 27) and to the development of the exo-erythrocytic forms (28, 29). Initial attempts to immunize rodents with killed sporozoites were unsuccessful. It was discovered, however, that attenuation of sporozoites could be produced with specific doses of X-irradiation (30).
These attenuated sporozoites could no longer cause a parasitaemia, and after

challenge with viable sporozoites, a number of animals were protected against parasitaemia. This discovery led to a long series of papers devoted to the subject of immunogenicity of sporozoites and the possibilities of using sporozoites as an anti-malaria vaccine.
These studies, together with some additional results from others, are extensively reviewed under four successive headings. Firstly, the phenomenon of protection will be discussed, then the humoral factors involved, next attention will be given to non-specific factors of protection and finally alternative approaches will be considered.

1.3.2 *Sporozoite induced protection*

Mice that received a previous inoculation with irradiated sporozoites showed a variable length of the prepatent period (the time lapse between inoculation and detection of the first circulating erythrocytic forms in blood smears after challenge with sporozoites). Animals developed either a delayed parasitaemia or no parasitaemia at all. This variation could not be standardized, but, in a single experiment, the number of animals that developed no parasitaemia after challenge with non-attenuated sporozoites appeared to be dose dependent to some extent. With 5000 sporozoites more than half of the animals got a parasitaemia after challenge, whereas with 75000 sporozoites only about 1/4 of the mice became patent (30, 31). Further increase of this single immunizing dose did not enhance the number of protected animals (32). The degree of protection decreases from 100-0% within 2 months (32). Protection is defined by them as survival without parasitaemia. Thus only an all or none effect is measured. Partially protected are considered to be those animals that develop a delayed parasitaemia and consequently die. Despite this fatal course of parasitaemias of *P. berghei* in mice, the prolonged prepatent period is indicative of some anti-sporozoite immune response.
Protection is consistently enhanced when the number of immunizing or booster inoculations with irradiated sporozoites is raised to 2-4, administered once or twice a week. But its degree varies. To obtain 100% protection it appeared to be sufficient to use 4 boosters with considerably less sporozoites than the initial dose (initially 75.000, boosters 5000-20.000) (31, 33). Challenges of these immune animals (i.e. animals completely protected), even with large doses of non-attenuated sporozoites did not result in parasitaemia (34), as compared with non-immunized control mice. This state

of being protected remained intact for some months (33). X-irradiated, infected mosquitoes can also induce a solid protection (37). Protection was best established by intravenous inoculations; intramuscular and intracutaneous inoculations were less effective (32, 35, 36).
In two out of several attempts, Nussenzweig succeeded in protecting rhesus monkeys, which had been immunized with multiple inoculations of large doses of irradiated sporozoites over a period of several months (53). Attempts at other laboratories were unsuccesful, probably due to insufficient numbers of sporozoites. At best a delayed prepatent period was found after challenge infections. This suggested that sporozoites, able to initiate tissue stages were reduced in number (39, 40).
Some of several human volunteers immunized by bites of X-irradiated mosquitoes, infected with *P. falciparum* or *P. vivax*, did not develop a parasitaemia for months after repeated challenge with homologous sporozoites (38, 41, 43).
However, it is still not a simple matter to establish protection against parasitaemias in primates, using attenuated sporozoites as a vaccine (38, 53).

X-irradiation has a clear effect on sporozoites. With 8000-10.000 rads no parasitaemias develop. However, at a dose of 8000 rads in one out of 11 livers some partially developed EEF were found (44). This indicates that irradiated sporozoites are able to penetrate liver parenchymal cells.
The infection is considered to be abortive. This single observation led to the hypothesis that attenuation does not necessarily abolish the penetration mechanism, nor the onset of multiplication. At lower doses of X-irradiation more retarded EEF are found in combination with prolonged prepatent periods (32). However, the viability of the inoculum is important; highly viable sporozoites need larger doses for attenuation (44).
These results have been confirmed in monkey malaria. Warren and Garnham (45,46) using X-irradiated sporozoites of *P. cynomolgi*, concluded that the development of EEF is influenced; the changes probably result from nuclear alterations. They also confirmed that the irradiation effects on development of EEF are dose dependent. A high dose of 10 krads resulted in no detectable EEF nor in a parasitaemia. Smaller doses initiated smaller EEF with few nuclei. Whether these were retarded or degenerative forms could not be established definitively.
The spleen appears to play no key role in the protective response.

Extirpation after 5 immunizing doses and before challenge hardly reduced the number of protected animals and immunization of splenectomized mice still led to 60% protected animals at challenge (32, 36).
Mice immunized with immature sporozoites (i.e. from oocysts or haemolymph) appeared not to be protected, when challenged with mature non attenuated salivary gland sporozoites (47).
Sporozoite-immunized mice remained totally susceptible to challenge with erythrocytic stages (31) and gametocytes developing in these animals were infective to mosquitoes that subsequently developed infective sporozoites again (47). This means that ookinete formation was also unhampered. Moreover, there was no evidence of interference by anti-sporozoite-immune serum *in vivo* development and morphology of isolated EEF, nor with the infectivity of originating primary merozoites (48). On the contrary active immunization with sporozoites or passive immunization with immune serum led to reduced numbers of EEF after challenge. The detectable EEF were smaller and less differentiated (49).
After challenge with heterologous sporozoites (i.e. *P. vinckei* and *P. chabaudi*) the immunized mice also appeared to be completely protected against these other rodent plasmodia (31, 33).
After immunization with huge numbers of mosquito borne sporozoites of both species, one volunteer appeared to be protected for 3 months to several strains of *P. falciparum* but not to *P. vivax*. Conversely, when he was protected for 6 months to strains of *P. vivax*, he was susceptible for *P. falciparum* (38).

1.3.3 *Humoral factors in the antisporozoite response*

Vanderberg and his colleagues (49) described a new *in vitro* approach for the detection of specific antibodies. Viable sporozoites incubated in a suspension medium with immune serum develop a thread-like precipitate on one end. Endpoint titres for these precipitins could be determined by incubation in various dilutions. The reaction was called circum-sporozoite precipitation (CSP) reaction.
The name is not quite adequate since the precipitate is not *circum*-sporozoite in viable sporozoites, but it is generated at the terminal apical cup (=conoid). Only freeze-thawed sporozoites show a real circum-sporozoite precipitate. Evenso, this name has been used for uniformity.
The reaction is sporozoite specific, since it could not be demonstrated in

serum of animals with a history of blood-induced parasitaemias. It was postulated that this CSP correlated with protection, and thus could serve as an *in vitro* method of demonstrating protection (49). Therefore, much work has been done to elucidate the merits of this test.

One intravenous immunizing dose with 75.000 irradiated sporozoites induces precipitates with non-irradiated sporozoites in sera, taken 35 weeks after inoculation. Larger doses initiate a more rapid appearance of CSP and result in higher titres (32). After a booster the CSP titre is detectable after 7 days and rapidly reaches a given level. Further boosters increase this level, which remains constant for more than 3 months (32). CSP reactions occur independently of complement factor C3 and perhaps, also of C1 and C2. But the reaction is apparently dependent on a cofactor present in immune as well as in normal serum. It is assumed that this serum factor stimulates antibody formation to the CSP antigen (32).

Though technically difficult, more information about the mechanism of precipitates may be obtained by absorption of sera with intact or disrupted sporozoites (Kagan, in discussion 34).

Intramuscular and intracutaneous inoculation also resulted in CSP reaction, but titres were lower than with intravenous inoculation. Sporozoites from oocysts produce very low CSP titres, those moving towards salivary glands produce higher titres, whereas salivary gland sporozoites give rise to the highest CSP titres. Heterologous rodent malaria sporozoites also form CSP after incubation in immune serum, but at lower titre than the homologous sporozoites (31). In monkeys and man, repeatedly inoculated with sporozoites of simian and human plasmodia respectively developed variable CSP antibody responses (38, 50).

It has previously been demonstrated that sporozoites elicit clear antibody responses in incompatible hosts (51). Rats appear to be very good anti-sporozoite antibody producers. Immunization with simian or human sporozoites to which rats are incompatible, nevertheless lead to strongly positive CSP reactions. However, positive reactions occur only with the homologous system, i.e. *P. falciparum* sporozoites showed no precipitate when incubated with anti-sporozoite sera of other primate plasmodia and vice versa. Only cross-reactivity between sporozoites from different strains of the same species could be demonstrated. It was suggested that, due to the absence of cross-reactivity a possibility exists for distinguishing various species of sporozoites, for example in epidemiological studies (52, 53).

This model of an anti-sporozoite immune response in an incompatible host has also been used to compare the antigenicity of the various developmental stages of sporozoites of simian plasmodia. Again it was found that salivary gland sporozoites are more immunogenic than oocyst sporozoites with regard to CSP reactions (54).

As stated above CSP reactions were at first thought to be indicative of protection, but CSP did not coincide with protection. After a single immunizing dose, some of the animals appeared to be protected after one week when challenged with non-attenuated sporozoites.

But CSP titres could not be demonstrated until 3 weeks after that immunizing dose (32, 36, 55). Moreover, immunization with heat-inactivated or fractionated, or irradiated sporozoites via the intraperitoneal route induced CSP antibody formation, but most of these animals appeared not to be protected. On the contrary, in animals immunized after splenectomy, virtually no CSP antibodies could be demonstrated, though 60% appeared to be protected. Recently it was found that immunization of thymectomized mice did not lead to protection, yet some CSP antibodies were demonstrable (56).

It was suggested that the spleen is a major site for CSP antibody production, but this type of antibody is not protective. Nevertheless, the possibility cannot be excluded that they may play an additional role in the *in vivo* clearance of sporozoites in immune hosts.

The circulation time of infective sporozoites has been used as an *in vivo* expression of anti-sporozoite activity. After intravenous inoculation of sporozoites blood samples were collected at regular intervals and sub-inoculated into normal recipients. The later development of a parasitaemia in these recipients was thus a sign for the presence of infective circulating sporozoites in the donor (57). After one hour apparently all infective sporozoites had disappeared from the circulation of normal donors, thus confirming former data from the human model (10). However, in mice that had been immunized with irradiated sporozoites the circulation time of non-irradiated sporozoites was much shorter and took no longer than 5-10 minutes. An accelerated clearance is thus taking place in immune animals. In mice that had received a passive transfer of immune serum, sporozoites also disappeared more rapidly from the circulation than in animals with a transfer of normal serum, when these passively immunized mice were challenged with sporozoites. All of them developed a parasitaemia.

The first erythrocytic stages were detected later than in controls, thus

these animals could be considered only partially protected. Even repeated transfers of immune serum fail to abolish infectivity of all sporozoites of a challenge inoculum (32, 34, 36). The observations indicated however, the probability of a humoral factor operating in the anti-sporozoite immune response. Protection was always partial. It means that other mechanisms must be involved as well.

Spitalny developed another method to detect humoral anti-sporozoite activity. Non-irradiated sporozoites were incubated *in vitro* in sera from immunized animals (33). After 45 minutes these sporozoites were inoculated into mice together with the incubation medium and the length of the prepatent period determined. Patency was generally delayed after incubation in immune serum. Thus the infectivity of these sporozoites was affected, and morphologically these sporozoites appeared to be altered.

The serum factor was called sporozoite neutralization activity (SNA). No total neutralization was found after incubation for periods shorter than 20 minutes. This was in agreement with the inactivation time indicated by *in vivo* clearance during circulation in the blood of immune animals (32). Seven days after a primary inoculation no SNA could be demonstrated in sera of those animals that were protected. After boosting, SNA was detected with increasing titre (up to 1:80 after 5 or more inoculations). Heat inactivation of the serum reduced this activity, which could not be restored by addition of whole complement. Moreover, complement factor C3 and the alternative pathway for complement activation were no prerequisites for SNA. Lysis of sporozoites is apparently not essential for neutralization and the antibodies involved may be heat labile.

Participation of complement factors C1 and C2 could not, however, be excluded as participating in neutralization activity. Since CSP could not be correlated with protection, the suitability of SNA as a parameter for protection was investigated. If SNA is an *in vitro* equivalent for protection its presence should coincide with protection, and without protection either no or low SNA should be detectable (32).

With regard to the first stipulation it was shown that SNA becomes detectable in a way similar to that of CSP: a single dose of sporozoites, by which some of the animals are protected, is not followed by the detectability of SNA, but this readily occurs after a booster inoculation, with titres up to 1:80.

Like CSP, detectable levels of SNA are not an essential component of protective immunity during the early stages of its development. Immunization with heat inactivated sporozoites induced CSP without protection, but hardly detectable titres of SNA. Animals that were protected despite splenectomy prior to immunization (60%), showed no CSP and had low SNA (32). Animals thymectomized before immunization, that were not protected, showed CSP but no SNA. After reconstitution with thymus cells, these animals again showed positive SNA. From these experiments it was concluded that some limited value can be placed on the SNA test as an *in vitro* indicator for the *in vivo* state of immunity (56).
The data on protection and serology pointed towards an additional mechanism, involved in the anti-sporozoite response. Thymus dependent mechanisms might play a role, as was suggested by the results with thymectomized and reconstituted mice. However, transfer of cells from various organs of immunized mice generally conferred no protection to normal recipients (53).

1.3.4 *Non-specific anti-sporozoite factors*

Since inocula of sporozoites from dissected salivary glands always contain contaminants of mosquito tissue and adherent microorganisms, it was thought that this debris might have an adjuvant effect (57). Repeated inoculation with salivary glands leads invariably to parasitaemia after sporozoite challenge. Before challenge these animals show no sign of CSP (49), nor do mice that were bitten by X-irradiated, but uninfected mosquitoes (47).
In contrast, when mice are bitten on several occasions by X-irradiated infected mosquitoes, they appear to be totally protected after intravenous challenge. By this way of immunization minimal contamination with mosquito antigen occurs (36). Eventual non-specific effects of mosquito debris were extensively studied, since Alger *et al.*(58) reported total protection of mice after intraperitoneal immunization with ground uninfected mosquitoes. Moreover, Nussenzweig had already established delayed patency and even protection after previous inoculation with *Corynebacterium parvum* (59). Measurements through clearance of injected carbon, revealed a short increase of non-specific phagocytosis after inoculation of uninfected salivary gland suspensions. After an optimum on day 2, phagocytic activity decreased to normal levels. Booster inoculations of mosquito tissue further promoted the phagocytic response; each time the carbon clearance became normal. When inocula of salivary glands contained sporozoites, the phagocytic

activity was significantly increased.
Spleen weights gave similar results. Unparasitized salivary glands induced a small increase after 2 days that could not be further increased with boosters. Inocula with irradiated sporozoites induced a more prominent increase of spleen weight, which decreased to normal levels after one week. Boosters gave the same effect; only short lasting increases, without cumulation. Also extirpation of the spleen reduced non-specific phagocytosis. There is thus no correlation between protective immunity and stimulation of the mononuclear phagocyte system. However, it cannot be excluded that this stimulation is important during the early stages of the anti-sporozoite response (57). In order to bypass these non-specific factors in this immune response, the described purification technique of sporozoites, using density gradient centrifugation might be of value (60).

1.3.5 *Alternatives for X-irradiation*

Various treatments of sporozoites were compaired with X-irradiation. γ-irradiation gave comparable results to X-irradiation, but immunization with heated or freeze-thawed sporozoites induced less protection (32, 35). Alger and colleagues reported also some experiments with heat-inactivated and freeze-thawed *P. berghei*-sporozoites (58). After 6 intraperitoneal inoculations, challenge resulted in 80 - 90 per cent protection! However, non-infected salivary gland tissue also induced 50 - 70 per cent protection. Further data would be of use, since others could not confirm these results. Their examination of the treated sporozoites by electron-microscopy revealed that the freeze-thawed sporozoites had been completely disrupted, whereas heat-inactivated sporozoites were very like the control sporozoites. The authors suggest that, in view of the comparable immunization result, the sporozoite protective immunogen is of stable molecular composition, perhaps of membrane origin (61).
Spitalny and Nussenzweig were not able to induce a high degree of protection with extracts of ultrasonicated or homogenized sporozoites, but CSP could be demonstrated in the serum of these animals (35). Young sporozoites from either oocysts or from the haemolymph that did not induce protection, led to CSP producing antibodies, but these precipitins were of another class to those specific for mature salivary gland sporozoites. Probably the antigens to which these antibodies are formed are functional in penetration of salivary glands, and of liver parenchymal cells, respectively (48).

Some attention was given to the immunogenicity of non-irradiated sporozoites. One experiment has been described in which adult rats received a single dose of either irradiated or non-irradiated sporozoites.
Longitudinally CSP reactions were determined. It appeared that titres of both groups rose after 20 days. Titres persisted in the rats that received irradiated sporozoites, but dropped to zero in the rats with non-irradiated sporozoites. From this result it was concluded that irradiated sporozoites are more immunogenic. This result was thought to be due to altered antigens, arrest of development, or fixation in other organs (32, 35, 36, 54). Booster inoculations with sporozoites induced high titres in both cases.
Strangely enough some reviewers misinterpretated this finding and concluded that non-attenuated sporozoites are not immunogenic (62, 63).
In the discussion (section 5.1) these results and conclusions are commented on in detail, because this single experiment has been an important encouragement and challenge for the present study.

In conclusion, we wholeheartedly agree with Nussenzweig's complaint that " there is a deeply rooted statement that sporozoites are not immunogenic ". The investigations of her group have convincingly demonstrated that sporozoites are immunogenic, indeed, and that immunization with this stage induces a solid protective response.
The studies reported below confirm and extend these admirably performed investigations.

References

1. Bafort, J., G.Timperman, T.Delbar (1968). Observations on tissue schizogony and sporogony of rodent malaria. Ann.Soc.belge Méd.trop. *48*, 535-540.

2. Vanderberg, J. and M.Yoeli (1965). Effect of temperature on sporogonic development of *Plasmodium berghei*. J.Parasit. *52*, 559-564.

3. Yoeli, M. J.Vanderberg, R.Nawrot, H.Most (1965). Studies on sporozoite-induced infections of rodent malaria. II. *Anopheles stephensi* as an experimental vector of *Plasmodium berghei*. Amer.J.Trop. Med.Hyg. *14*, 927-930.

4. Sterling, C.R., M.Aikawa, J.P.Vanderberg (1973). The passage of *Plasmodium berghei* sporozoites through the salivary glands of *Anopheles stephensi*: An electron microscope study. J.Parasit. *59*, 593-605.

5. Killick-Kendrick, R. (1974). Parasitic protozoa of the blood of rodents: a revision of *Plasmodium berghei*. Parasitology *69*, 225-237.

6. Vanderberg, J.P. (1975). Development of infectivity by the *Plasmodium berghei* sporozoite. J.Parasit. *61*, 43-50.

7. Vanderberg, J. (1974). Studies on the motility of *Plasmodium* sporozoites J.Protozool, *21*, 527-537.

8. Beckman, H. (1963). The nature of *Plasmodium gallinaceum* host specificity I. Apparent site of sporozoite arrest in the non-susceptible species. Amer.J.Trop.Med.Hyg., *12*, 519-523.

9. Beckman, H. (1965). Survival of *Plasmodium gallinaceum* sporozoites in previously excised skin of mouse and chick. Proc.Soc.Exp.Biol.Med., *119*, 525-527.

10. Fairley, N.H. (1947). Sidelights on malaria in man obtained by subinoculation experiments. Trans.R.Soc.trop.Med.Hyg., *40*, 621-676.

11. Nussenzweig, R.S., J.P. Vanderberg, Y.Sanabria, H.Most (1972). *Plasmodium berghei:* accelerated clearance of sporozoites from blood as part of immune-mechanisms in mice. Exp. Parasit., *31*, 88-97.

12. Bafort, J.M. (1971). The biology of rodent malaria with particular reference to *Plasmodium vinckei vinckei* Rodhain 1952. Ann.Soc.belge Méd.trop., *51*, 1-204.

13. Yoeli, M., R.S.Upmanis, J.Vanderberg, H.Most (1966). Life cycle and patterns of development of *Plasmodium berghei* in normal and experimental hosts. Mil.Med., *131*, 900-914.

14. Killick-Kendrick R. (1974). Some morphological aspects of malaria. In: Basic research on malaria. Techn.Rep. ERO-5-74, 3-19.

15. Garnham, P.C.C. (1972). Comments on plasmodial ultramicroscopy. Proc.Helm. Soc.Wash., *39*, 194-197.

16. Vanderberg, J.P., R.S.Nussenzweig, H.Most (1968). Further studies on the *Plasmodium berghei - Anopheles stephensi-* rodent system of mammalian malaria. J.Parasit., *54*, 1009-1016.

17. Boyd, M.F. (1949). Malariology. Saunders Company, Philadelphia.

18. Mulligan, H.W., P.F.Russell, B.N. Mohan (1940). Specific agglutination of sporozoites. J.Mal.Inst.India *3*, 513-524.

19. Russell, P.F., H.W.Mulligan, B.N.Mohan (1942). Active immunization of fowls against sporozoites but not trophozoites of *Plasmodium gallinaceum* by injection of homologous sporozoites. J.Mal.Inst. India *4*, 311-319.

20. Russell, P.F. and B.N. Mohan (1942). The immunization of fowls against mosquito-borne *Plasmodium gallinaceum* by injection of serum and of inactivated homologous sporozoites. J.Exp.Med. *76*, 477-495.

21. Garnham, P.C.C. and R.S.Bray (1956). The influence of immunity upon the stages (including late exo-erythrocytic schizonts) of mammalian malaria parasites. Rev. Bras. Malar. *8*, 152-160.

22. Garnham, P.C.C. (1966). Immunity against the different stages of malaria parasites. Bull.Soc.Path.exot. *59*, 549-557.

23. Richards, W.H.G. (1966). Active immunization of chicks against *Plasmodium gallinaceum* by inactivated homologous sporozoites and erythrocytic parasites. Nature, *212*, 1492-1494.

24. Graham, H.A., N.C.Palczuk, L.A.Stauber (1973). Immunity to exo-erythrocytic forms of malaria. II. Passive transfer of immunity to

exo-erythrocytic forms. Exp.Parasit. *34*, 372-381.

25. Holbrook, T.W., N.C.Palczuk, L.A.Stauber (1974). Immunity to exo-erythrocytic forms of malaria. III. Stage specific immunization of turkeys against exo-erythrocytic forms of *Plasmodium fallax*. J.Parasit. *60*, 348-354.

26. Nussenzweig, R.S., R.Herman, J.Vanderberg, M.Yoeli, H.Most (1966). Studies on sporozoite-induced infections of rodent malaria. III. The course of sporozoite induced *Plasmodium berghei* in different hosts. Amer.J.Trop.Med.Hyg. *15*, 684-689.

27. Most, H., R.S.Nussenzweig, J.Vanderberg, R.Herman, M.Yoeli (1966). Susceptibility of genetically standardized (JAX) mouse strains to sporozoite-and blood-induced *Plasmodium berghei* infections. Mil.Med. *131*, 915-918.

28. Yoeli, M. and H.Most, (1965). Studies on sporozoite-induced infections of rodent malaria. I. The pre-erythrocytic tissue stage of *Plasmodium berghei*. Amer.J.Trop.Med.Hyg. *14*, 700-714.

29. Yoeli, M., R.S.Upmanis, J.Vanderberg, H.Most (1966). Life cycle and patterns of development of *Plasmodium berghei* in normal and experimental hosts. Mil.Med. *131*, 900-914.

30. Nussenzweig, R.S., J.Vanderberg, H.Most, C.Orton (1967). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. Nature, *216*, 160-162.

31. Nussenzweig, R.S., J.P.Vanderberg, H.Most, C.Orton (1969). Specificity of protective immunity produced by X-irradiated *Plasmodium berghei* sporozoites, Nature *222*, 488-489.

32. Spitalny, G.L. (1973). Immunological aspects of sporozoite-induced resistance in rodent malaria. Ph.D. thesis New York University.

33. Nussenzweig, R.S., J.Vanderberg, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. Mil.Med. *134*, 1176-1182.

34. Nussenzweig, R.S., J.Vanderberg, Y.Sanabria, H.Most (1972). *Plasmodium berghei*: accelerated clearance of sporozoites from blood as part of immune-mechanisms in mice. Exp.Parasit. *31*, 88-97.

35. Spitalny, G.L. and R.S.Nussenweig (1972). Effect of various routes of immunization and methods of parasite attenuation on the development of protection against sporozoite-induced rodent malaria. Proc.Helm.Soc.Wash. *39*, 506-514.

36. Nussenzweig, R.S., J.Vanderberg, G.L. Spitalny, C.I.O. Rivera, C.Orton, H.Most (1972). Sporozoite-induced immunity in mammalian malaria. A review. Amer.J.Trop.Med.Hyg. *21*, 772-728.

37. Vanderberg, J.P., R.S.Nussenzweig, H.Most (1970). Protective immunity produced by the bite of X-irradiated mosquitoes infected with *Plasmodium berghei*. J.Parasit. *56* (II), 350.

38. Clyde, D.F., V.C.McCarthy, R.M.Miller, W.E.Woodward (1975). Immunization of man against falciparum and vivax malaria by use of

attenuated sporozoites. Am.J.Trop.Med.Hyg. *24*, 397-401.

39. Collins, W.E. and P.G.Contacos (1972). Immunization of monkeys against *Plasmodium cynomolgi* by X-irradiated sporozoites. Nature New Biol. *236*, 176-177.

40. Ward, R.A. and D.E.Hayes (1972). Attempted immunization of rhesus monkeys against cynomolgi malaria with irradiated sporozoites. Proc. Helm.Soc.Wash. *39*, 525-529.

41. Clyde, D.F., H.Most, V.C.McCarthy, J.P.Vanderberg (1973). Immunization of man against sporozoite-induced falciparum malaria. Amer.J. med.Sci. *266*, 169-177.

42. Clyde, D.F., V.C.McCarthy, R.M.Miller, R.B.Hornick (1973). Specificity of protection of man immunized against sporozoite-induced falciparum malaria. Amer.J.med.Sci. *266*, 398-403.

43. Rieckmann, K., P.Carson, R.Beaudoin, J.Cassels, K.Sell (1974). Sporozoite-induced immunity in man against an Ethiopian strain of *P. falciparum*. Trans.R.Soc.trop.Med.Hyg. *68*, 258-259.

44. Vanderberg, J.P., R.S.Nussenzweig, H.Most, C.G.Orton (1968). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. II. Effects of radiation on sporozoites. J.Parasit. *54*, 1175-1180.

45. Warren, M. and P.C.G.Garnham (1968). The effect of X-irradiation of sporozoites of *Plasmodium cynomolgi* as seen by abnormal development in the liver. Trans.Roy.Soc.trop.Med.Hyg. *62*, 460.

46. Warren, M. and P.C.C.Garnham (1970). *Plasmodium cynomolgi*; X-irradiation and development of exo-erythrocytic schizonts in *Macaca mulatta*. Exp.Parasit. *28*, 551-556.

47. Vanderberg, J., R.S.Nussenzweig, Y.Sanabria, R.Nawrot, H.Most (1972). Stage specificity of anti-sporozoite antibodies in rodent malaria and its relationship to protective immunity. Proc.Helm.Soc. Wash. *39*, 514-525.

48. Vanderberg, J.P. (1973). Inactivity of rodent malaria anti-sporozoite antibodies against exo-erythrocytic forms. Amer.J.trop.Med.Hyg. *22*, 573-577.

49. Vanderberg, J., R.S.Nussenzweig, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. Mil.Med. *134*, 1183-1190.

50. Nussenzweig, R.S., J.Vanderberg, H.Most, C.Orton (1970). Immunity in simian malaria induced by irradiated sporozoites. J.Parasit. *56*, II, 252.

51. Corradetti, A., F.Verolini, A.Sebastiani, A.M.Proietti, L.Amati (1964). Fluorescent antibody testing with sporozoites of plasmodia.Bull. Wld Hlth Org. *30*, 747-750.

52. Nussenzweig, R.S., W.Montuori, G.L.Spitalny, D.Chen (1973). Antibodies against sporozoites of human and simian malaria produced in rats. J.Immunol. *110*, 600-601.

53. Nussenzweig, R.S. (1975). Sporozoite immunization. WHO/MPD/SGIM/WP/75.7 (mimeographed).

54. Nussenzweig, R.S. and D.Chen (1974). The antibody response to sporozoites of simian and human malaria malaria parasites: its stage and species specificity and strain cross-reactivity. Bull. Wld Hlth Org., *50*, 293-297.

55. Spitalny, G.L. and R.S.Nussenzweig (1973). *Plasmodium berghei*.Relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. Exp. Parasit., *33*, 168-178.

56. Spitalny, G.L., J.P.Verhave, J.H.E.Th.Meuwissen, R.S.Nussenzweig (1975). T-cell dependence of sporozoite-induced immunity in rodent malaria. Submitted for publication.

57. Rivera-Ortiz, C. and R.S.Nussenzweig (1972). Increased phagocytic activity of the reticulo-endothelial system during immunization with X-irradiated sporozoites of *Plasmodium berghei* in mice. Proc.Helm.Soc.Wash., *39*, 497-506.

58. Alger, N.E., J.A.Harant, L.C.Willis, G.M.Jorgensen (1972). Sporozoite and normal salivary gland induced immunity in malaria. Nature *238*, 341.

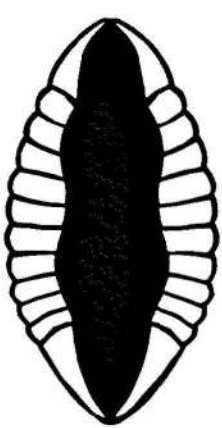
59. Nussenzweig, R.S. (1967). Increased non-specific resistance to malaria produced by administration of killed *Corynebacterium parvum*. Exp.Parasit., *21*, 224-231.

60. Krettli, A., D.H.Chen, R.S.Nussenzweig (1973). Immunogenicity and infectivity of sporozoites of mammalian malaria isolated by density-gradient centrifugation. J.Protozool., *20*, 662-665.

61. Jakstys, B.P., N.E.Alger, J.A.Harant, P.Silverman (1974). Ultrastructural analysis of *Plasmodium berghei* sporozoite antigens prepared by freeze-thawing and heat inactivation. J.Protozool., *20*,344-348.

62. Phillips, R.S. (1974). Antigenic character of the plasmodia. Proc.Third Int.Congr.Parasit., *2*, 1106-1107.

63. Voller, A. (1974). Immunopathology of malaria. Bull. Wld Hlth Org., *50*, 177-186.



CHAPTER II

MATERIALS AND METHODS

2.1 The choice of experimental animals

Female Wistar rats (TNO Zeist, The Netherlands), 2-3 months old, were the main experimental hosts for the malaria parasite in this study. Their body weight was 150-200 gram and batches of rats varied at arrival less than 5% in individual weight. Generally guinea pigs were used for additional feeding to maintain a mosquito colony. Six to eight week old female Swiss mice (TNO, Zeist) were used for the daily infection of mosquitoes. This strain of Swiss mice is rather insusceptible to sporozoite induced infections in the sense that the number of developing EEF in the liver remains relatively low in comparison with other strains and species of host animals (1). Therefore Swiss mice are not the appropriate hosts for quantitative studies on the infectivity and development of pre-erythrocytic stages. A/J strain mice are more susceptible, but these animals were not available in sufficient quantities. Young rats, however, exceed mice of the A/J strain in susceptibility to sporozoite induced infections (2). This determined the choice of the experimental animal. Inbred B10.LP mice in which the thymus-deficiency factor (nude) was back crossed (TNO, Zeist) were used for immunization studies, together with normal littermates. Tree rats *(Thamnomys sp)* were obtained from Dr.I.Landau, Paris. They originated from the Central African Republic and are indicated as the Ippy-strain (3). All animals were housed under standard conditions in the Central Animal Laboratory of the Medical Faculty (head Dr.W.J.I. van der Gulden).

2.2 Rearing of mosquitoes

The development of *Plasmodium berghei* in their mosquito hosts requires the

fig. 2. View inside the rearing room. Newly emerged adult mosquitoes are collected with a sucking machine containing a mosquito cage (right).

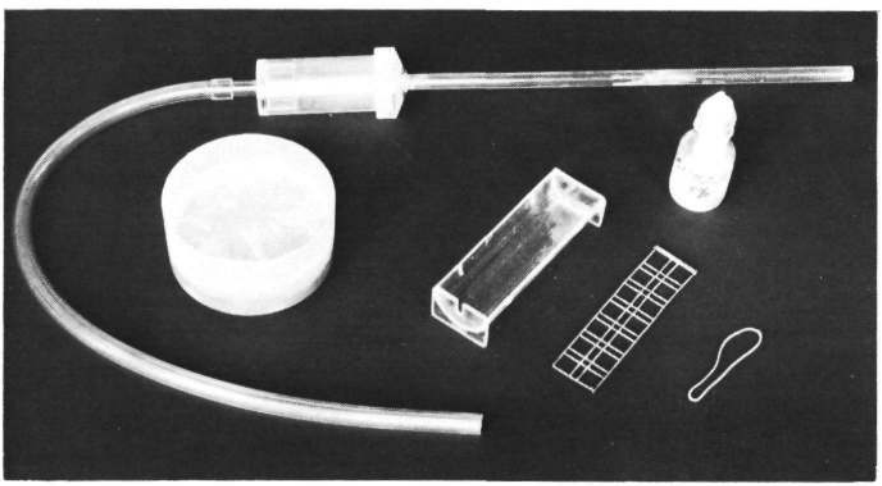


fig. 3. Some accessories for mosquito-rearing: a bottle with cotton-wool and glucose; a holding cage with wireframe and rubber band; a beaker for egg laying; an aspirator.

relatively low temperature of 18-21°C (4). Rearing of mosquitoes is optimal at higher temperatures. Therefore, two separate temperature and humidity controlled rooms were in use. *Anopheles stephensi* was obtained through the courtesy of Dr.J.M.Bafort, Antwerp, and reared according to a technique, composed from various descriptions (5-8). The animals were maintained at 27-28°C and 85% relative humidity, with a daily cycle of 12 hours of light and 12 hours of darkness. Adults were fed 5% glucose solution. Females were allowed to feed biweekly on guinea pigs and laid their eggs after 2-4 days on filterpaper, put on a wet sponge in a shallow beaker. Eggs were put into rearing pans inside a floating paper square in order to prevent eggs from sticking to the inner walls of the pans and drying up by evaporation. Larvae were fed successively with hay infusion, yeast powder and a mixture of ground dog pellets (Hope farms) and cereal baby food (Bambix). Rearing pans were covered with a netting. Newly emerged adults were collected with a sucking machine and transferred directly to cages (fig. 2).

2.3 Plasmodial strain and infection of mosquitoes

The strain of *P. berghei* (ANKA) was supplied by Dr.Bafort. It was kept in mice by weekly transfer of infected blood. After a maximum of 5 blood-transfers the strain was cyclically transmitted. Several authors described the optimal conditions for the cyclical transmission of this rodent parasite (2, 9-13). The infected animals and the infected mosquitoes were kept at 21°C and 85% relative humidity. Mice were used for infection of mosquitoes, three days after intraperitoneal inoculation of diluted infected blood (plm. 10^7 parasitized erythrocytes), when first erythrocytic forms, including gametocytes, were detectable in blood slides.
This was done on 2 subsequent days per batch of mosquitoes. Mice were suitable for mosquito infection for about 4 days. After that time parasitaemias are high and though many gametocytes circulate in the blood, mosquitoes fail to become infected (11). The *P. berghei* infection in Swiss mice is lethal and most of the animals die within 7-10 days.
The abdomen of inoculated mice was shaved, and in order to facilitate biting they were put into half cyclindrical perspex holding cages. The cages could be closed with a wireframe and fixed with a rubber band (fig. 3 and 4, design and construction by Mr.J.van Munster, Institute of Medical Parasitology). These cages have an important advantage over other devices in that one technician can easily immobilise animals for the feeding of mosquitoes

without the use of anaesthetics. There are also larger models for rats and guinea pigs.

2.4 Harvest and inoculation of sporozoites

Salivary glands of infected mosquitoes contain sporozoites about 14 days after taking the infective bloodmeal. At that time the percentage of positive mosquitoes was determined (generally approximating 100%) and the batch was used as soon as possible, i.e. 15-20 days after biting (10). Small numbers of mosquitoes were collected with a mouth aspirator (14). Before dissection legs and wings of the female mosquitoes were removed, under light chlorophorm anaesthesia, and the bodies were kept in a moist atmosphere to prevent premature death by dessication.

Salivary glands of ten to twelve mosquitoes were dissected out into one drop of medium on a glass slide (9, 15). Perspex- and siliconized slides did not suit our purpose. The salivary glands were flushed into an all-glass homogenizer placed in crushed ice, using a 2 ml syringe filled with chilled medium and fitted with a medium sized needle. Grace's Insect Tissue Culture medium (Gibco) with the pH adjusted to 7 was used as the dissection and suspension medium (16). Occasionally TC medium 199 (10) was also used.

After dissection of the whole batch of mosquitoes the collected glands were homogenized and the suspension centrifuged for 3-5 min. at 700 r.p.m. (160 g) to remove the bulk of mosquito debris.

The sediment usually contained 5-10% of the total sporozoite numbers, most of which could be separated from the debris by repeated grinding and centrifugation. Sporozoites in the supernatant were counted in a haemocytometer and adjusted to the required number per unit volume of medium.

As calculated from 60 different suspensions the number of salivary gland sporozoites ranged between 15.000-35.000 with a mean of 25.000 per mosquito.

Sporozoite suspensions were inoculated without delay into a tail vein of rodent hosts. Intravenous inoculation has been reported to ensure maximal infections and induction of immune responses (2, 17, 18).

Rats were lightly anaesthesized with aether and tails were soaked in warm water for exposure of the veins. 0,5 ml was inoculated per rat, and 0,1 ml per mouse. The whole procedure from dissection of one hundred mosquitoes to inoculation of twenty rats took about two and one half hours. Inoculations were done with assistance of one or two biotechnicians.

The inocula were never standardized for exact numbers of sporozoites and a

comparable order of concentrations was considered satisfactory. However, control groups of untreated animals were always included. The effect of treatment in the experimental group was assessed on account of the control group. Experimental animals were injected with one or more sporozoite suspensions, hereafter called inoculations or immunizing doses. The first immunizing dose contained about 150.000 sporozoites. whereas following "booster" inoculations contained about 50.000 sporozoites. This procedure of a large initial dose and smaller boosters was suggested previously (19, 20). The time-lapse between immunizing doses was generally one week. The final inoculation, hereafter called the challenge, again contained higher doses of sporozoites, which was also administered to control animals.

2.5 Parasitological examination of blood slides

For the estimation of parasite densities, blood films were prepared from tail blood. After drying they were fixed in methanol and stained with a Giemsa dilution for 45 min. (9, 15).
The Giemsa stock solution was prepared as follows:
Giemsa powder (Merck) 7,5 g in 650 ml of methanol and 350 ml of glycerol, shaken with glass beads for at least 48 hr. For use a 2,5% solution of Giemsa stock dilution was prepared in a buffer solution of 1,28 g $Na_2HPO_4.IH_2O$ and 0,72 g KH_2PO_4 in 4 l demineralized water (pH 7.2).
In the smears 25 microscopic fields per smear were examined, each field with oil immersion objective containing approximately 250 erythrocytes. The number of parasitized cells was expressed per 10.000 erythrocytes.

2.6 Histological procedures

In this study density of exo-erythrocytic forms (EEF) was used as a measure for numbers of sporozoites that escaped natural and acquired immunity. Since the maturation time of EEF of the Anka strain of *P. berghei* is about 48 hours (11, 21), autopsy or biopsy of the liver was carried out at 45 hours after inoculation. These were fixed in a modified Carnoy's solution (10 ml glacial acetic acid, 30 ml chloroform, 60 ml ethanol 96%) for 5 hours with a change of the fixative after 1 hour. The fixative was then replaced by ethanol 96%, which was also changed once. Pieces of liver were automatically passed in transit through acetone, methylbenzoate and toluene into a mixture of equal parts of paraffin and paraplast. 5 µm sections were cut with a microtome,

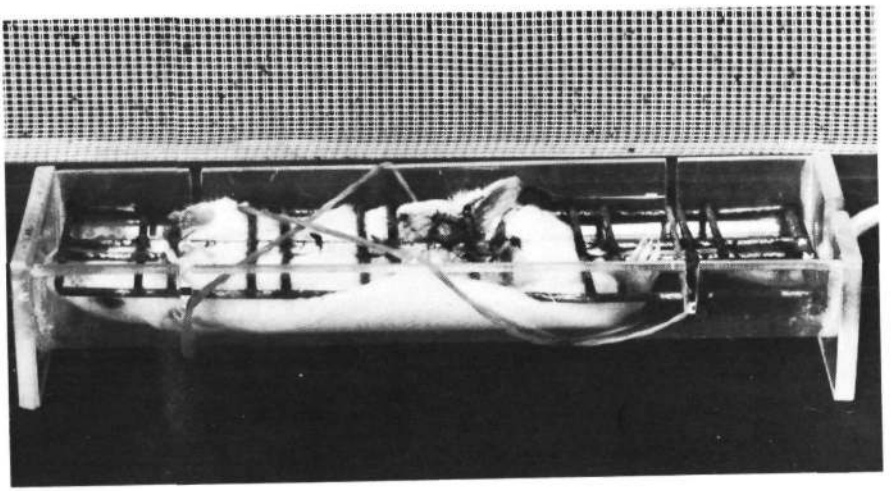


fig. 4. A mouse in a holding cage with mosquitoes feeding on its shaven belly.

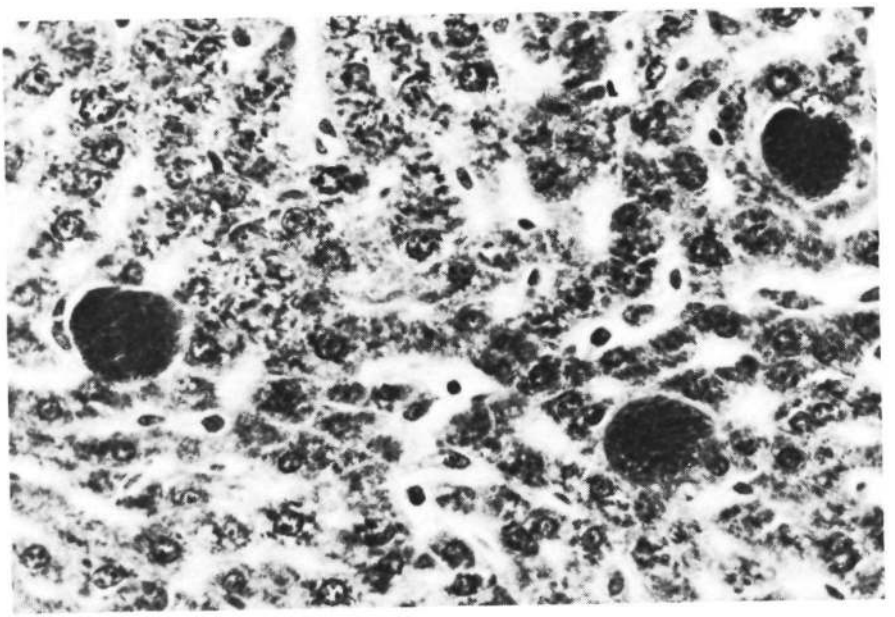


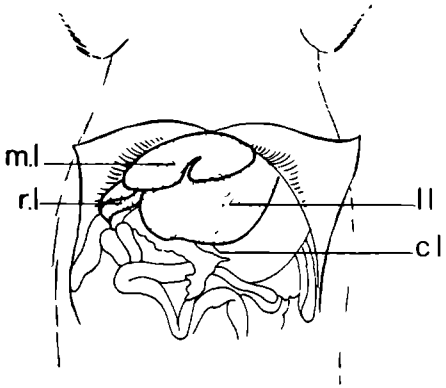
fig. 5. Rat liver tissue with three maturing EEF.

and selected according to methods described below.
The sections were mounted on slides with white gelatine (Merck) and after drying stained with the Giemsa-colophonium method (9, 15), as follows. Wax was removed from the sections by dipping the slides in xylene; subsequently the slides were dipped in alcohol and water. The tissue was stained in a mixture of 15 ml Giemsa stock solution (see section 2.5) 10 ml acetone, 10 ml methanol and 100 ml buffer. The same buffer was used as that for staining blood slides. For differentiation the slides were washed with tap water and, while still wet, rinsed with colophonium resin (15% solution in acetone) until green colour no longer came off. The colophonium was washed off with a mixture of 30% acetone and 70% xylene and the slides were placed in xylene before being mounted with Euparal.

2.7 Processing of liver sections and counting of EEF

If not stated otherwise the median lobe of the liver was processed and divided into 3 pieces: one cut in line with the deep fissure, the other halving the bigger part. The 3 pieces were embedded together in paraffin wax, in an arbitrary sequence. Serial sections of 5 µm were prepared, and three of them (A, B and C) selected as follows: after a first section A a ribbon of 10 subsequent sections was discarded and a section B was chosen from the next 5 sections (generally the middle one) for the third section C this procedure was repeated. This guaranteed a mutual distance between A, B and C of at least 50 µm (50-90 µm). Since the maximal diameter of mature EEF in rats is up to 40 µm (11, 21), the same EEF could not be counted twice in these 3 sections. The total number of EEF were counted in the three slices of liver tissue on each slide A, B and C (9 slices). The procedure is depicted in fig. 6. Next the surface areas of tissue on slide B was determined by projecting this slide together with a point screen. The points within the tissue areas were counted (N) and used also as a reference for the tissue areas on slided A and C (fig. 7).
For conversion of the number of points to mm^2 a conversion factor P was determined by counting the number of points within the projected area of a coverglass of a measured surface.

$$P = \frac{\text{surface coverglass in } mm^2}{\text{surface coverglass in number of points}}$$

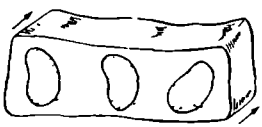


liver lobes of a rat in situ

r l right lobe
m l median lobe
l l left lobe
c l caudal lobe

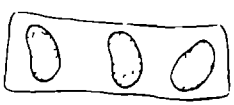
median lobe

one cutting line along the fissure
and another halving the bigger part



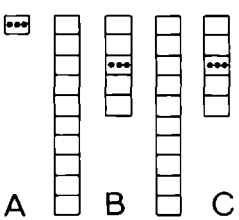
three parts embedded

in wax (paraffin and paraplast 1 1)



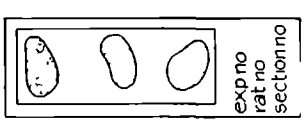
one section of 5µm

with slices of 3 parts



selection of sections from ribbons

A no 1
B no 14 (12-16)
C no 30 (28-32)



microscopic slide

with mounted section and
cover glass

fig. 6. Schematic presentation of the preparation of selected sections of liver tissue.

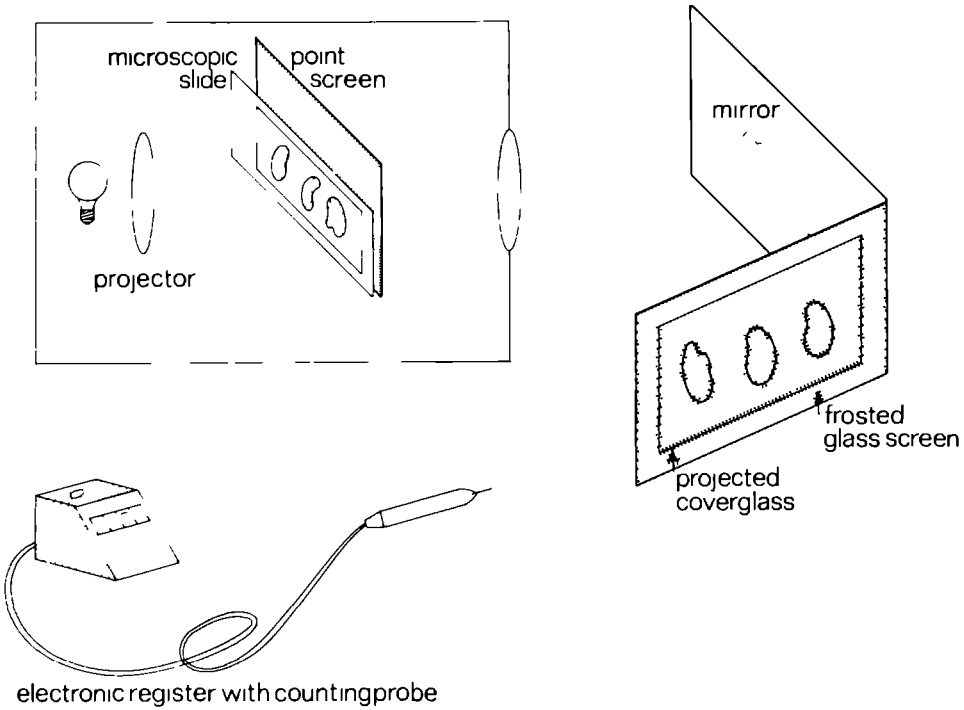


fig. 7. Schematic presentation of the method to determine the surface area of slices of liver tissue.

The surface area of liver tissue in slides A, B and C (S) was now calculated as

$$S = 3 \times N \times P \text{ mm}^2$$

For a measure of density of EEF (M) the number of EEF on slides A, B and C was determined and expressed per mm^2 of liver tissue.

$$M = \frac{\text{number of EEF}}{S}$$

In addition, serial tissue sections were made of the median lobe and also of other parts of liver, for the study of EEF distribution and for the evaluation of the described measure used to assess EEF density (see chapter III).

2.8 Serological techniques

2.8.1 *Circumsporozoite precipitation (CSP).*

Qualitative determination of specific anti-sporozoite antibodies was performed by Dr.G.L.Spitalny using the CSP reaction (22). In short, the procedure is as follows: Harvested sporozoites were mixed in a drop of serum on a microscopic slide and overlaid with a coverslip. The preparation was incubated for 30 min. at 37°C and 20 sporozoites examined for the characteristically long precipitate.
Serum samples were considered positive when at least 2 out of 20 sporozoites showed the CSP reaction.

2.8.2 *Sporozoite neutralization activity (SNA)*

This test was also performed by Dr.Spitalny, as described (20). 0,5 ml of pooled sera were mixed with 0,5 ml of a sporozoite suspension and incubated for 45 min at room temperature with vigorous shaking. After this time 0,1 ml of the mixture, containing 3.000 sporozoites was injected i.v. into normal A/J mice. Serum samples were considered to possess SNA, when no more than 3 out of 7 recipients developed a parasitaemia, compared to 7 out of 7 of the controls.

2.8.3 *Interferon production*

In order to find out if after inoculation with sporozoites and with parasitized red blood cells interferon was produced, sera of inoculated rats were incubated on a tissue culture of rat embryo cells and subsequently the inhibition of plaque formation by vesicularstomatitis virus (VSV) was determined. For the assessment of interferon activity Wagner's plaque-inhibition method was used, as described by Jahiel et al. (23). The test as we used it needs improvement, and therefore, only the general procedure is given here.
17-18 days old rat embryos were minced and incubated with a trypsin solution. Free cells were collected and grown at 37°C in enriched Hanks BSS.
The cells were transferred to plastic Petri dishes (Ø 60 mm) and placed in a humidified CO_2 incubator at 37°C. The serum samples were diluted to 1:20 and incubated with the monolayers of cells (two dishes per dilution). After 5 hours of incubation and washing, the cells were incubated with an appropriate dilution of VSV, warranting about 100 plaques forming units (PFU) after absorbtion for 1 hour. Finally the VSV dilution was mixed with agar, containing neutral red. The plaques were counted after 48 hours.

As a control for the test some rats were injected intravenously with an artificial interferon inducer, a complex of polyriboinosinic and polyribocytidylic acids (rI : rC), and diethyl-aminoethyl (DEAE) -dextran in PBS. The sera were collected by retro-orbital bleeding before inoculation, 2, 4, 8 hours afterwards, and twice on subsequent days.

References

1. Yoeli, M., H.Most, G.Boné (1965). The natural history of *Plasmodium berghei* in the field and under experimental conditions. Ann.Soc.belge Méd.trop., *45*, 267-274.
2. Wéry, M. (1968). Studies on the sporogony of rodent malaria parasites. Ann. Soc.belge Méd.trop., *48*, 1.
3. Landau, I (1973). Diversité des méchanisms assurant la pérennité de l'infection chez les sporozoaires cocciomorphes. Mem.Mus.Nation.Hist.Nat. (Nouv.Sér.)A, *77*, 1-62.
4. Yoeli M., J.Vanderberg, R.Nawrot, H.Most (1965). Studies on sporozoite-induced infections of rodent malaria. II. *Anopheles stephensi* as an experimental vector of *Plasmodium berghei*. Amer.J.Trop.Med.Hyg., *14*, 927-930.
5. Gerberg, E.J., J.W.Gentry, L.H.Diven (1968). Mass rearing of *Anopheles stephensi* Liston.Mosquito News, *28*, 342-346.
6. Gerberg, E.J. (1970), Manual for mosquito rearing and experimental techniques. Am.Mosq.Contr.Ass., Bull. 5.
7. Thomson, E.G. and L.H.Bell, (1968). Laboratory studies on the biology of *Anopheles stephensi* Liston.Mosquito News, *28*, 639-642.
8. Stahler, N., D.C.Seeley (1971). Effect of age and host on oviposition of *Anopheles stephensi* in the laboratory. J.Ec.Ent., *64*, 561-562.
9. Shute, P.G. and M.Maryon (1966). Laboratory on technique for the study of malaria. 2nd Ed. Churchill, London.
10. Vanderberg, J.P., R.S.Nussenzweig, H.Most (1968). Further studies on the *Plasmodium berghei-Anopheles stephensi*-rodent system of mammalian malaria. J.Parasit, *54*, 1009-1016.
11. Bafort, J.M. (1971). The biology of rodent malaria with particular reference to *Plasmodium vinckei vinckei* Rodhain 1952. Ann.Soc.belge.Méd. trop., *51*, 1-204.
12. Killick-Kendrick, R. (1971). The collection of strains of murine malaria parasites in the field, and their maintenance in the laboratory by cyclical passage. Symp.Brit.Soc.Par. (eds. AER.Taylor & R.Muller), *9*, 39-64.
13. Fink, E. (1972) Kausalprophylaktische Wirkung von Standard-Malariamitteln bei der Nagetiermalaria (*Plasmodium berghei yoelii*). Z.Tropenmed.Parasit., *23*, 35-47.
14. Colluzzi, M. and V.Petrarca (1973). Aspirator with paper cup for collecting mosquitoes and other insects. WHO/MAL/73.794/VBC73.426 (mimeographed).
15. Garnham, P.C.C. (1966). Malaria parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford.

16. Fink, E. and E.Schicha (1969). Influence of synthetic insect T.C.medium on the survival of malaria sporozoites *in vitro* (*Plasmodium berghei yoelii* and *P.cathemerium*) Z.Parasitenk., *32*, 93.

17. Wěry, M. and R.Killick-Kendrick (1967). Differences in the numbers of tissue schizonts of *Plasmodium berghei yoelii* in the livers of individual mice, and a comparison of two routes of inoculation of sporozoites. Trans.R.Soc.Trop.Med.Hyg., *61*, 447-448.

18. Spitalny, G.L. and R.S.Nussenzweig (1972). Effect of various routes of immunization and methods of parasite attenuation on the development of protection against sporozoite-induced rodent malaria.Proc.Helm.Soc.Wash., *39*, 506-514.

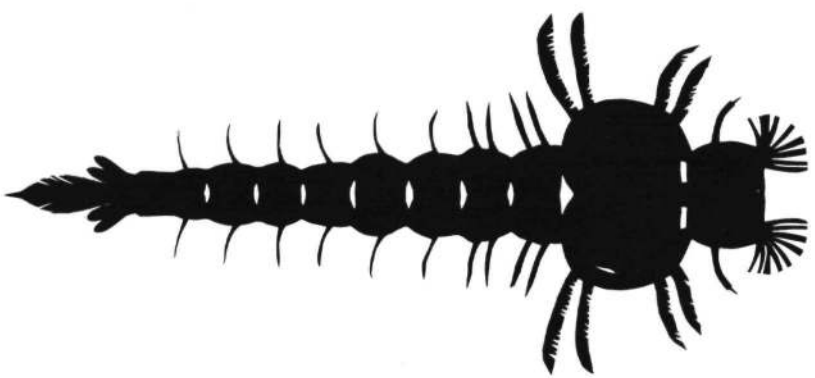
19. Nussenzweig, R., J.Vanderberg, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. Milit.Med., *134*, 1176-1182.

20. Spitalny, G.L. (1973). Immunological aspects of sporozoite-induced resistance in rodent malaria. Thesis, New York University.

21. Killick-Kendrick, R. (1974). Parasitic protozoa of the blood of rodents: a revision of *Plasmodium berghei*. Parasitology *69*, 225-237.

22. Vanderberg, J., R.Nussenzweig, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. Milit.Med., *134*, 1183-1190.

23. Jahiel, R.I., R.S.Nussenzweig, J.Vilček, J.Vanderberg (1969). Protective effect of interferon inducers on *Plasmodium berghei* malaria. Amer.J.Trop. Med.Hyg., *18*, 823-835.



C H A P T E R III

ACCOUNT OF THE METHOD FOR QUANTITATIVE EEF ASSESSMENT

(in collaboration with Th.M. de Boo,
Department of Statistical Consultation,
University of Nijmegen)

3.1 Introduction

The length of the prepatent period can be used to obtain information about the infectivity of sporozoites in a host model system. This period begins at the moment of inoculation of sporozoites, continues with their migration to the liver, includes the time needed for the development of EEF in liver parenchymal cells, and then the subsequent liberation of liver merozoites in the blood. It ends when the first infected erythrocytes are detected in blood-smears. Thus, the length of the prepatent period is the ultimate outcome of a complex series of events, and each of these is liable to alter with various influences. Therefore, the prepatent period, as a measure for infectivity, has important limitations.
Another, more direct measure is the density of EEF in the liver. Earlier attempts for this histological approach were made, but the determination of this density is laborious and statistical assumptions have to be made. None of these techniques are practical in experiments involving large numbers of animals, but the directness of the approach is attractive. Thus an attempt to develop a reliable method for the estimation of this parameter was made. In the following sections the limitations and merits of this modified counting technique are evaluated and possible applications tested.

3.2 Statistical considerations

3.2.1 *Distribution of EEF in liver lobes*

At first sight the hypothesis of a uniform distribution of EEF throughout an infected liver or liver lobe seems acceptable. To test this hypothesis, median lobes of sporozoite-infected animals, inoculated on different occasions, were completely sectioned. Slices were selected at mutual intervals, with a distance of ± 75 µm. If a uniform distribution existed, the numbers of EEF per slice would follow a Poisson distribution. A χ^2 test (1) was used to check the validity of this hypothesis, (α = 0,05; table 3.2.1).

Table 3.2.1

Rat	part of median lobe	hypothesis of uniform distribution	$\bar{M}$
A	a	not rejected	0.024
	b	rejected (p< 0.025)	0.024
	c	not rejected	0.021
B	a	rejected (p<0.005)	0.287
	b	rejected (p<0.005)	0.386
	c	rejected (p<0.005)	0.311
C	a	not rejected	0.041
	b	not rejected	0.041
	c	not rejected	0.034

The data show that a uniform distribution could not always be demonstrated. Among other things, this was owing to technical factors in preparing histological sections. Serial sections through a liver lobe should show a gradual change in the size of their surface areas, since they follow the natural shape of this lobe.

Instead of a gradual change in size, irregularities were sometimes found. The possibility was considered that the source of this variation may have been due to attaching freshly cut sections to glass slides with warmed gelatin

adhesive, and thus stretching the sections on the slides. This would directly influence the surface size of the tissue. Even if this fact was accounted for, other unexplained differences remained. Thus, it was concluded that although a uniform distribution of EEF may occur in the median lobe with low densities of EEF, this could not be assumed generally. Therefore, the results obtained by the counting method used, were assessed irrespective of a specific distribution of EEF through the median liver lobe.

3.2.2 *Properties of M (relative EEF density)*

As stated in section 2.7, M is the number of EEF/mm^2 of liver tissue surface. It is used as a measure for comparing EEF densities in the livers of individual rats. It also provides a relative measure for the infectivity of the batch of sporozoites used. An account is given of the properties of M.
It is impossible to cut each median lobe into three parts exactly along the along the same lines (see fig. 6). Therefore, define a region of about 2 mm on both sides of theoretical perpendicular cutting-planes. This region is large enough to contain all possible realisations (sections). The actual sections can then be considered as a random sample from the population of sections in this region. Consequently, $\underline{M}$ is stochastically distributed over this region. However, only the distribution of $\underline{M}$ over a given population of rats is of interest, if a comparison of two or more populations is wanted. Let this distribution for population i be denoted by F_i, its mean by μ_i and its variance by σ^2_i. If a rat is randomly chosen from population i, sections taken as described above and M_i computed, then the sources of variation in M_i are:
1) Biological variation between rats of population i, σ^2_{bi}
2) Variation caused by sampling from the liver region defined above, σ^2_{ri}
3) Variation caused by shortcomings in the experimental procedure, σ^2_{ei}.
These three variances are mutually independent, so they may be added; the sum of them gives just $\sigma^2_i = \sigma^2_{bi} + \sigma^2_{ri} + \sigma^2_{ei}$.
An assumption has to be made as a result of the circumstance that a sample of sections is taken from an anatomically defined region, instead of being taken at random from the whole liver. Since the value of M has to be representative for the EEF density in the whole liver, density in the defined region should be a monotonically increasing function of the density in the whole liver. If, for example, the decision would be to accept the hypothesis:

$\mu_i > \mu_j$ (i.e. the mean of population i is greater than that of population j), then acceptance of the given assumption implies that animals from population i have a higher density of EEF in their livers than those of population j, which is what is required. The given assumption seems to be a natural one. It is impossible to prove that it is always true, but an illustration of its correctness is given in the following:
From each of the four lobes of 17 rats (from 3 experiments) 3 sections were taken as described in chapter II. In the median lobe they were taken from the defined region, in the other lobes they were selected at random. The EEF density for each of the lobes was determined. The mean, say D, of these four values is then an estimator for the mean EEF density in the whole liver. Thus, there were 17 different D-values, ranging from 0.09 to 1.9. Now the assumption implies that M is a monotonically increasing function of D. To verify this, the correlation coëfficient between M and D was computed. This proved to be 0.996, which implies that the relation between M and D is positive and almost linear. The regression-equation was M=0.017 + 1.042 D, which shows that (for the given animals, in the given range) the value of M is a bit larger than the mean value D (see also section 3.2.5).

3.2.3 *Tests of hypotheses*

The aim of many of the following experiments is the comparison of 2 or more groups of rats, drawn at random from different populations. Therefore, it was necessary to know the distribution of $\underline{M}$ for those populations.
It appears that this distribution depends on the population from which the sample of rats was drawn. For instance, if a group of rats is almost completely immunized, the distribution of $\underline{M}$ will approach the limiting distribution $P(\underline{M}=0)=1$, because in that case there are hardly any or no EEF left in the liver. On the other hand it could be that the normal distribution is a fairly good approximation, if the population from which the rats are drawn consists of heavily infected rats. Between these two extremes, many mixed distributions are possible. To avoid this multitude of unknown distributions, distribution-free methods were used. Where a comparison between two samples, drawn from populations i and j was wanted Wilcoxon's test (2) was used.
The null hypothesis is H_o: $F_i = F_j$, in other words, the distribution of $\underline{M}_i$ and $\underline{M}_j$ are equal. If H_o is rejected then, strictly speaking, the only conclusion can be that $F_i \neq F_j$. It is, however, justified to extend

this conclusion to $\mu_i \neq \mu_j$, if one of the distributions approaches the above-mentioned limiting distribution; if neither of the distributions approaches the limiting one and both (sample) variances are not too different, the assumption is, that we are dealing with two equal, but translated distributions.
It often occurred that in one sample a large group of equal observations appeared (e.g. several null-values). In such cases a correction should be applied to the test-statistic of Wilcoxon. This, however, was never done, but in not doing this, the computed tail-probabilities (p-values) are usually conservatively compared with the real p-values and consequently our conclusions are rather more than less safe.
Finally, it should be noted that, in most cases, it was impossible to construct confidence intervals for $\mu_i - \mu_j$ on account of the dissimilarity of F_i and F_j.

If more than two groups had to be compared, there were two possibilities: that the sample sizes were either unequal or equal. In the first case Wilcoxon's test was repeatedly used for each comparison. In the latter case Steel's test was used (3). All the following tests had $\alpha=0.05$ unless otherwise stated. If the name of the test is not given then Wilcoxon's test was used; in all the tests the p-values are given.
In order to give a quantitative impression of differences in EEF densities the mean of the sample from population i was also expressed as a percentage of the mean in the other sample from population j (generally that of the control group). It should be stressed that such percentages only relate to the samples and do not give exact information of the magnitude of the differences between the populations. Thus, the values of these percentages is limited.

3.2.4 *The number of slices per liver and the number of animals per group*

In the normal routine 3 x 3 slices of liver per animal were examined for the determination of M_i as an estimator for μ_i. It is not simple to find out which number of slices would give an acceptable approximation of μ_i. The accuracy of M_i as indicated before (see section 3.2.2), is disturbed by three sources of variation. The probability that M_i approximates μ_i, increases when more rats are included per group, when more slices are examined per animal and when technical failures are minimal.

To get some insight into the significance of the differences between the variances σ^2_{bi} and σ^2_{ri}, consider the following as an illustration.
It is assumed that for the below mentioned rats the variance $\sigma^2_{ei} = \sigma^2_e$.
We may divide σ^2_e into two parts, one of which is added to the biological variance σ^2_{bi} and the other to the region-sampling variance σ^2_{ri}.
The first part could be called the "induced biological variance" and contains the variance caused by such technical errors as for instance unsuccessful injection. The latter could be called the "induced region-sampling variance" and contains the variance caused by errors such as compressing and stretching of tissue slices. The notations: σ^2_{bi} and σ^2_{ri} will not be changed.

A piece of liver from the defined region in the median lobe of one rat was cut into 50 serial sections and numbered from 1 to 50.
To compute M, there are 26 trios of sections available, e.g. {1, 13, 25}, {2, 14, 26}, ..., {26, 38, 50}. In the normal procedure, a trio was selected at random. Thus, the variance of these 26 M-values is an estimator, say s^2_r, for σ^2_r ($s^2_r = 7881_2 \times 10^{-8}$). The mean of the M-values was 0.068.
In order to compare σ^2_r with σ^2_b, it seemed logical to use for comparison those groups (samples), whose mean $\bar{M}$ did not deviate too much from 0.068.
As a result, 17 groups from the following experiments were used with $\bar{M}$ ranging from 0.048 to 0.088.

the experiments:	4.1.3. C	4.1.10. C	4.2.7. E2
(chapter IV)	4.1.4. C	4.2.3 E	4.2.8. E
	4.1.7A E1	4.2.5. C1	4.3.2. C
	4.1.7A C1	4.2.5. C2	4.3.3. C
	4.1.7B E2	4.2.6. C4	4.3.4. P
		4.2.7. E1	4.3.5. P

For each of these groups the estimation s^2_i of σ^2_i is known. To be able to analyze statistically, the following assumptions are made:

1) The above mentioned set of 26 $\bar{M}$-values is a sample, drawn from a normal distribution. Also the 17 groups are samples from normal populations.
2) The region-sampling variances σ^2_{ri} are equal for all the populations i (i = 1, 2,.., 17) and equal to σ^2_r.

re 1: Even if this is not the case, then the actual distributions are probably reasonably well approximated by normal distributions.

re 2: This assumption seems feasible.

With assumption 1 the stochastic variable $\frac{\underline{s}^2_i/\sigma^2_i}{\underline{s}^2_r/\sigma^2_r}$ has the F-distribution with $\nu_i = n_i - 1$ (n_i is the number of rats in group i) and 25 degrees of freedom.

The consequence of assumption 2 is, that $\frac{\sigma^2_i}{\sigma^2_r} = \frac{\sigma^2_{bi} + \sigma^2_r}{\sigma^2_r} = 1 + \frac{\sigma^2_{bi}}{\sigma^2_r}$

Now we are able to construct 95% - one sided confidence intervals for the ratio σ^2_{bi}/σ^2_r, as follows:

$$P\left(\frac{\underline{s}^2_i/\sigma^2_i}{\underline{s}^2_r/\sigma^2_r} < F_{0.95}\right) = 0.95 = P\left(\frac{\sigma^2_i}{\sigma^2_r} > \left(F_{0.95} \times \frac{\underline{s}^2_r}{\underline{s}^2_i}\right)^{-1}\right)$$

$$= P\left(1 + \frac{\sigma^2_{bi}}{\sigma^2_r} > \left(F_{0.95} \times \frac{\underline{s}^2_r}{\underline{s}^2_i}\right)^{-1}\right)$$

$$= P\left(\frac{\sigma^2_{bi}}{\sigma^2_r} > \left(F_{0.95} \times \frac{\underline{s}^2_r}{\underline{s}^2_i}\right)^{-1} - 1\right)$$

If $\underline{a}_i = \left(F_{0.95} \times \underline{s}^2_r/\underline{s}^2_i\right)^{-1} - 1$, then the 17 confidence intervals are thus determined by: $P(\sigma^2_{bi}/\sigma^2_r > \underline{a}_i) = 0.95$ for i = 1,....,17.

The 17 computed values for a_i are (smallest to largest):

0.84; 1.78; 2.06; 2.84; 3.32; 4.73; 4.97; 5.14; 5.30; 5.71; 5.83; 7.68; 8.53; 14.98; 17.14; 18.76; 23.12.

From these results it can be seen that, in general, the biological variance is (much) greater than the region-sampling variance: even when the largest values are disregarded, σ^2_b is (generally) at least 4 to 5 times as large as σ^2_r.

With some caution it might be stated that a similar effect will probably be apparent with other (not too small) values of M.

These and some other considerations were made using already available data obtained by the described method of sampling.

From all this it appears that not much is gained by taking more than, say, 3 x 3 slices per animal, at considerable expense of labour. Thus the number

of 9 was used in all experiments. Much more information would be gained by increasing the number of animals per group. This number, however, is restricted by experimental circumstances; 7 or 8 animals were used in most cases.

3.2.5 *Densities of EEF in other liver lobes*

The question of densities in other lobes of the liver was intriguing, though knowledge of this point was not necessary for this study, in which only median lobes were used for the assessment of EEF density.
But, as stated before, the number of EEF found per given surface area of liver tissue, cannot be generalized for the other lobes. The four lobes of a liver are anatomically different, making it impossible to prepare sections from comparable regions. Therefore, the M of the median lobe is incomparable with the values found in other lobes. However, there is a way to compare the densities in the 4 lobes in general, and that is to take sections at random from each of the lobes. This was done with four infected rats, by examining 10 sections of 3 slices each, taken at random from the four lobes. For each of the rats the hypothesis was tested that there was no difference in EEF density between the lobes (with one-way analysis of variance). In none of the cases (with densities ranging from 0.12-0.32) the hypothesis was rejected ($p<0.50$ for all of them), which implies that no significant difference could be demonstrated.
Another question that one may ask, is, whether or not there exists a systematic higher (or lower) EEF density in one lobe than in the others (although, as we saw, the actual differences for these 4 rats are not significant).
Using Friedman's test (3) it was found that no such indication existed ($p>0.10$).
However, remembering the outcome of the regression equation in section 3.2.2, the same test was applied to the there-mentioned 17 rats. In this case, a slight indication was found ($0.05<p<0.10$) that the density of the median lobe was systematically higher than that of the caudal lobe.
The values of the other lobes lay in-between.
The point was not further investigated.

3.2.6 *The limited sensitivity of the counting method*

It was mentioned that under certain circumstances the number of EEF in the

liver might approach zero, i.e. no EEF being demonstrated with the counting technique.

Indications for subdetectable EEF can be found by two methods. The first includes the complete sectioning of the liver, which is very laborious. Therefore an approach was made by sectioning the complete median lobe of a rat in which no EEF had been found with the routine method, the rat was taken from a group where the other animals had shown detectable numbers of EEF. In 53 sections (3x53=159 slices) at mutual distances of 75 µm, a total of 4 EEF could be detected. This indicates, qualitatively, that the counting technique has its limitations. The other method is by performing biopsies while keeping tha animals alive, to allow the eventual development of parasitaemias. Even if EEF were not detected, erythrocytic stages might appear. This method also gives a qualitative assessment of the sensitivity of this counting method.

Not withstanding these limitations in the sensitivity of the counting method, difference between populations could be readily detected by a statistical analysis. In these studies this property was used to demonstrate mechanisms that interfere with EEF development.

3.3. Application of the counting method

3.3.1 *Dose of sporozoites.*

To investigate the relationship between numbers of inoculated sporozoites and densities of EEF different dilutions of a suspension were inoculated into groups of two month old rats. Results are given in table 3.3.1, (exp. 1A).

The correlation coefficient r between the dose of sporozoites and the number of EEF per mm^2 of surface was 0,83.

This experiment was repeated with dilutions of another sporozoite suspension. A correlation of r= 0,94 was found. Data of both experiments are given in figure 8. It can be seen that in both experiments a higher dose results in a higher density of EEF, within the range of the given doses. Regression lines can be constructed through these points; the relationship between dose and density is not necessarily linear, however, especially not in doses which fail to induce detectable densities in all animals (exp. 1B).

From the figure it becomes clear that both regression lines are not identical, implying that results from different experiments cannot be compared. This was confirmed by the attempt to compare numbers of mature EEF of 21 independent sporozoite inoculations. The variable sporozoite doses were plotted against the numbers of EEF per mm^2 of liver tissue (fig. 9).

In this case a correlation coefficient r=0,51 was found. It means that the correlation between doses of sporozoites from different batches and densities of EEF is very weak. It can be seen that a high dose from one batch does not necessarily result in a high EEF density. Thus the density resulting from a sporozoite dose of another batch of mosquitoes cannot be predicted.

Table 3.3.1*

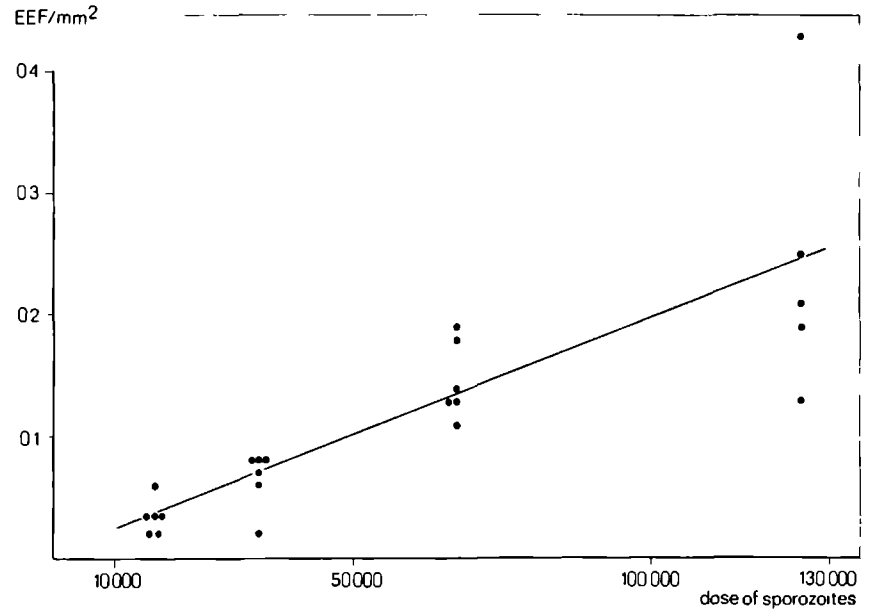
Exp. 1A

	inoculated sporozoites			
	125.000	67.500	34.400	17.000
	0,13	0,11	0,02	0,02
	0,19	0,13	0,06	0,02
	0,21	0,13	0,07	0,04
	0,25	0,14	0,08	0,04
	0,43	0,18	0,08	0,04
		0,19	0,18	0,06
$\bar{M}$	0,24	0,15	0,06	0,04
s.d.	0,11	0,03	0,02	0,01

Exp. 1B

	inoculated sporozoites				
	300.000	215.000	107.000	27.000	7000
	0,214	0,102	0,026	0,0	0,0
	0,248	0,103	0,026	0,004	0,0
	0,272	0,148	0,051	0,004	0,0
	0,313	0,199	0,057	0,018	0,007
$\bar{M}$	0,262	0,138	0,040	0,006	0,002
s.d.	0,042	0,046	0,016	(0,008)	(0,004)
R				0,0-0,018	0,0-0,007

* In this and the following tables the values of M (number of EEF/mm^2 of liver tissue), the mean value $\bar{M}$ and the standard deviation s.d. are given. When s.d. < $\bar{M}$, the range R is also given.



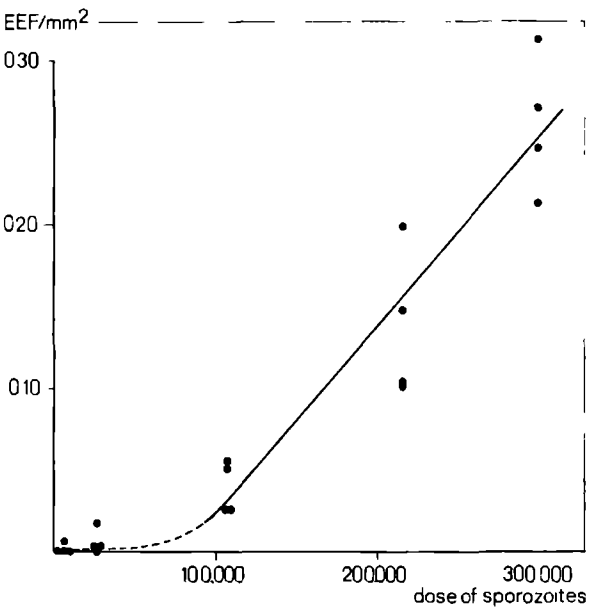


fig. 8. The relationship between numbers of sporozoites from one batch of mosquitoes, and resulting EEF densities. Upper graph exp. 1A, lower graph exp. 1B.

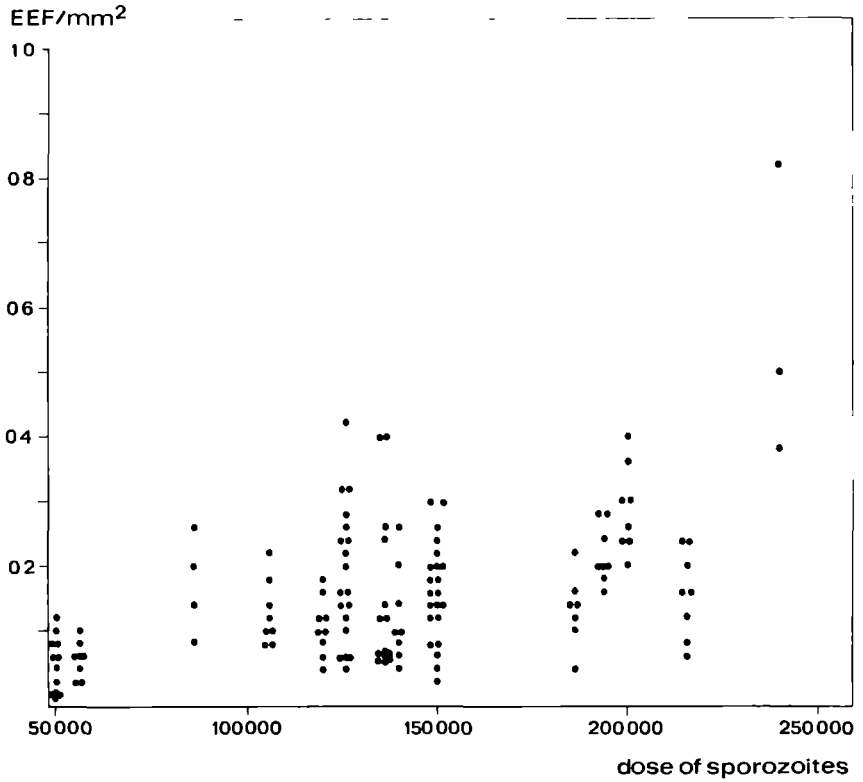


fig. 9. The relationship between numbers of sporozoites from different batches of mosquitoes, and resulting EEF densities.

3.3.2 *Preservation of infective sporozoites in vitro*

Sporozoites tend to loose their infectivity rapidly in vitro. Since it takes at least two hours to dissect a hundred mosquitoes, and to prepare and inoculate the sporozoite suspension, a medium is needed which preserves their infectivity. In a small experiment (2A), we tried to assess the influence of delayed inoculation, and preservation *in vitro* at 4^{o} in two media: M199 and Grace's insect TC medium. Rats received 120.000 sporozoites, suspended in one of these media, directly after preparation and after one and two hours. The resulting numbers of EEF (table 3.3.2) with Grace's TC medium tend to be somewhat higher than those with M199, which may indicate a better preservative effect of Grace's medium on sporozoites. The influence of time on the

Table 3.3.2

Exp. 2A

time of inoculation (hours)	Grace	M 199
0	0,20	0,13
	0,11	0,10
1	0,21	0,08
	0,16	0,06
2	0,14	0,07
	0,10	0,05

Exp. 2B

	original Grace pH6, directly inoculated	original Grace pH6, inoculated after 90 min.	Grace pH7 inoc. 90 min.	Grace pH7 10% calve serum inoc. 90 min.
	0,16	0,02	0,04	0,16
	0,18	0,06	0,13	0,18
	0,21	0,12	0,14	0,18
	0,21	0,12	0,16	0,18
	0,21	0,12	0,19	0,19
	0,24	0,15	0,22	0,22
	0,28	0,17	0,24	0,23
	0,28	0,20	0,29	0,23
$\bar{M}$	0,22	0,12	0,17	0,19
sd	0,03	0,05	0,07	0,02

loss of infectivity of sporozoites kept in Grace's insect TC medium and in modifications of it was investigated (exp. 2B).
A sporozoite suspension was divided into 4 parts. Two parts remained untreated, to the third part 10% calve serum was added and the acidity of this and the fourth part adjusted to pH 7. Volumes and numbers of sporozoites of the four parts were equal. One of the untreated batches was inoculated immediately after preparation (i.e. 2 hours after start of dissection). The three other batches were inoculated 90 minutes thereafter.

Using Steel's test, differences between the four groups were investigated. The results of both untreated batches differed significantly ($p<0,05$), so did those of the second untreated batch (90 minutes), and the batch adjusted to pH 7 with additional serum.
This indicates that the infectivity of sporozoites in unmodified Grace's medium (pH 6) is relatively badly preserved. pH 7 and additional serum prevent the sporozoites from significant loss of infectivity during a time lapse of $1\frac{1}{2}$ hour. In further studies the pH was adjusted to 7, to preserve infectivity during the dissection and preparation of the sporozoites suspension. Serum has generally not been used, to avoid the danger of serum sickness.

3.3.3 *Influence of species and age on susceptibility to sporozoites*

Initially 6-8 weeks old Swiss mice were used in experiments.
It appeared, however, that mice are very unsuitable hosts for sporozoites: mice got (lethal) parasitaemias, but prior numbers of EEF were very low.
B 10. LP and A/J mice were also tested. Only the A/J mice were more susceptible to sporozoites than the Swiss strain, but could not be obtained in large numbers. Moreover in Wistar rats better results were consistently obtained. About 20 times more EEF were found in two months old rats than in Swiss mice, when inoculated according to their body weight. But in contrast to mice there are indications that susceptibility in rats diminishes with age. The ultimate effect of these variable influences was compared, including those on the tree rat (*Thamnomys sp.*). The results are summarized in table 3.3.3.

Table 3.3.3.

Experimental animals	number	Mean body-weight	sporozoite inoculum	EEF/mm^2 liver tissue
4 month rats	5	230 g	1.00 ml	0,20 ± 0,11
2 month rats	3	125 g	0,55 ml	0,40 ± 0,14
Thamnomys	3	40 g	0,18	0,21 ± 0,15
Swiss mice	5	23 g	0,10 ml	0,003 ± 0,005

From these results it was concluded that rats are relatively susceptible hosts for pre-erythrocytic stages of *P. berghei*, and that young adult rats especially show high densities of mature EEF. Clear differences between the means of the young rats and those of tree rats were not found.

3.3.4 *Causal prophylactic effect of chloroquine*

In the studies presented in chapter 4.1. the effect of the immunogenic capacity of sporozoites had to be distinguished from effects caused by the erythrocytic stages developing from mature EEF of previous sporozoite inocula. Since there are practically no quantitative data on the influence of a parasitaemia, or its subsequent immune response on the course of reinoculation with sporozoites, chloroquine was used to suppress the development of parasitaemias. Though there are no indications in the literature that suggest a causal prophylactic effect of chloroquine, the influence of the drug was tested several times by its addition to drinkingwater. A representative experiment is described: a group of ten young rats received chloroquine in their drinkingwater over ten days (Nivaquine, 1,0 ml/l). Following these ten days sporozoites were inoculated (250.000/rat), while chloroquine treatment continued. Results from this treated group and a control group, both consisting of 10 rats, showed no difference (p=0,4,table 3.3.4). Hence chloroquine was used in experimental and control groups in immunization studies.

Table 3.3.4	(chloroquine)	(control)
	0,065	0,050
	0,168	0,162
	0,184	0,168
	0,200	0,239
	0,222	0,249
	0,267	0,273
	0,315	0,292
	0,340	0,323
	0,433	0,332
	0,504	0,429
$\bar{M}$	0,270	0,252
sd	0,131	0,105

3.4 Conclusions

a) Generally,the hypothesis of a uniform distribution of EEF in the median lobe had to be rejected. The true distribution was not investigated, since it was not considered important for the evaluation of results.

b) EEF densities were indicated as follows. Numbers of EEF in 9 slices of liver tissue from a defined region of the median lobe were expressed per surface area of the tissue slices. It was assumed that densities in defined regions of the median lobes, are a monotonically increasing function of the densities in the livers.

c) The sensitivity of the counting technique was limited, but sufficient to compare EEF-densities in different populations of rats.

d) There was an indication of a linear relationship between numbers of inoculated sporozoites and developing EEF. It seemed unlikely that a clear relationship exists between doses from different batches of mosquitoes, in different experiments.

e) In comparative studies it was found that two months old rats are susceptible hosts for pre-erythrocytic stages and that the infectivity of sporozoites is fairly well preserved in Grace's insect TC medium at pH 7.

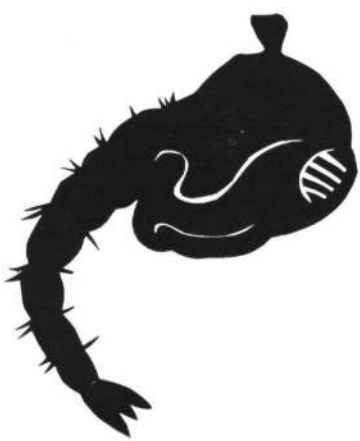
f) It was confirmed that chloroquine, administered with the drinkingwater, has no effect on the number of developing EEF.

References

1. Rao C.R. (1952). Advanced statistical methods in biometric research. J.Wiley & Sons, Inc. New York.

2. Owen, D.B. (1962). Handbook of statistical tables. Addison-Wesley Publ. Company, Inc. Reading.

3. Miller, R.G. (1966). Simultaneous statistical inference. McGraw-Hill Book Company, New York.



CHAPTER IV

THE EXPERIMENTAL WORK ON ANTI-SPOROZOITE RESPONSES

4.1 Studies on the immunization with sporozoites.

The aim of these studies was to investigate whether non attenuated sporozoites of P.berghei induce a response, which subsequently inhibits the invasion and development within liver parenchymal cells, after a succeeding inoculation of sporozoites.
Therefore a series of experiments was started with animals that were protected against following parasitaemias by chloroquine. This drug affects exclusively the erythrocytic stages and leaves the pre-erythrocytic stages undisturbed (see section 3.3.4). In these experiments, animals received varying doses of sporozoites, and responses were recorded as altering densities of EEF after a challenge with sporozoites.

Experiment 1 The effect of a single previous inoculation with sporozoites on a sporozoite challenge.

15 Two month old rats were intravenously inoculated with sporozoites (75.000/rat). Chloroquine was subsequently added to their drinking water. 60 Hours afterwards 8 animals (E1) received a sporozoite challenge (105.000/rat) that was also administered to 8 control rats (C1). After 45 hours median liver lobes were fixed; EEF were counted and expressed per mm^2 of covered tissue. The 7 remaining rats (E2) were challenged after one week (85.000/rat), 7 control rats (C2) received the same inoculum, and after 45 hours as above their livers processed as described. The results are tabelled below (table 4.1.1). The means of groups E1 and E2 differed significantly from the means of their controls ($p<0.001$, resp. $p<0.002$); the mean number of EEF in group E1 and E2 were 17% and 15% respectively of that of C1 and C2. In 2 animals of the group E2 no EEF could be demonstrated at all.

Table 4.1.1

	E1 (60 h)	C1 (control)	E2 (1 week)	C2 (control)
	0,011	0,080	0,0	0,037
	0,015	0,095	0,0	0,099
	0,016	0,102	0,004	0,126
	0,017	0,104	0,004	0,150
	0,020	0,120	0,014	0,153
	0,025	0,149	0,040	0,161
	0,060	0,187	0,080	0,223
	0,100	0,230		
$\bar{M}$	0,023	0,133	0,020	0,136
sd	0,015	0,052	0,030	0,058

Experiment 2 The effect one and two previous inoculations.

The experiment was extended with a group to which a second inoculum was administered before challenge. 15 Animals (E1 and E2) received a primary inoculum of sporozoites (110.000/rat) and after 4 days 7 of them (E2) got a second dose (100.000/rat). 12 Days after the first inoculation both groups were challenged with 150.000 sporozoites/rat and 8 control rats (C) received the same dose. From the results of this experiment (table 4.1.2) it was concluded that the first group E1 did differ significantly from the control C($p=0,01$). The mean number of EEF in group E1 was 69% of the control group C. The mean of the twice inoculated group E2 differed from that of the control group at a highly significant level ($p<0,001$) and here the mean number of EEF was only 6% of that of control group C. Furthermore the difference between groups E1 and E2 was highly significant ($p<0,001$).

Table 4.1.2

	E1 (1 dose)	E2 (2 doses)	C (control)
	0,099	0,0	0,149
	0,100	0,006	0,151
	0,108	0,010	0,187
	0,143	0,012	0,197
	0,150	0,013	0,207
	0,156	0,015	0,215
	0,164	0,027	0,218
	0,210		0,315
$\bar{M}$	0,141	0,012	0,205
sd	0,038	0,008	0,052

Experiment 3 The effect of two previous inoculations.

The previous experiment was repeated in a slighty different way. A group of 8 rats (E1) received 220.000 sporozoites per animal and after 5 days another group of 6 (E2) received a primary dose of 120.000 sporozoites per animal. Simultaneously, animals of the first group (E1) received a second dose of 60.000 sporozoites. Both groups were challenged a week later (135.000/rat) and 8 control (C) rats were also inoculated with this dose. The results (table 4.1.3) show a quite distinct outcome, when compared with the earlier experiments. In the once inoculated group E2 only 2 animals showed EEF, and in the animals (E1) that had received 2 inoculations no EEF were detected.

The differences with the control group are highly significant ($p<0,001$)

Table 4.1.3

	E2 (1 dose)	E1 (2 doses)	C (control)
	0,0	0,0	0,057
	0,0	0,0	0,058
	0,0	0,0	0,063
	0,0	0,0	0,066
	0,008	0,0	0,068
	0,009	0,0	0,071
		0,0	0,073
		0,0	0,128
$\bar{M}$	0,003	0,0	0,073
sd	(0,004)		0,023
R	0,0-0,009		

The results of experiments 1-3 were consistent with the observed phenomenon of reduced densities of EEF after one or two previous inoculations with sporozoites. However, the range of reduction as assessed by the counting method varied widely. The results from experiment 2 and 3 indicated that a second dose further reduced the density of EEF originating from the challenge inoculation. Therefore the question was how many booster inoculations would be necessary in order to achieve observed absence of EEF development induced by the challenge inoculation. For this purpose two experiments were done in which 1-4 boosters were administered after a primary sporozoite inoculation. The first dose always contained more sporozoites than the following ones.

Experiment 4 Effect of repeated doses of sporozoites on a sporozoite challenge.

4 groups of 4 animals were immunized with varying doses of sporozoites. 4 animals served as the control (group C). A first group (E1) received an immunizing dose (150.000/rat). After one week this group was boosted with 50.000 sporozoites per rat and at the same time a second group (E2) was inoculated with the primary immunizing dose (150.000/rat). The next week a third group E3 received the same primary dose, whereas the others were boosted with 50.000 sporozoites. The fourth week experimental group E4 got the primary dose, and the other groups E1-E3 got their booster inoculations, thus creating series E1-E4 of animals treated with 4-1 sporozoite doses. One week after the last dose all animals were challenged (100.000/rat) and the median lobes were fixed 45 hours later.
The results are given in table 4.1.4. Only in 2 out of 4 animals of group E4 could some EEF be demonstrated and even in them the mean number was below that of the control animals; in none of the other animals EEF could be detected.

Table 4.1.4

	E1 (4 doses)	E2 (3 doses)	E3 (2 doses)	E4 (1 dose)	C (control)
	0,0	0.0	0,0	0,0	0,019
	0,0	0,0	0,0	0,0	0,045
	0,0	0,0	0,0	0,009	0,064
		0,0	0,0	0,012	0,108
$\bar{M}$	0,0	0,0	0,0	0,005	0,059
sd				(0,006)	0,037
R				0,0-0,012	

Experiment 5 Effect of repeated doses.

The previous experiment was repeated with immunizing doses of about 300.000 sporozoites per rat and boosters of about 60.000 per rat. 5 groups of three animals each E1-E5 were immunized with 5-1 doses. The challenging dose consisted of 200.000 sporozoites and was administered one week after the last dose to all animals. In none of the immunized animals could a single EEF be demonstrated as can be seen in table 4.1.5

Table 4.1.5

	E1 (5 doses)	E2 (4 doses)	E3 (3 doses)	E4 (2 doses)	E5 (1 dose)	C (control)
	0,0	0,0	0,0	0,0	0,0	0,153
	0,0	0,0	0,0	0,0	0,0	0,202
	0,0	0,0	0,0	0,0	0,0	0,250
$\bar{M}$	0,0	0,0	0,0	0,0	0,0	0,202
sd						0,048

From the experiments 1-5 we got the impression that an immunizing dose, followed by 2 boosters prevented the development of EEF in liver parenchymal cells in all rats after challenge.

Experiment 6 Effects of previous exposures to infected mosquitoes on intravenous challenge.

Attempts were made to immunize animals via bites of infected mosquitoes. 7 Rats (E1 and E3) were each exposed to 50 female, *P. berghei* infected anophelines, three times at intervals of one week.
Three other rats (E2) were bitten once. Ten days later the latter group and 3 rats of the former group (E1) were challenged i.v. (700.000/rat), together with animals that had been bitten previously by non-infected mosquitoes (C1). The four remaining animals (E3) were challenged after 11 months (200.000/rat). Group C2 animals similarly exposed to uninfected anophelines, served as controls for the latter group. Results of both challenges are given in table 4.1.6. In none of the 3x exposed animals (E1) EEF were detected, whereas the group E2 differed significantly from group C1 (p=0,05). These results suggest that animals can be immunized adequately with naturally transferred sporozoites. The mean EEF number of group E2 was 5% of that in group C1. After about one year the interfering effect is still present as judged by the significant difference (p=0.03) between the results of groups E3 and C2; the mean number of EEF in group E3 was 21% of that in control group C2.
Another group of animals was immunized via bites of 10 mosquitoes per rat. Through a technical failure the chloroquine had been kept away too early and all animals developed parasitaemias after the fourth booster-biting. This indicates that these animals were not protected by 3 exposures to 10 mosquito bites with intervals of one week.

Table 4.1.6

	E1 (3 exposures)	E2 (1 exposure)	C1 (control)	E3 (3 exposures)	C2 (control)
	0,0	0,008	0,128	0,023	0,067
	0,0	0,010	0,150	0,026	0,134
	0,0	0,021	0,514	0,032	0,203
				0,034	
$\bar{M}$	0,0	0,013	0,264	0,029	0,135
sd		0,007	0,217	0,005	0,068

Experiment 7 Influence of contaminants in the sporozoite doses, used for immunization.

As indicated by the results of the previous experiment, there were no obvious reasons to suppose that the reduction in EEF-density was a non-specific effect, due to repeated contact with saliva of biting mosquitoes. However, by the artificial way of intravenous inoculation, suspensions of triturated salivary glands were used. These inocula always contain some debris, despite centrifugation. The possible effects of responses to these contaminants were studied.

5 Animals (group E1) were immunized with suspensions of salivary glands. These suspensions had been prepared by dissecting out the glands of 25 non-infected mosquitoes and by suspending them in a homogenizer. The suspensions were gently centrifuged and from the supernatants, containing little particulate material, 0,5 ml was injected per animal, three times at weekly intervals. Two weeks after the last dose the animals were challenged with sporozoites (90.000/rat) prepared in the similar, normal way, whereas nontreated rats of the same age got the same dose (group C1). In table 4.1.7 A the results are listed. A significant difference could not be demonstrated, thus the distributions of both populations are considered equal. The mean of samples in group E1 is 80% of that in group C1.

In addition a group of 8 rats (E2) was immunized with supernatants of

centrifuged suspensions of complete triturated thoraces. These supernatants still contained considerable amounts of mosquito debris and microorganisms. On 3 subsequent weeks these suspensions were inoculated i.v. and one week after the last dose, the animals and those of control group C2 were challenged with similarly treated suspensions containing sporozoites (125.000/rat). Results are given in table 4.1.7 B. It was observed that the means of the immunized and control groups differed significantly (p=0,05). The mean number of EEF in group E2 was 55% of that in the control group C2.

Table 4.1.7

Experiment 7A		Experiment 7B	
E1 (salivary glands)	C1 (control)	E2 (thoraces)	C2 (control)
0,03	0,02	0,0	0,054
0,04	0,04	0,025	0,067
0,04	0,05	0,027	0,092
0,06	0,09	0,052	0,093
0,08	0,11	0,058	0,107
		0,065	0,107
		0,090	0,124
		0,110	0,131
$\bar{M}$ 0,050	0,062	0,053	0,097
sd 0,020	0,037	0,036	0,026

Results of both parts of experiment 7 indicate that to avoid nonspecific interference, care should be taken to use sporozoite suspensions that are as purified as possible. Thus, in this study salivary glands dissected from infected mosquitoes were always used.

Experiment 8 Longitudinal effect of immunization with sporozoites.

Next the question of the maintenance of the preventive effect was studied. 23 Rats received three doses of sporozoites with intervals of one week, (100.000, 65.000 and 35.000/rat); one week after the last dose 5 of these animals (E1) were challenged (135.000/rat) and 6 control rats (C1) received the same dose. This procedure was repeated every following week, using 6 of the immunized rats. The challenging doses were respectively 215.000, 140.000 and 125.000 sporozoites per rat. The results are given in table 4.1.8. It became obvious that the prevention of EEF development was not induced in the expected way (see experiment 1-5). The fourth week challenge caused detectable EEF in all animals, but in the groups challenged earlier, varying numbers of animals were found to show no parasites. Nevertheless, in each experimental group a significant ($p<0,05$) difference was demonstrated with the mean number of EEF in respective control groups.
The mean number of EEF in the group E1-E4 were respectively 1%, 6%, 3% and 7% of that of the respective control groups C1-C4.

Table 4.1.8

E1 (ch.1 week)	C1 (control)	E2 (ch.2 weeks)	C2 (control)
0,0	0,12	0,0	0,063
0,0	0,14	0,003	0,076
0,0	0,24	0,003	0,127
0,0	0,26	0,007	0,163
0,01	0,40	0,009	0,168
	0,41	0,040	0,197
			0,236
			0,242
$\bar{M}$ 0,002	0,262	0,010	0,162
sd (0,004)	0,124	(0,015)	0,068
R 0,0-0,01		0,0-0,040	

	E3 (ch.3 weeks)	C3 (control)	E4 (ch.4 weeks)	C4 (control)
	0,0	0,044	0,003	0,070
	0,0	0,070	0,004	0,072
	0,0	0,092	0,008	0,073
	0,0	0,104	0,011	0,108
	0,011	0,106	0,011	0,117
	0,012	0,149	0,017	0,147
		0,273		0,149
				0,201
				0,233
$\bar{M}$	0,004	0,130	0,009	0,130
sd	(0,006)	0,075	0,005	0,058
R	0,0-0,012			

Experiment 9 The protective aspect of the anti-sporozoite response.

The preventive effect of immunization with sporozoites on developing EEF after a challenge was further explored. 5 animals (E) received an immunizing dose of 200.000 sporozoites per rat and 3 subsequent booster inoculations at intervals of one week (60.000/rat). A week after the last dose of sporozoites chloroquine was removed from the drinking water. One month later these animals and the control group C were challenged with 125.000 sporozoites and after 45 hours a liver biopsy was performed. The immunized and control groups were kept alive and daily bloodsmears were made over a fortnight. One immunized rat showed a patent infection, two days after the mean day of patency of the control group; in the other 4 animals no parasitaemia was induced. The five control animals became patent, indicating that the time lapse between the end of chloroquine treatment and challenge was enough to minimalize the suppressive effect. The results of counting EEF in the biopsies are given in table 4.1.9. EEF could not be demonstrated in experimental group E, and apparently 4 of 5 immunized animals were fully protected against parasitaemia.

Table 4.1.9

	E (imm.)	C (control)
	0,0	0,008
	0,0	0,010
	0,0	0,012
	0,0	0,020
	0,0	0,022
$\bar{M}$	0,0	0,014
sd		0,006

Experiment 10 Further extent of challenge

The interval between challenge inoculum and immunizing dose was extended further to in the following experiment. 4 animals (E) received 4 booster inoculations (± 75.000/rat) after an immunizing dose (160.000/rat). They were challenged 3 months after the last booster with 100.000 sporozoites, together with 5 control animals (C) of the same age. As can be seen in table 4.1.10, no EEF could be detected in the immunized animals (p=0,03)

Table 4.1.10

	E (imm.)	C (control)
	0,0	0,018
	0,0	0,047
	0,0	0,054
	0,0	0,063
		0,098
$\bar{M}$	0,0	0,056
sd		0,029

Experiment 11 Anti-sporozoite responses in rats of different ages.

Animals of different ages were immunized, for the study of age dependency in anti-sporozoite immune response of rats.

Groups of 3 week (E1), 2 (E2) and 4 month (E3) old rats, consisting of 5 animals each, got an immunizing dose of 120.000 sporozoites and 2 booster inoculations (44.000 and 75.000/rat) at intervals of one week.
Groups of 3 (C1, C3) and 2 rats (C2) served as controls. All animals were challenged 2 weeks after the last booster with 75.000 sporozoites. Results are given in table 4.1.11, body-weights and liver-volumes are also given.
It seems that the E2 and E3 group were especially well immunized. In both groups only one positive case was found, with a low number of EEF.
The means were 1%, resp. 2% of that of the controls. This contrasted sharply with the young group, in which EEF were easily found in all animals. In this case the mean number of EEF was 16% of that of the control group C1. Though this is a considerable reduction, this small experiment gives an indication that in adult rats the reductions are more pronounced than in young animals and probably not in proportion to increase in body-weight and liver-volume.

Table 4.1.11

	young rats		young rats		old rats	
body-weight (g)	101,5±5,4		157,3± 14,6		185,9± 8,2	
liver-volume (ml)	2,3±0,2		2,9± 0,2		3,1± 0,2	
	E1	C1	E2	C2	E3	C3
	0,008	0,180	0,0	0,029	0,0	0,007
	0,021	0,198	0,0	0,120	0,0	0,025
	0,034	0,249	0,0		0,0	0,139
	0,048		0,0		0,0	
	0,061		0,007		0,007	
$\bar{M}$	0,034	0,209	0,001	0,074	0,001	0,057
sd	0,021	0,036	(0,003)	0,064	(0,003)	(0,0072)
R			0,0-0,007		0,0-0,007	0,007-0,139

The results of this series of experiments show that the development of EEF can be prevented in rodents by repeated inoculation with sporozoites of *P. berghei*. This appeared to be a gradually developing mechanism.
One previous inoculation caused already a decrease in the density of EEF, but the effectiveness of a single immunizing dose of sporozoites to a challenge appeared variable. The preventive effect gathered strength with increasing numbers of booster inoculations, and finally resulted in the failure to detect EEF. The number of inoculations to achieve this response also varied, but generally 2 to 3 boosters after one immunizing dose were sufficient to reduce the number of detectable EEF to zero.
The counting technique has limitations which became obvious when animals were kept alive after biopsy. Occasionally, a delayed parasitaemia could appear, even though no EEF had been detected in the biopsy. Further studies with standardized inocula are needed to establish a procedure which leads to a completely protective response.
The preventive effect was found to be maintained in the experimental animals for several months, though not always at the same level. Even after a year considerable reduction could still be detected.
The preventive effect could also be demonstrated after intravenous challenge with sporozoites, following exposures to infected mosquitoes.
It was shown that the small quantities of mosquito contaminants, inoculated with sporozoites did not cause such an effect. Only when the animals were repeatedly inoculated with considerable amounts of mosquito tissue and subsequently challenged with sporozoites in heavily contaminated suspensions a significant decrease of EEF was established.
It was found that adult rats of different ages can be immunized equally well. Young rats, however, show a much smaller reduction of EEF.
Whether this is related to their optimal susceptibility to sporozoites remains unclear.

4.2 Studies of mutual short-term interference of pre-erythrocytic stages.

During the pre-erythrocytic stages interference of sporozoite induced infections was observed in animals that had been challenged 60 hours after one previous immunizing dose of sporozoites (see section 4.1, exp. 1). At that time virtually all EEF had already completed schizogony, and merozoites had been liberated before the sporozoite challenge. Therefore the question arose as to whether such an interference could also be demonstrated in challenge infections given before complete maturation of EEF from the primary dose of sporozoites.

Experiment 1 Effect of one previous sporozoite infection on the infectivity of sporozoites, inoculated after 24 hours.

Three groups of 3 rats were used in a pilot attempt. Groups C1 and E were inoculated with sporozoites (250.000/rat) and after 24 hours groups E and C2 received a second inoculum (300.000/rat). Group C1 served as a control of the first inoculum. Livers were fixed after 45 hours. Group E was the experimental group, whereas group C2 served as control for the second inoculum. Livers of groups E and C2 were fixed 45 hours after the second inoculum.
Results are listed in table 4.2.1 and those of E and C2 were compared. The mean values indicate a possible difference between the groups ($p=0.10$). The mean of E was 22% of that of C2. The experiment was repeated with larger groups of animals.

Table 4.2.1

	C1 (control 1st inoc.)	E (twice inoc.)	C2 (control 2nd inoc.)
	1,54	0,13	1,33
	1,28	0,40	2,07
	0,87	0,29	0,33
$\bar{M}$	1,230	0,273	1,243
sd	0,338	0,136	0,873

Experiment 2 Infectivity at reinoculation after 24 hours.

3 Groups C1a, C1b and E, each consisting of 7 rats received a primary inoculation with sporozoites (150.000/rat). After 24 hours a second sporozoite inoculum (150.000/rat) was administered to groups E and C2. Group C1a was used as a control for the first inoculation. The median lobes of these animals were fixed after 45 hours. Animals of group C1b were killed after 69 hours, together with those of groups E and C2. The results of group C1b would give information about a possible persistance of EEF from the first inoculum. Animals of the twice inoculated group E were killed 45 hours after the second inoculation together with the control animals of group C2. The results are listed in table 4.2.2. No EEF could be demonstrated in group C1b, but numerous cell infiltrations indicated that merozoites had been liberated. Numbers of EEF in group E were thus considered to originate from the second inoculum of sporozoites. The mean of this group was found to be significantly differed from that of control group C2 (p=0,01) and the mean of E was 35% of that of C2.

Table 4.2.2.

	C1a (control 1st inoc.45 h.)	C1b (control st inoc.69 h)	E (twice inoc.)	C2 (control 2nd inoc.)
	0,06	0,0	0,003	0,03
	0,08	0,0	0,01	0,05
	0,14	0,0	0,02	0,08
	0,16	0,0	0,04	0,12
	0,25	0,0	0,05	0,13
	0,27	0,0	0,05	0,16
	0,31	0,0	0,09	0,18
$\bar{M}$	0,181	0,0	0,038	0,107
sd	0,097		0,030	0,056

Experiment 3 Infectivity at reinoculation after 20 hours.

In addition to experiment 1 and 2 the time lapse between both inoculations was shortened to 20 hours. The first dose (200.000/rat) was administered to groups C1 (4 rats) and E (6 rats), whereas groups E and C2 (4 rats) got a second sporozoite inoculum (85.000/rat). Results are expressed in table 4.2.3. Means of groups E and C2 again differed significantly (p=0,01) and the mean of E was 35% of that of C2.

Table 4.2.3

	C1 (control 1st inoc.)	E (twice inoc.)	C2 (control 2nd inoc.)
	0,21	0,03	0,09
	0,24	0,03	0,14
	0,26	0,05	0,21
	0,31	0,06	0,27
		0,07	
		0,13	
$\bar{M}$	0,255	0,062	0,177
sd	0,042	0,037	0,079

Experiment 4 Infectivity at reinoculation after 16 and 8 hours.

The interval between the first and the second inoculation was further shortened to 16 and to 8 hours. Groups C1a, b, c and E1,2 got a primary inoculation of sporozoites (120.000/rat). Animals of groups E1 and C2 were reinoculated after 8 hours (200.000/rat) and those of groups E2 and C3 received their second dose 16 hours after the first one (240.000/rat). Animals of group C1a were killed after 45 hours, and gave positive information about the infectivity of sporozoites of the first inoculation. 4 Rats of group C1b were killed after 8 + 45 = 53 hours, together with E1 and C2.

3 Other rats (C1c) were killed after 16 + 45 = 61 hours, together with E2 and C3. The results of both groups C1b and C1c served as indicators for a possible persistence of EEF from the first inoculum.In table 4.2.4 the results are given. The presence of EEF in C1b rats, killed after 53 hours together with groups E1 and C2, means that persistence of some EEF is also likely in animals of group E1, but this number is considered negligible, compared with the numbers of EEF due to the second inoculation. The means of E1 and C2 appeared to differ significantly (p=0,01) and E1 was 50% of C2.

After 61 hours the C1c rats failed to show EEF. Therefore presence of persistent EEF of the first inoculum in group E2 animals was considered unlikely. The means of E2 and C3 showed a significant difference (p=0,01), expressed as 41%.

	C1a (controls 45 h)	C1b 1st inoculation) 53 h	C1c 61 h	E1 (2x inoc.) 8 h	C2 (control) 8 h	E2 (2x inoc.) 16 h	C3 (control) 16 h
	0,13	0,004	0,0	0,06	0,25	0,04 ±	0,39
	0,13	0,005	0,0	0,07	0,30	0,07	0,50
	0,16	0,005	0,0	0,10	0,37	0,17	0,58
	0,19	0,008		0,10	0,40	0,24	0,83
				0,15		0,24	
				0,21		0,25	
				0,29		0,26	
				0,32		0,63	
$\bar{M}$	0,152	0,005	0,0	0,162	0,330	0,237	0,575
sd	0,029	0,002		0,100	0,068	0,180	0,187

Experiment 5 Infectivity at reinoculation after 36 hours.

Interfering influence of one previous inoculation was also studied over a period of 36 hours. Groups C1 and E each containing 5 rats, were inoculated with sporozoites (120.000/rat) and after 36 hours groups E and C2 received a second inoculum (100.000/rat). Results are given in table 4.2.5. An indication for difference (p=0,10) could be demonstrated between the means of group E and C2; the mean of E was 52% of that of C2.

Table 4.2.5

	C1	E	C2
	0,048	0,020	0,022
	0,059	0,022	0,045
	0,079	0,035	0,057
	0,104	0,041	0,127
	0,105	0,046	
$\bar{N}$	0,079	0,033	0,063
sd	0,026	0,011	0,045

Experiment 6 Influence of the sporozoite dose of the first inoculum on infectivity of reinoculated sporozoites.

In order to study the interfering capacity of the first inoculation, sporozoite suspensions of different concentrations were prepared and administered to three groups of 7 animals (E1:300.000/rat; E2:30.000/rat; E3:3.000/rat) 2 Animals of each (C1, C2 and C3) group were killed after 45 hours, to control infectivity. The remaining animals together with 8 controls (C4) received second sporozoite inoculum after 20 hours (55.000/rat).

Results are given in table 4.6.6. The means of groups E1, E2 and E3 all differed significantly from that of group C4 and the mean numbers of E1, E2 and E3 were respectively 13, 47 and 44% of that in C4.

Mutually the subgroups differed as follows:

E1	- E2	p=0,032	E2	- E3	p=0,29 (n.s)
E1	- E3	p=0,047	E2	- C4	p=0,017
E1	- C4	p=0,001	E3	- C4	p=0,02

(n.s.= non significant)

As indicated by the similar results of the control groups C2 and C3, some irregularity might have occurred with the sporozoite dilutions. The equal means of E2 and E3 may be due to this presumed technical failure. Nevertheless the comparison of E1 and E2, E3 with C4 indicate a dose dependent interference on the second sporozoite inoculum after the first injection. The experiment was repeated.

Table 4.2.6

	E1	E2	E3	C4
	0,0	0,001	0,0	0,027
	0,0	0,001	0,003	0,029
	0,0	0,020	0,014	0,048
	0,0	0,021	0,035	0,066
	0,010	0,031	0,042	0,066
	0,020	0,056	0,047	0,069
		0,071	0,048	0,094
				0,101
$\bar{M}$	0,006	0,024	0,024	0,062
sd	(0,011)	(0,026)	0,021	0,029
R	0,0-0,027	0,001-0,071		

	C1	C2	C3
	0,126	0,010	0,008
	0,282	0,054	0,056

Experiment 7 Dose related interference.

The initial inocula consist of similar numbers of sporozoites and the second inoculum was administered after 24 hours (110.000/rat). Results are given as in experiment 6, in table 4.2.7.

Mutual difference and levels of significance were as follows.

E1 - E2	p=0,31 (n.s)	E2 - E3	p=0,04
E1 - E3	p=0,036	E2 - C4	p<0,01
E1 -C4	p=0,01	E3 - C4	p=0,07 (n.s)

The mean numbers of E1, E2 and E3 were respectively 26, 33 and 58% of the mean in controlgroup C4. With some care it can be concluded that the interference with infectivity of the second inoculum is dependent on the number of previously inoculated sporozoites.

Table 4.2.7

	E1	E2	E3	C
	0,0	0,027	0,053	0,125
	0,0	0,028	0,095	0,298
	0,047	0,046	0,103	0,308
	0,060	0,071	0,112	0,311
	0,088	0,080	0,121	
	0,129	0,083	0,167	
	0,163	0,161	0,241	
		0,212	0,320	
$\bar{M}$	0,069	0,088	0,151	0,260
sd	0,054	0,054	0,083	0,083

	C1	C2	C3
	0,192	0,035	0,006
	0,247	0,055	0,009

Experiment 8 Influence of salivary gland tissue on infectivity of sporozoites, inoculated shortly afterwards.

A control experiment was performed to exclude the possibility that the interference was due to the mosquito tissue, inoculated with the first dose.

Triturated salivary glands of non-infected mosquitoes were inoculated into 8 animals (group L) which were reinoculated after 24 hours with sporozoites (50.000/rat) at the same time as control rats (C2). The results are given in table 4.2.8. No indication for a difference between groups L and C2 could be found.

Table 4.2.8

	L	C
	0,007	0,003
	0,007	0,011
	0,036	0,012
	0,037	0,013
	0,050	0,055
	0,065	0,076
	0,087	0,090
	0,100	0,110
$\bar{M}$	0,049	0,046
sd	0,034	0,042

Experiment 9 Influence of a reinoculation on number and size of EEF of the previous infection.

Finally, it was investigated as to whether the development of EEF of the first inoculum might be influenced by the second. Thus, two groups of animals C1 and E were inoculated with sporozoites (200.000/rat). Group E was reinoculated after 20 hours (85.000/rat) and animals of both groups killed 45 hours after the first inoculum. At that time, it is still impossible to detect developing EEF of the second dose. No difference could be established between the mean numbers of EEF in both groups.

Table 4.2.9

	E	C
	0,01	0,21
	0,11	0,24
	0,26	0,26
	0,30	0,31
	0,30	
	0,32	
$\bar{M}$	0,217	0,255
sd	0,127	0,042

Width and length of 20 randomly chosen EEF were measured in 4 animals of both groups. It was supposed that the size of EEF within each of the groups was comparable, and that the distribution of cutting places of EEF in both groups was equal. Using the Wilcoxon test for a normal approximation and correction for ties, no significant difference was found between the sizes in both groups ($p=0,50$). This means that a reinoculation of sporozoites does not interfere with number and size of the maturing EEF of the first inoculum.

4.2.1 *Conclusions*

A limited study was initiated to see whether sporozoites, inoculated shortly after each other, would exert a mutual influence.
Results showed a decrease in the numbers of EEF, when the second inoculation was given 24 hours after the first dose of sporozoites.
This would mean that the first 24 hours of EEF development was influenced by already present EEF, induced by the primary dose, now in their last 24 hours of maturation and rupturing at the end of their maturation period.
Further investigations along this line confirmed the result of the pilot experiment. Inoculations at different times during the 48 hours period of EEF maturation, following the first dose of sporozoites, resulted consistently in lower numbers of EEF. The reduction in the various groups, following re-inoculation after 8-36 hours, generally was more than twofold and dependent on the number of sporozoites in the first inoculum.
A reverse effect on the developing EEF of the first inoculum was not demonstrated. A possible effect of non-infected salivary glands on subsequently inoculated sporozoites could be excluded. It is still impossible to explain the partially preventive effect of this non-specific inhibitor on EEF development (see also section 4.4 on the possible effect of interferon).

4.3 Studies of interference by present and past parasitaemias.

An attempt was made to repeat earlier studies on the influence of parasitaemia upon EE development. The quantitation of EEF on four occasions parasitaemias were induced in 3-4 month old rats, by intraperitoneal inoculation of infected blood containing 5.10^7 parasitized erythrocytes. Peaks of less than 10% were reached about one week after inoculation, and there-after parasite densities decreased rapidly to levels below 0,5% within another week. The courses of these parasitaemias are depicted in fig. 10. The infected animals received an intravenous inoculation of sporozoites at different stages of the parasitaemia.

Experiment 1. Infectivity of sporozoites at the onset of parasitaemia.
At the onset of parasitaemia (± 8 parasitized erythrocytes/10.000), 5 days after infection, 8 rats (group P) were inoculated with sporozoites (80.000/rat). Forty five hours after this challenge, median lobes were obtained at autopsy. At that time parasite density averaged 85 parasitized erythrocytes per 10.000.
Lobes of 6 control rats (group C) inoculated with the same dose were also collected. Results are given below (table 4.3.1). Not a single EEF was observed in the animals with a parasitaemia. This differed, at a highly significant level ($p<0,001$), with the results of controls, which showed a mean number of 0,014 EEF/mm^2 liver tissue.

Table 4.3.1

P (parasitaemia)	C (control)
0,0	0,006
0,0	0,008
0,0	0,014
0,0	0,017
0,0	0,020
0,0	0,020
0,0	
0,0	$\bar{M}$ 0,014
	sd 0,006

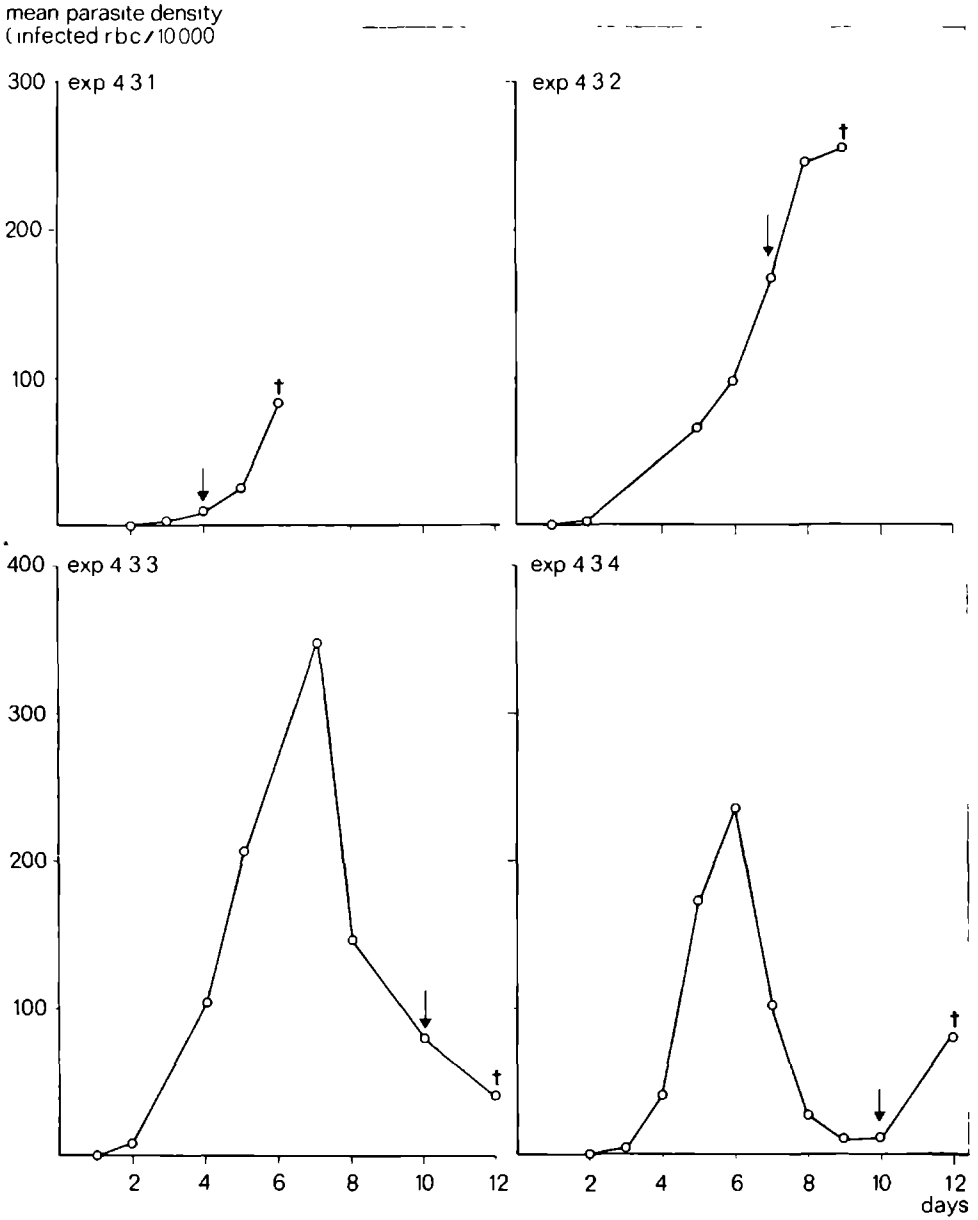


fig. 10. Courses of parasitaemias in rats, superinfected with sporozoites. ↓ day of inoculation with sporozoites; † day of autopsy.

Experiment 2. Infectivity of sporozoites at a raised parasitaemia.

At an increasing parasitaemia (± 128 parasitized erythrocytes/10.000) 7 days after infection, another group of 8 rats (P) received a sporozoite inoculation (10.000/rat). Results are given in table 4.3.2. Forty five hours later no EEF were found in 5 out of 8 animals, whereas in 3 rats very low numbers were found. The results of the whole experimental group averaged 0,004 EEF/mm^2 and the control group (C) of 8 rats had a mean of 0,052 EEF/mm^2. The means differed at a highly significant level ($p<0,001$). The mean numbers of EEF in group P was 6% of that in group C.

Table 4.3.2

	P (parasitaemia)	C (control)
	0,0	0,007
	0,0	0,020
	0,0	0,029
	0,0	0,046
	0,008	0,065
	0,008	0,070
	0,012	0,071
		0,108
$\bar{M}$	0,004	0,052
sd	(0,005)	0,033
R	0,0-0012	

Experiment 3. Infectivity of sporozoites at a lowering parasitaemia.

A group of 8 rats (P), in which the parasite density was decreasing after a peak parasitaemia (83 parasitized erythrocytes/10.000), was challenged with sporozoites (105.000/rat) on day 10 after infection.

Results are given in table 4.3.3. Forty five hours later no EEF were found in 3 of these rats. The results of the whole group averaged 0,017 EEF/mm^2 and that of the control group 0,064 EEF/mm^2.

The difference of these means was significant (p=0,01). The mean number of EEF in group P was 23% of that in group C.

Table 4.3.3

	P (parasitaemia)	C (control)
	0,0	0,014
	0,0	0,026
	0,0	0,031
	0,015	0,051
	0,016	0,058
	0,028	0,088
	0,029	0,111
	0,051	0,132
$\bar{M}$	0,017	0,064
sd	(0,018)	0,042
R	0,0-0,051	

Experiment 4. Infectivity of sporozoites at a decreased parasitaemia.

After peak parasitaemias in a group of 7 rats the levels of parasite density had come down to below 50 parasitized erythrocytes/10.000 on day 8. On day 10, with a mean parasite density of 6 parasitized rbc/10.000, the rats (P) were challenged with sporozoites (75.000/rat) simultaneously with 4 controls (C). At autopsy all parasite densities had been increased to a mean of 82 parasitized erythrocytes/10.000. Comparison of the results in both groups does indicate a significant difference (p=0,055); the mean number of EEF in group P was 50% of that in group C.

Table 4.3.4

	P (parasitaemia)	C (control)
	0,009	0,056
	0,028	0,120
	0,042	0,157
	0,042	0,197
	0,068	
	0,099	
	0,175	
$\bar{M}$	0,066	0,132
sd	0,056	0,060

After this series of experiments on the interfering capacity of parasitaemias, subsequent experiments were done in animals with past parasitaemia. The susceptibility to sporozoites was studied in animals with a solid immunity to the erythrocytic stages of the homologous strain of *P. berghei*.

Experiment 5. Infectivity of sporozoites in animals immune to bloodstages.

Rats that survived a first infection with parasitized erythrocytes were boosted after 2 months. Another fortnight later, when no parasitaemia could be detected, 6 of these rats (P) and 5 controls (C) received a sporozoite inoculum (110.000/rat). A significant difference was found between the means of experimental and control groups ($p=0,04$); the number of EEF in group P was 44% of that in the control group C.

Table 4.3.5

	P (immune)	C (control)
	0,0	0,084
	0,027	0,174
	0,103	0,178
	0,108	0,270
	0,109	0,284
	0,176	
$\bar{M}$	0,087	0,198
sd	0,064	0,081

Experiment 6. Infectivity of sporozoites during a chronic low parasitaemia.

10 animals that previously had survived a blood-induced parasitaemia, were boosted with erythrocytic stages after 2 months. They developed a chronic infection with parasite densities of less than 20 parasitized erythrocytes/ 10.000. These animals (P) were inoculated with sporozoites (45.000/rat)., together with their infected controls (C). Results are given in table 4.3.6. The difference of means of groups P and C appeared to be significant (p=0,01) and the number of EEF in group P was 36% of that in the control group C. This confirmed thus the indications found in the previous experiment.

Table 4.3.6

	P (immune)	C (control)
	0,0	0,005
	0,005	0,013
	0,005	0,014
	0,006	0,014
	0,009	0,020
	0,010	0,032
	0,015	0,035
	0,016	0,039
	0,020	0,047
	0,026	0,054
		0,064
$\bar{M}$	0,011	0,031
sd	0,008	0,019

Experiment 7. Immunization with sporozoites under protection of differently induced liver-blood barriers.

An attempt was made to immunize rats with sporozoites whereby in part of the animals a liver-blood barrier was induced by stimulation of the immune mechanism against stages that infect the erythrocytes. No chloroquine was given with the drinking water. 8 Rats (P) were immunized against the erythrocytic stages by a blood induced infection, followed one month later by similar a booster. Another 5 weeks later no erythrocytic stages could be detected and a first sporozoite inoculum was administered (120.000/rat). After a week a sporozoite booster inoculation was given (75.000/rat). 8 Normal rats (E) were protected with chloroquine against erythrocyte infection and were immunized with sporozoites at the same time as group P. Together with 8 control rats (C), the 2 groups of immunized animals were challenged one week later with 145.000 sporozoites. Results of this experiment are given in table 4.3.7. The means of both groups immunized with sporozoites (P and E) differ highly significantly from that of the control C. ($p<0,001$). In 7 out of 8 blood immune animals (P) no EEF were found, compared with 5 out of 8 chloroquine protected animals (E). The mean number of EEF in P and E was 1%, resp. 4% of that in C.

Table 4.3.7

	P (blood imm.)	E (Chlor.)	C (control)
	0,0	0,0	0,057
	0,0	0,0	0,059
	0,0	0,0	0,077
	0,0	0,0	0,094
	0,0	0,0	0,110
	0,0	0,007	0,128
	0,0	0,008	0,151
	0,007	0,019	0,195
$\bar{M}$	0,001	0,0045	0,109
sd	(0,002)	(0,007)	0,048
R	0,0-0,007	0,0-0,019	

4.3.1 *Conclusions*

These results indicate that parasitaemia in some way interferes with the development of EEF in liver parenchymal cells. In all experiments, densities of EEF in rats with a present or past parasitaemia were lower than in controls, always at a significant level.
Primary parasitaemias especially caused considerable reductions of developing EEF during the rising and decreasing phases. No EEF could be detected at all in 17 out of 25 rats (68%) used in the three first experiments.
It is not clear, however, if the observed effect is correlated with parasite densities, or with other changes provoked by parasitaemia.
Indications were found that animals, immune to erythrocytic stages, offer also a less favourable environment for infective sporozoites.
But this immunity is obviously not able to prevent exo-erythrocytic development completely. To achieve this effect, it is necessary to immunize with sporozoites.(For a possible role of interferon, see section 4.4).

4.4 Preliminar observations on some mechanisms of resistance

4.4.1 *Serological findings*

20 rats were immunized under different protective regimens. 6 rats (Group N) were treated as normal with chloroquine, another 6 (Group M) were fed with PABA-deficient milkpowder and the remaining 8 (Group B) were left untreated during immunization. An immunizing dose of 150.000 sporozoites were administered per animal and 3 subsequent booster inoculations at weekly intervals (65.000, 20.000 and 160.000/rat).
5 rats of group B succumbed to their parasitaemias within two weeks, whereas in the 3 remaining animals and those of the other groups no erythrocytic stages were found in blood smears three weeks after the immunizing dose.
Anti-sporozoite antibodies were determined in sera of these animals, obtained 6 days and 7 weeks after the last booster. CSP reactivity was determined by counting 20 sporozoites and indicating the number showing precipitates;

Results of these determinations are listed in table 4.4.1. About three quarter of the sporozoites that were incubated in the sera, obtained shortly after immunization showed precipitates. There was a tendency that the number of sporozoites with precipitates slichtly decreased over the period of 6 weeks, but this should be affirmed in titration experiments. All of the pooled sera (✓) from immunized rats showed SNA, in contrast with the pre-immunization sera.

The results indicate that treatment with chloroquine, induces similar CSP and SNA antibody levels as are detected in untreated animals.

Table 4.4.1

Group		1 week before first dose		5 days after last booster		7 weeks after last booster	
		CSP	SNA	CSP	SNA	CSP	SNA
	1	0+	✓	8+	✓	15+	✓
	2	0+	✓	19+		13+	✓
N	3	0+	✓ Neg	18+	✓ Pos	11+	✓ Pos
	4	0+	✓	14+	✓	12+	✓
	5	0+	✓	18+	✓	15+	✓
	6	0+		18+	✓	6+	
mean±sd		0		15,7±4,1		12,0±3,3	
	7	0+		16+		14+	
	8	0+		16+		16+	
M	9	0+		16+		7+	
	10	0+		17+		12+	
	11	0+		18+		17+	
	12	0+		16+		12+	
mean±sd		0		16,5±0,8		13,0±3,6	
	14	0+		16+	✓	5+	✓
B	16	0+		17+	✓ Pos	13+	✓ Pos
	20	0+		6+	✓	14+	✓
mean±sd		0		13,0±6,1		10,7+4,9	

✓ indicates the serum samples, pooled for detection of SNA

4.4.2 *A pilot study on the production of interferon*

There are indications that malaria parasites induce the production of interferon and other data pointing to a deleterions effect of interferon to malaria parasites (both the pre-erythrocytic and erythrocytic stages). The presence of circulating interferon would give an explanation for the short-term interference of sporozoite infectivity by a previous sporozoite inoculation (see section 4.2) and partially for the observed interference by parasitaemias (see section 4.3).

Three groups of two month old rats were inoculated with 10^8 erythrocytic stages, with 200.000 sporozoites per animal and with the artificial interferon inducer r I:r C respectively. Sera were obtained at regular intervals. The number of plaque-forming units (PFU) was determined on a monolayer of rat embryonic cells, incubated with these sera and overlayed with VSV virus.

The results of the sera from rats treated with r I:r C were consistent. Within 4 hours after inoculation 80% reduction of PFU was observed, while after about 30 hours the numbers of PFU approached again the control values.

In the sera of rats inoculated with erythrocytic stages PFU counts fluctuated but never showed a 50% inhibition as compared with control plaque counts. The parasitaemias, which were lethal, apparently caused no induction of circulating interferon at detectable levels. The sera of animals, inoculated with sporozoites showed no uniform pattern. In the series of two rats fluctuating results were obtained, without a sign of difference after appearance of parasitaemias. In one of the series we observed a 40% reduction of PFU after 48 hours, which might correlate with the rupture of liver parenchymal cells containing mature EEF. Some of the results are depicted in figure 11. It should be admitted that our experience is still insufficient and the performance of the test needs improvement. However, since the results of the artificial inducer were consistent, we report here, on these preliminary results.

4.4.3 *Immunization of normal and athymic mice*

In order to obtain insight into the role of the thymus in anti-sporozoite responses inbred B10.LP mice, which had acquired the thymus deficiency factor (nude) by repeated backcrossing were immunized. Groups of athymic (nu/nu) and

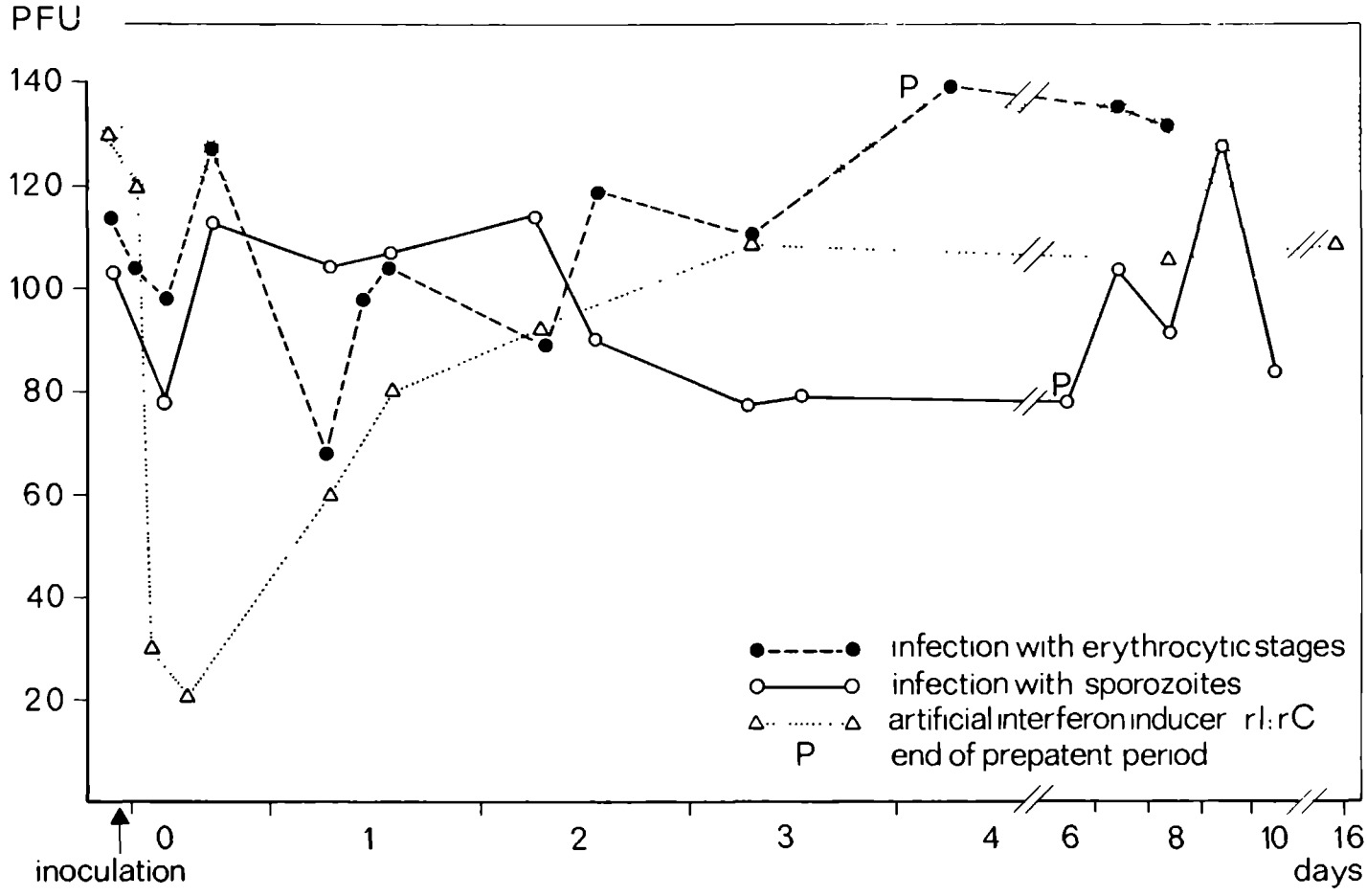


fig. 11. Longitudinal observation of interferon levels in three groups of rats, infected with erythrocytic stages, with sporozoites, or treated with an artificial interferon inducer.

normal (nu/+) littermates received 4 doses of sporozoites, at weekly intervals (100.000, 130.000, 30.000, 40.000/mouse). After challenge (30.000/mouse) 3 weeks after the last booster daily blood smears were made to monitor parasitaemias. It was found that the immunized nu/nu animals invariably developed parasitaemia within a week, as did the non-immunized nu/nu and nu/+ controls. Only in the immunized nu/+ group no parasitaemia was found until 16 days after challenge. These results could be reproduced in a second attempt, in which 120.000, 20.000, 15.000 and 32.000 sporozoites/mouse were administered at weekly intervals.

Two weeks after the last booster a challenge of 80.000 sporozoites/mouse was given. The results are expressed in table 4.4.3. In the serum of the immunized nu/+ mice from the second attempt CSP reactivity could be demonstrated. 8 animals of this group were rechallenged with sporozoites after an only one animal developed a parasitaemia. Additionally, it was observed that sporozoite-induced parasitaemias in nu/+ and nu/nu mice developed similarly, but most nu/nu mice survived longer and some even recovered.

Table 4.4.3

Exp No.	Experimental Groups		Protection No.Animals Protected	Mean Day Patency
I	Immunized	nu/nu	0/4	6,5
		nu/+	8/8	-
	Control	nu/nu	0/1	5,0
		nu/+	0/10	4,5
II	Immunized	nu/nu	0/9	4,5
		nu/+	12/12	-
	Control	nu/nu	0/7	4,1
		nu/+	0/6	3,9

4.4.4 *Transfer of immune spleen cells*

One animal of a group of 8 two month old inbred Wistar rats was exposed to infected mosquitoes 3 times a week, during a month. Another animal was simultaneously exposed to non-infected mosquitoes. Both received chloroquine in their drinking water. After the immunization procedure the spleen cells of each of these animals were suspended and transferred intravenously to 2 groups of 3 rats (group S receiving cells of the sporozoite immunized rat and group M those of the non-immunized rat; $1{,}5.10^7$ cells per animal).
5 hours later both groups received an intravenous sporozoite challenge (260.000/rat), and the median lobes were fixed 45 hours after inoculation. Results are given in table 4.4.4. In group S considerably less EEF were found, and the mean number was 29% of that in group M.

Table 4.4.4

	S (imm. cells)	M (control cells)
	0,073	0,144
	0,084	0,335
	0,111	0,456
$\bar{M}$	0,089	0,312
sd	0,017	0,157

4.4.5 *Conclusions*

A few preliminar conclusions can be drawn from this section on mechanisms of resistance.

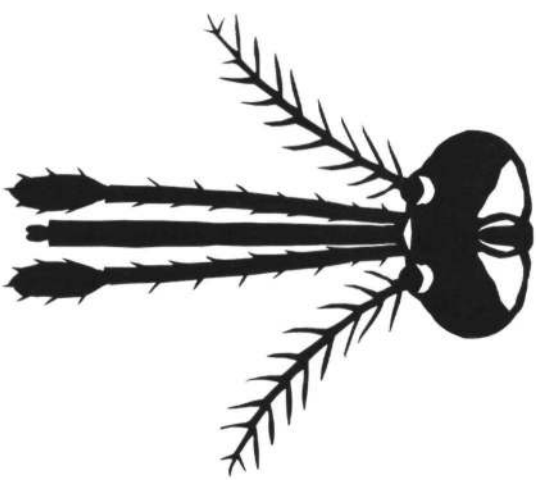
a) Viable sporozoites induce considerable amounts of specific antibodies. Their production is neither influenced by chloroquine treatment, nor by parasitaemias. There is a tendency that CSP reactivity slightly decreases during 6 weeks. Neutralization activity probably indicative for the protective response, is demonstrable in all of the pooled sera except for the

pre-infectious sera.

b)Clear levels of interferon are neither detectable in sera from rats with sporozoite-induced infections, nor in sera from rats with blood-borne infections, thus contrasting with the consistent reduction of the number of PFU after inoculation with the artificial interferon inducer r I : r C.

c)Mice immunized with viable sporozoites develop a protective immunity, as judged by the absence of parasitaemias after challenge. But all congenitally athymic mice, simultaneously immunized, show parasitaemias after sporozoite challenge. This indicates that the thymus or T-cells are indispensable for the upbuilding of a protective response.

d)Spleen cells from two donors, repeatedly exposed to infected and non-infected mosquitoes, were transferred to inbred recipients, which were subsequently challenged with sporozoites. The recipients of sporozoite-immune cells showed considerably lower EEF-density than those of non-immune cells. Since no separation of the leucocytes was performed it remains unclear which population actually caused the reduction. T-cells may be involved, as well as B cells and macrophages. The experiment was described to show the usefulness of the counting technique as a possible *in vivo* measure for immunological studies of the anti-sporozoite response.



CHAPTER V

DISCUSSION

5.1 Quantitative assessment of EEF-numbers in liver tissue

Garnham and Bray (1) were the first to quantitate numbers of EEF in liver biopsies for the estimation of the relative density. They counted the numbers of EEF found in a fixed area (50 mm^2) of biopsy sections. The final result was expressed as the number of EEF per mm^2. In another approach they counted the number in 20-50 sections of known area and thickness, and measured the average diameter of the EEF. From these data the number of EEF/cm^3 was computed. The differences found between these two methods were negligible. Their study was performed with a few monkeys, and they noticed that it was necessary to carry out experiments on a larger scale, and that despite this quantitative approach, the results could only be used as a crude measure. In these and subsequent studies (2) it was tacitly assumed that the relative EEF density in a given biopsy is representative for the whole liver. The place of biopsy was not defined, however. In our present studies with rats, we have always taken a counting sample from a defined region of the liver, since we could not consistently demonstrate a uniform distribution of EEF. Thus, one may only assume that the density in a defined region is correlated in some way with the density in the whole liver.

Also Yoeli *et al.* (3) found that the distribution seems to be irregular. They found less EEF adjacent to the liver capsule and at the periphery of the lobules. The majority of parasites was situated at the center or sides of the lobules, in cells near the vessels. However, they did not quantitate their results.

Wéry did use a quantitative approach in his studies on rodent plasmodia. As

a general routine, he determined the number of EEF in 50 or 100 serial sections and assumed that the average number of EEF per section gives an indication of the intensity of infection in a piece of liver. Initially a fixed area of 60 mm^2 was searched per section (4), but later on this method was usually omitted and exclusively used to compute the number of EEF in a whole liver or per unit volume (5).

We have several comments to make concerning Wéry's approach.

The surface are of tissue sections is a variable unit:

a) Sections, arbitrarily taken from different lobes of a given liver and those from corresponding lobes in the livers of other animals differ in size.

b) The outlines of sections are sometimes irregular and bloodvessels and artefacts may influence the actual tissue surface.

c) The shape of a liver lobe may cause a gradual change of the surface of serial sections.

To our opinion, the mere comparison of mean numbers of EEF per section is a very inaccurate approximation and we emphasize that the surface of the sections is an essential component for comparing relative EEF densities.

The use of serial sections implies the occurrence of identical EEF in several sections.

We have selected our sections, to avoid double counting. A hundred counted serial sections may give a more precise result than the 9 selected sections, used in our studies; but a search of large numbers of serial sections may not be necessary to get "an indication of the intensity of infection", in view of the accuracy with which Wéry contented himself. The optimal number of sections depends on this intensity and cannot be given beforehand, but we found that the mean number of EEF/mm^2 from 9 sections did not deviate from that in 100 serial sections. We therefore preferred the less laborious approach.

From more or less equal numbers of EEF per unit volume in two lobes of a few rats, Wéry concluded a uniform distribution of EEF in the liver. We could not consistently prove such a distribution; this matter deserves further attention, before extrapolation from small samples to whole lobes or livers is acceptable.

Apart from these remarks, it must be admitted that Wéry's study has not been equalled, and that he has gathered a mass of information on sporozoite-host interrelationships in rodent malaria.

Generally, investigators appear to shrink from an histological approach for quantitating sporozoite infectivity, and the indirect, but less laborious approach of determining the prepatent period is chosen. Among others Fink has done so; he justified this choice by pointing to the precondition of statistical analysis of the results and the unfitness of his *P. yoelii*-mouse system for such an approach (6).

Only occasionally has the histological method been used. Vanderberg (7, 8) repeatedly used the second method of Wéry, and Bafort (9) once determined the average number of EEF in 3 sections at a distance of 100 µm in a few rats. A qualitative approach has been used in some studies on the influence of drugs (10, 11, 12). In the opinion of Most (personal communication) there is no need to count EEF in studies on causal prophylactic activity, provided the controls have an abundant number of readily detectable parasites. This is, however, an unreliable factor, since inocula may contain variable numbers of infective sporozoites. Counting techniques have their limitations, especially if the only interest is the presence or absence of EEF. But it may be of use when a partial effect of drugs on EEF is studied in animals that are susceptible to this stage of the parasite. In relatively unsusceptible hosts the all or none effect as measured by the onset of a parasitaemia remains the only suitable method, whereby an attempt to quantitate the effect of EEF can be made by determining the length of the prepatent period.

5.2 Application of the counting method

5.2.1. *Dose of sporozoites*

An impression of the infectivity of sporozoites in a vertebrate host can be obtained by two methods:

a. quantitative assessment of developing EEF
b. duration of the prepatent period

For use of the second method a relationship between the number of inoculated sporozoites and EEF has to be assumed. If that is so, then the time lapse between inoculation and appearance of parasites in the blood serves as a measure for the degree of infectivity in a batch of sporozoites. It is a relatively easy technique, which gives satisfactory results. This is the method of choice in those hosts which have a high innate resistance to sporozoite infection. Nussenzweig (13) and Vanderberg (7) and their coworkers found that, when the dose of sporozoite of *P. berghei* was increased, the prepatent period of

inoculated mice became shorter. This was confirmed by Gregory and Peters (14) who demonstrated a linear relationship between log inoculum of sporozoites and the mean time required to develop a 2% parasitaemia. However, doses which failed to produce a 100% group patency required a similar time for development of two percent parasitaemias, thereby demonstrating a breakdown in the linear relationship. Fink also found a similar linear relationship with *P. yoelii* sporozoites in mice, using 0,2% parasitaemia as a measure for the prepatent period (15). Difficulties with regard to the reproducibility of prepatent periods and individual variation were, however, regularly observed. He therefore proposed determining the mean infective dose of sporozoite suspensions (6). All these approaches presuppose a direct relation between sporozoite doses and numbers of EEF.

Our attempts to demonstrate a correlation between inoculated sporozoites and numbers of EEF substantiated the presupposition of others. The correlation between higher doses is not necessarily valid for the lower doses. Mathematically a negative dose is suggested for the groups in which some of the animals were found to be negative. The impossibility of this substantiates the results of Gregory and Peters (14), who demonstrated that the linear relationship between sporozoite-dose and prepatent period breaks down when the inoculum is insufficient to give a 100 per cent group patency. Obviously our failure to detect EEF in animals which received the low doses, does not imply a total absence of this stage in the liver.

The correlation coefficients of the data per experiment were unequal, indicating that there was a difference in the number of infective sporozoites present in different batches of mosquitoes. This appeared to be very clear when our data from several experiments were combined, in order to find a correlation between dose of sporozoites from different batches and the number of developing EEF. It then appeared that there was no linear relationship between numbers of sporozoites and EEF. This means that under these experimental conditions it is not permissible to extrapolate from different sporozoite-batches to numbers of EEF. Though Vanderberg *et al.* (7) using the counting technique of Wéry, reported considerable variation in "viability" between different pools of sporozoites and in susceptibility of individual rats and mice, they nevertheless concluded that by increasing the number of injected sporozoites, the number of observed EEF could be increased. It is our opinion, however, that the amount of increase is completely unpredictable.

5.2.2 *In vitro preservation of infectivity*

We confirmed the findings of others that sporozoites of *P. berghei* very rapidly lose their infectivity *in vitro*. A measure for this has generally been the length of the prepatent period or time lapse whereafter a certain parasite density is recorded. Another, more direct, measure is the number of EEF, as was suggested above. We demonstrated that *in vitro* incubation of sporozoites for one to two hours on ice halves the density of EEF (see section 3.3.2). This reduction can be minimalized when media, which preserve the sporozoites better, are chosen. Grace's insect TC medium suited our purpose best. Fink and Schicha (17) suggested its use and they found it at least as good as TC medium 199. The pH of the medium may vary from 5 - 7,6 without ill effect (Fink, personal communication). However, at present he uses pure calve serum instead.

We found that sporozoites, kept for two hours in Grace's medium at pH 7 and additional calve serum gives rise to significantly more mature EEF than when kept at pH 6. The recent publication by Vanderberg (18) of the preservative effect of bovine serum albumin (BSA) on infectivity and motility of sporozoites adds significant progress to the *in vitro* maintenance of this vulnerable stage. However, BSA and serum cannot be used in experiments wherein animals are immunized by repeated inoculations of sporozoite suspensions. We found that adjustment of pH to 7 already suited our purpose reasonably well. When care is taken to keep the sporozoites in suspensions for a short time, the loss of infectivity is minimal and under those circumstances other media like TC 199 can be used with satisfactory results.

5.2.3 *Species and age of vertebrate host*

Yoeli *et al.*(19) found indications of differences in susceptibility of rodents to sporozoites of *P. berghei*. They found 2-4 weeks old rats more susceptible than tree rats, whereas mice were by far the least susceptible.

These results were quantitated on the basis of prepatent periods and parasitaemias by Nussenzweig *et al.* (13), who confirmed a relatively high innate resistance of mice to sporozoites. Vanderberg *et al.* (7) in their attempts to quantitate EEF, reported that tree rats were more susceptible than young white rats, which in turn were more susceptible than mice (A/J). Nussenzweig *et al.* (13) also demonstrated a clear influence of age in the susceptibility of rats, 2-3 week old rats were far more susceptible than 2 month old rats.

Apparently, the innate resistance rises with age. Wéry (5) found that the susceptibility of mice to sporozoites of *P. berghei* is very disappointing. In 3 infected mice he examined 50 liver sections and he found only once an EEF. He found young albino rats 25-50 times more susceptible than mice. With our counting method of EEF we could confirm most of the observations cited above. It was very difficult to obtain sizable numbers of EEF in the livers of mice. Older rats were less susceptible than young rats, whereas *Thamnomys sp.* appeared to have a susceptibility comparable to that of young Wistar rats. Our studies on sporozoite immunization were carried out in two month old Wistar rats.

5.2.4 *Effect of cloroquine on sporozoites and EEF*

Several authors have studied the causal prophylactic activity of chloroquine in rodent plasmodia. Most *et al.* (10, 11), Gregory and Peters (14) and Fink (6, 16) found no indication for such an effect. It is not effective against pre-erythrocytic stages, but it exhibits potential suppressive activity on the erythrocytic stages. In other studies chloroquine was used to avoid side effects of parasitaemias (21); and it was recently used in attempts to immunize with *P. fallax,* to inhibit the development of erythrocytic stages, making it possible to judge the effect of immunization with merozoites (22). This encouraged us to administer this drug during our immunization procedure using sporozoites. We confirmed the lack of causal prophylactic activity in our studies, since no significant difference of EEF density in the liver could be demonstrated between chloroquine treated and control animals (see section 3.3.4). Erythrocytic stages, on the other hand, are effectively suppressed. Nevertheless, it may be possible that chloroquine influences the immune response. Holtz *et al.* (23) reported that it can interfere with antigen-antibody reactions. It was found in this study that, after immunization with sporozoites, chloroquine treated animals showed comparable antisporozoite antibody activity, as milk-fed or nontreated animals (see section 4.4.1). Houba and Adam (24) found no effect of chloroquine on primary and secondary antibody response to streptococcal antigens in rabbits.
On the other hand Panayi *et al.* (25) have shown that longterm chloroquine therapy can suppress the nonspecific stimulation of lymphocytes. In our experiments control groups always received the same chloroquine regimen, and the observed effects of immunization were always compared with these controls.

In conclusion of this part of the discussion, we wish to emphasize our preference for the use of quantitating relative EEF densities as a measure for sporozoite-infectivity. In our hands the described method proved to be a reliable and relatively easy technique, applicable in a variety of studies on the pre-erythrocytic stages of malaria.

5.3 Immunization with sporozoites

Our results indicate that previous inoculations with sporozoites control a challenge infection with sporozoites considerably, as assessed by the quantitation of EEF in liver parenchymal cells after the challenge.
Possible anti-sporozoite effects of parasitaemias are excluded both in the approach of Nussenzweig (irradiated sporozoites) and in our method (chloroquine treatment). But the following differences are obvious. In our method healthy EEf come forward from the non-attenuated infective sporozoites. These stages mature unimpeded inside liver parenchymal cells and, while rupturing, they liberate their merozoites. This is followed by an immediate cellular reaction of the host. This reaction has not been observed during the immunization with irradiated sporozoites.
We demonstrated (section 4,1, exp.1) that rats challenged 60 hours and 7 days after one previous inoculation showed about 86% reduction of EEF in both cases. Whether this affect was due to mobilized phagocytes in the liver granulomas, or to another type of response remains as yet unknown.
Rivera-Ortiz and Nussenzweig (26) have drawn attention to another phenomenon in which phagocytosis is involved. They demonstrated an increased clearance of colloid carbon in mice after the inoculation of an irradiated sporozoite suspension. An increase after one day, an optimum after two days and a gradual decrease during the following 9 days was found. The possibility of a phagocytic mechanism being involved in the reduction of EEF from a second inoculum, that we have obtained cannot be excluded. The mononuclear phagocyte system might have been activated both by the earlier inoculation of sporozoites, and by the subsequent rupture of mature EEF.
Generally, no detectable EEF were observed in rodents that received one inoculation of irradiated sporozoites. The authors presumed that the penetration mechanism of the sporozoites remained intact after irradiation, since rare, degenerating EEF could be demonstrated.
Invasion of these attenuated sporozoites thus led to the induction of an abortive infection of the host cells (27, 30). It is not unrealistic to presume

that many of the irradiated sporozoites are phagocytosed by macrophages. The number of these sporozoites has never been assessed. On the other hand inocula of non-irradiated sporozoites will also contain numbers of non-infective, and even non-viable sporozoites (5,7). Garnham especially pointed out the degenerate forms in populations of *P. berghei* sporozoites (31). It is unknown what role phagocytosed sporozoites play in the upbuilding of an anti-sporozoite immune response, and whether irradiated and non-irradiated sporozoites have different immunogenic capacities.

Serological differences after inoculation with irradiated versus viable sporozoites have incidentally been sought for. In a single attempt, Nussenzweig and Spitalny (28, 32) demonstrated persistent titres of CSP antibodies in rats that had received one inoculation of irradiated sporozoites. In rats inoculated with non-irradiated sporozoites CSP titres were also found, but after a short optimum the titres again decreased to normal levels. In both cases the antibody responses could be boosted and titres then remained high for weeks. From this single experiment, in which the numbers of rats was not mentioned, the far reaching conclusion was drawn that, though viable sporozoites are immunogenic, irradiated sporozoites have greater immunogenic capacity. This conclusion has been repeatedly quoted by others in an incomplete and inaccurate way.
However, they did not give data concerning the development of parasitaemias, and its consequences for the detectability of CSP antibodies. An experiment was performed in collaboration with Spitalny, to study the influence of a concomitant parasitaemia on the formation of CSP antibodies (see section 4.4.1). We found that the CSP-activity was comparable to that of chloroquine protected animals inoculated with viable sporozoites and therefore, apparently a parasitaemia does not interfere with the induction of an anti-sporozoite antibody response. Nevertheless, the conclusion of lower immunogenicity of viable sporozoites needs further exploration.

We noticed that the degree of reduction of EEF numbers after a single previous inoculation varied considerably from experiment to experiment. These results, thus, confirm those of Nussenzweig, who found variable numbers of mice protected against subsequent parasitaemias (33). It is therefore not surprising that Yoeli could not find a reduction in EEF after one previous sporozoite induced infection of *P. berghei* in rats (3, 34). Similar negative results were obtained by Garnham (1, 2) using one to three previous sporo-

sporozoite induced infections of *P. cynomolgi bastianellii* in rhesus monkeys. In our experiments, the administration of a second or third sporozoite inoculum boosted the initial effect; the numbers of EEF after sporozoite challenge were further reduced, and frequently no EEF could be detected at all with the counting technique. Further increase in the number of booster inoculations almost invariably induced a response which prevented the development of EEF. Strictly speaking, we should not use the term prevention, since only a small counting sample was taken from the median lobe. However, the single experiment in which parasitaemias were allowed to develop indicated that 4 out of 5 rats immunized with 4 doses were completely protected against induction of a parasitaemia resulting from a sporozoite challenge.
Similarly complete protection was found in intact mice. The effects last for several months, and a certain, not protective response remains detectable for at least one year. These results confirm those on protection in mice that have been accumulated by Nussenzweig's group (35). Our immunizations, obtained with viable sporozoites and chloroquine treatment, have recently been confirmed in mice by Strickland and Beaudoin (personal communication).

Increase in age of the challenged rats might bias the results. Therefore we always used animals of the same age in experimental and control groups. The increasing significance of innate resistance in rats to sporozoites has repeatedly been described. We got preliminary evidence for such an effect (see section 3.3.3). In an unreported attempt, meant to repeat the experiments of Nussenzweig and coworkers (36) on the time of sporozoite clearance in normal and immune mice, we inoculated a 1 month old and a 4 month old rat with doses of sporozoites, adjusted to their bodyweight. At several times after the inoculation, blood from these animals was subinoculated into other 1 month old rats, and the onset of parasitaemia assessed. To our opinion highly susceptible recipients are preferable to less susceptible animals like (A/J) mice. Those rats that received blood from the young one developed parasitaemias, even when the blood was transferred 1 hour after inoculation. Further delay of blood-transfers did not result in parasitaemias. This agrees with Nussenzweig's results in mice. However, none of the animals receiving blood from the old rat became patent, not even when transfer was done 10 minutes after the inoculation. This effect could be due to the activity of the mononuclear phagocyte system in older animals. The increasing importance of this form of innate resistance should be kept in mind as immunized rats grow older. However, the

results of challenge of immunized 2 and 4 month old rats (see section 4.1, exp. 11) were comparable: both groups showed almost complete reduction of detectable EEF after two previous sporozoite inoculations. This means that the degree of innate resistance is already established in 2 month old rats. Apparently the specific anti-sporozoite response is more important than the innate resistance. Our results indicate that young rats behave quite distinctly from older animals in that reduction, though impressive could not match that of the older groups. This may be due to a incompletely developed immune system in young rats.

Another possibility was also considered: in these highly susceptible young animals, a greater number of viable sporozoites are infective than older rats. These sporozoites might then be eliminated from the immunity-inducing process after invading parenchymal cells of the liver.

This consideration brings us back to the question of what actually induces the anti-sporozoite immune response. Is it the sporozoite itself, do infective sporozoites actually participate in these processes, or are only the phagocytosed sporozoites immunogenic? At present this question cannot be answered, yet it may have practical consequences, since the relative numbers of both categories of sporozoites might have an impact on mounting the specific response.

Garnham thought that too few sporozoites are phagocytosed to provide a sufficient quantity of antigen to stimulate the production of antibodies. Nevertheless, he considered non-viable sporozoites capable of provoking an agglutination or lysin response.

In our studies, unlike those of Nussenzweig, we have not attempted to use uniform immunizing doses and challenges. Only approximately similar numbers of infected mosquitoes were dissected and the counting of sporozoites was generally performed after inoculation.

This was done for three reasons:

a) to keep sporozoites *in vitro* for a short a time as possible, ensured minimal loss of infectivity (37)

b) varying infectivity in doses of sporozoites obtained at different times, from different batches of mosquitoes (see section 3.3.1)

c) reliability of results depends on the use of sufficient numbers of animals of the same age in the experimental and control groups. It was impossible to guarantee that for each inoculation the required number of sporozoites would

be available for all animals in a given experiment.

However, our controls might be considered inadequate, since they only give information on the infectivity of sporozoites and do not reflect the reactions to non-infective sporozoites in experimental animals exactly. In the light of a probable dose dependency of the anti-sporozoite response and the mentioned possibility that non-infective sporozoites might be the main inducers at this response, we now endorse the use of predetermined numbers of sporozoites specially in the primary immunizing doses and at challenge.

Regarding the nature of the observed interference of EEF development, some preliminary findings were reported. Circum sporozoite precipitins were readily observed in sera of rats and mice, immunized with non-attenuated sporozoites. Sporozoite neutralization activity could also be demonstrated (see section 4.4.1).

The induction of complete protection appeared to be thymus dependent, as indicated by our failure to induce protection in athymic mice (see section 4.4.3). These results are in line with those of Spitalny who found the same to be true in thymectomized mice (38).

In not reported attempts to perform *in vitro* lymphocyte transformation of spleen cells from immune rats with cleaned sporozoite suspensions and non infected salivary gland suspensions, we only found stimulation with the latter antigen. Purified and sterile antigens should be used, prepared by the technique described by Krettli and Nussenzweig (39).

In a small experiment spleen cells from an immunized donor rat were transferred to normal inbred recipients (see section 4.4.4). After challenge of these recipients a considerable reduction of EEF numbers were found. This approach gave no clear information about the type of immunogenic cells taking part in the anti-sporozoite immune response and previous suggestions of T-cell dependency are premature (40). However, nobody has reported, as yet, the induction of protection by cell transfers and this preliminary result indicates that our EEF counting method can act as a tool for the measurement of the anti-sporozoite response in transfer experiments with classified populations of immune cells, and with immune sera.

Though Nussenzweig was unable to transfer immunity with macrophages from the liver and the peritoneal cavity, we obtained preliminary evidence that macrophages play a significiant role in the arrest of sporozoites. We recently found that opsonized sporozoites seem to be more readily phagocytosed by

peritoneal macrophages than sporozoites, incubated in normal serum; macrophages from sporozoite-immune mice appear the most active in this respect.
Finally we wish to discuss the possible non-specific effects of mosquito debris, that contaminates the sporozoite suspensions. We tried to study these effects as reported in section 4.1, exp. 7. Generally, no reducing effect of immunization by non-infected salivary gland suspension could be demonstrated on the number of EEF after challenge. These results confirm those of Nussenzweig (26). She and her coworker Rivera-Ortiz, in fact, demonstrated some increase in phagocytic activity and spleen weight after inoculation with suspensions of non-infected salivary glands.
Repeated inoculations induced no potentiation of this mononuclear phagocytic activity. Each time the phagocytic activity decreased to normal values after a short optimum on the second day.
These results permitted us to use normal rats as controls, without simultaneous immunization with non-infected salivary glands in each separate experiment. However, by loading the immune mechanisms with repeated intravenous inoculations of triturated thoraces of non-infected mosquitoes, we found a significant reduction of EEF when these animals were challenged with triturated thoraces of infected mosquitoes. The numbers by no means approached the zero-level and these results are thus quite contrary to those of Alger *et al.* (41), who reported complete protection in half of the treated mice after immunization with salivary gland material via the intraperitoneal route. Russell and colleagues, (42) who were the first to immunize with sporozoites, using ground infected thoraces and challenged via the bite of infected mosquitoes. We suppose that at least part of the described responses was due to a non-specific effect caused by the repeated inoculation of an equivalent of 200 mosquito thoraces.

In conclusion we have strong indications that a stage-specific immune response underlies the phenomenon of complete and incomplete reduction of developing EEF after challenge of rodents that previously had been immunized with suspensions of viable sporozoites (43). The effect of simultaneous immunization with debris in the inocula appears to be insignificant, though it is not completely excluded.

5.4 Interference by one previous sporozoite inoculation

In a series of experiments (see section 4.2) we demonstrated that a considerable part of sporozoites inoculated shortly after one preceding inoculation is inhibited in the course of their further development. In animals with

parasitized liver parenchymal cells newly introduced sporozoites give rise to less EEF compared with controls. This phenomenon has not been described before, nor is it understood. In our opinion, there is no question of a true immune mechanism in the sense of a specific cellular or humoral response. The time period of less than 48 hours seems too short for the induction and implementation of such a response.

There are two approaches of attack: the sporozoites before they invade parenchymal cells, and the early phase of intracellular EEF development. An effect that might interfere with the extracellular sporozoites, could be the non specific stimulation of the mononuclear phagocytic system. Rivera-Ortiz and Nussenzweig (26) demonstrated that phagocytosis could be stimulated by intravenous inoculation of salivary gland suspensions with and without X-irradiated sporozoites.

The effect was measured by changes in spleen weight and by clearance of colloid carbon. Both spleen weight and clearance rates showed a rapid increase within 24 hours, and a peak at 2 days after inoculation. Thereafter the reactions gradually returned to normal values. They noted that maximal clearance rates and spleen weights were consistently lower in animals inoculated with the non-infected salivary glands. Indications can be derived from their results that carbon clearance increases two fold, two days after salivary gland inoculation, and an eight-fold increase was found two days after inoculation with infected salivary glans, as compared with control values. We were not able to demonstrate a significant difference in numbers of EEF when the inoculation was preceded 24 hours earlier by an inoculation with non-infected salivary glands. In the light of the above results it may be that the slight increase in phagocytic activity induced by non-infected salivary glands after one day is not refelected by change in EEF numbers. The considerable increase in this activity after administration of infected salivary glands may correlate with our findings of a more than two-fold reduction of EEF numbers. However, we must bear in mind that the host systems were different.

We observed that when the reinoculation was given 60 hours after the first one, the reduction of EEF numbers was more pronounced than after $\pm$ 24 hours. This might be correlated with the cellular reactions at the time of liberation of the merozoites originating from the first infection ($\pm$ 48 hours). Sporozoites that enter the liver might be phagocytosed at higher rates by the polymorphs and macrophages, that have been mobilized and are engaged in the

engulfement of merozoites. Sporozoites that are inoculated before the rupture of parasitized parenchymal cells do not meet these infiltrates and the risk of being phagocytosed before entering parenchymal cells is less pronounced.

The other mechanism that is seriously considered, has also been suggested by Nussenzweig's work. She has demonstrated that pre-erythrocytic stages are susceptible to interferons (44, 45). When these agents are induced by a virus or otherwise, either non or delayed parasitaemias are observed (46). The peak interference was demonstrated when interferon production was induced 24 hours after inoculation of sporozoites (47, 48). This means that interferon influences the EEF development; no retarded EEF have been observed, but at that time the parasites are rather small for detection in histological sections.

To explain our findings of short term interference, we wondered if the parasite itself could induce the production of interferon in its host. It had been claimed that blood stages of *P. berghei* are able to do so (49). The results of our preliminary attempts to demonstrate circulating interferon (see section 4.4.2) were successful in rats that had received the artificial interferon-inducer rI:rC. However, the results in rats inoculated with sporozoites were not consistent. Only in one out of three animals a reduction of plaque-forming units was demonstrated after 48 hours, thus coinciding with the liberation of merozoites and the immediate cellular reactions in the liver. Further research is in progress along these lines.

Thus the results of this series of experiments point to a certain phenomenon of interference, the nature of which still waits elucidation. Theoretically, the production of a chalon, specific for EEF could also offer an explanation for these observations (50), though the occurrence of such agents produced by dividing cells and regulating subsequent multiplication is unknown in malaria.

5.5 Interference by present and past parasitaemia.

As reported in section 4.3, sporozoites inoculated during a primary parasitaemia succeeded, to a lesser extent, in developing into EEF than in animals without experience of a parasitaemia. In 8 out of 23 rats in the acute stage of the infection (section 4.3, exp. 1-3) no liver schizonts could be detected at all.

In animals with lowered parasite densities and in animals immunized with

erythrocytic stages mean numbers of EEF were below that of controls (exp. 4-6). Though further experimental data are needed to confirm and extend these findings, this phenomenon of interference has never been described before. To our knowledge, only Yoeli has published some results on sporozoite infections, following parasitaemias in rats. Shortly after his discovery of the optimal conditions of sporozoite induced infections (51, 52, 53) he tried to discern if sporozoites could develop into EEF in rats immune to erythrocytic stages (3, 34). Maturing EEF were found in such animals, but no subsequent parasitaemia could be detected. He stated that acquired immunity to sporozoite or trophozoite induced parasitaemias is not extended to sporozoites. The only mechanism involved is, what was called the hemo-hepatic barrier. This liver-blood barrier would prevent merozoites originating from EEF penetrating erythrocytes. Our results confirmed the effectiveness of the liver-blood barrier, since animals immune to erythrocytic stages, need no protection with chloroquine during a subsequent immunization with sporozoites.

Bafort (9) has also published observations on the influence of previous parasitaemias on sporozoites. In two thicket rats that had survived a sporozoite-induced (?) parasitaemia of *P. vinckei*, the acquired immune response was repeatedly boosted with erythrocytic stages. After a subsequent sporozoite inoculation no EEF were found in 50 serial sections of a liver biopsy. After a further sporozoite inoculation via mosquito bites EEF could also not be detected. It remained unclear whether this result was due to a non-specific, or to a species- or stage specific response. But it seems to contrast with the findings of Yoeli. The immune response to the erythrocytic stages apparently prevented the development of EEF, whereby the induced liver-blood barrier was not challenged.

Our results are intermediate between those of Yoeli and Bafort. The general statement of Yoeli that in bloodstage-immune animals sporozoites are able to develop into EEF was confirmed, however, with one important restriction. We found a significant, two-fold reduction in the number of EEF.

As was stated before, Garnham (1, 2) found EEF after reinoculation with sporozoites in monkeys that had previously developed a sporozoite induced parasitaemia of *P. cynomolgi*. Similar results were claimed for *P. vivax* (54) and *P. malariae* (55) and it was stated that the strong immunity against the blood forms of the homologous strain was completely without influence on the

EEF.

We confirm that if there is EEF development, their shape is not different from that in animals without such immunity. Since antibodies are not able to gain access to the intracellular parasites, this is to be expected.

But before the entry of host cells, sporozoites are vulnerable and somehow the number of EEF may be reduced.

This is, however, difficult to demonstrate in experiments with primate malarias, since inclusion of the required controls for the demonstration of reduction is sometimes an unattainable luxury.

We feel, therefore, that the conclusions of these authors should be reconsidered in the light of Nussenzweig's findings in mice and primates, and our results with rodents.

As to the explanation of interference of present and past parasitaemias, there are some mechanisms that are worth considering.

a) *Circulating interferon.*

Nussenzweig presented evidence that pre-erythrocytic stages of *P. berghei* are susceptible to interferon (44-47). At the same time Huang *et al.* (49) found that during a parasitaemia of *P. berghei* serum titres of interferon were increased, and that, on the other hand, the onset of parasitaemia could be delayed with interferon (56). Others could not confirm this effect in human malaria (57). In a preliminary attempt we were also unable to show demonstrable interferon production in rats with a trophozoite-induced parasitaemia.

b) *Interstage cross-reacting antibodies.*

It has been demonstrated that sporozoites and EEF react in fluorescent antibody tests with sera from animals with a parasitaemia, and *vice versa* (58-62). As has been stated, a functional cross-protection is unlikely (63).

c) *Soluble antigen-antibody complexes.*

During a parasitaemia soluble antigen-antibody complexes are circulating (58). These complexes, involved in the pathophysiology of malaria, activate the complement system. It should be considered that soluble antigens or complexes possibly may adhere to sporozoites. Thus burdened sporozoites may then be readily phagocytosed, especially through the interaction of complement factors; other activated complement components may cause lysis of sporozoites.

d) *Increase of phagocytic activity.*

There is a considerable evidence that colloid carbon is cleared faster in animals with a parasitaemia, directly after its onset; results of several other approaches have also pointed to the involvement of the mononuclear phagocyte system (65-70). The number of macrophages in the liver increases rapidly and Kupffer cells can be observed loaded with plasmodial pigment. Kupffer cells and other macrophages lining blood sinuses, that are thought to remove sporozoites of a primary inoculation, might be activated by T lymphocytes that are specifically stimulated during parasitaemia. We previously found indications for stimulation of T cells in monkeys and mice after bloodborne infections (71, 72). Evidence for T cell-induced nonspecific activation of phagocytosis and intracellular killing in macrophages of other parasites is growing (73, 74).

e) *Hepatic dysfunction.*

In a review on liver involvement in acute mammalian malaria, Maegraith (75) pointed to several pathogenic effects. Acute *P. berghei* parasitaemia causes lesions in the livers of rodents; sinusoids are dilated and congested; large numbers of swollen Kupffer cells are present and the intrahepatic bloodflow is reduced; glycogen disappears from parenchymal cells, mitochondria are damaged and a fatty degeneration of these cells is apparent. Since parenchymal cells are the sites of multiplication of pre-erythrocytic stages, the considerable reduction of EEF during parasitaemias might be explained by a deterioration of the environment. Another report (12) points towards the inability of sporozoites to develop into EEF and subsequent parasitaemia, in an ethionine-induced fatty liver. Thus by deposition of fat, the capacity of degenerating parenchymal cells to support intracellular growth of EEF might be affected. The diminished number of maturing EEF after a sporozoite inoculation during a parasitaemia, might be caused by a combined action of more than one of the above mentioned mechanisms. The described results could only be obtained through the use of the quantitative method to determine relative EEF-densities.

5.6 Epidemiological considerations on anti-sporozoite responses

There appears to be no question of a total protective response to sporozoites in nature. People in endemic areas are regularly exposed to infected mosquitoes, and they show parasitaemias, which not likely originate only from relapses. To our knowledge serious attempts to demonstrate the occurence of natural

sporozoite specific antibodies in man have never been performed.
This may be due to obvious difficulties, such as the problem to obtain mosquitoes, infected with human plasmodia, for the preparation of sporozoite antigen required for the serological test, and the problems of crossreactivity in the fluorescent antibody test with antibodies induced by the erythrocytic stages. Moreover, the non-pathogenic pre-erythrocytic stages have always recieved little attention, especially as sporozoites are repeatedly said not to be immunogenic.
However, since the describing, by Vanderberg and his coworkers (76), of a precipitate forming at one end of sporozoites placed in serum from immunized rodents, a specific *in vitro* anti-sporozoite test has been available. Applying this test, CSP activity was demonstrated in a wild-caught monkey (77). In the serum of monkeys and volunteers immunized with irradiated sporozoites such precipitins could also be demonstrated but they only occur after several large boosters. The experimentally induced protection and the detectability of antibodies lasts in vaccinated primates at least for months. The apparent difficulties to induce protective anti-sporozoite responses in monkeys and men and the requirement of considerable numbersof sporozoites might reflect why the natural response to mosquito-borne sporozoites is not effective at first sight.
When we would succeed in developing a method for detection of antisporozoite antibodies in man, and assuming that antibodies attribute to neutralization under natural circumstances in endemic malarious areas, then a wide area is open for studies of great practical importance.
In areas with season dependent transmission the period of effective antisporozoite immunity should then be determined. And when a natural response exists, but would be not sufficient to bridge the transmission periods, the question would arise, whether the natural response can be potentiated artificially. Such attempts to increase the natural response by sporozoite vaccines would then certainly also be applicable in areas with a constant pattern of transmission. For this kind of vaccination the infants and youngsters should be considered in the first place. We should keep in mind, however, that vaccination remains an idle dream as long as there are no methods for *in vitro* cultivation of parasites.

If we extrapolate the findings of our experimental study to the situation in the field, then the following possibilities should be considered.
a. Frequent inoculations of sporozoites may cause a response that partially

prevents the development of EEF, reducing the number of merozoites entering the circulation.

b. Present and past parasitaemias may also interfere with the invasion of liver parenchymal cells by sporozoites. This would also reduce the number of generated merozoites.

Such a reduction of the number of merozoites would mean that reinfection of the blood starts with a lower parasite density. This in turn would imply a less severe course of the desease and less immuno-pathological damage since the amount of soluble antigen remains lower.

It is a wellknown fact that the partial immunity to erythrocytic stages in man is established after years of intensive contact with parasites from the local strains. The hypothetical reduction of the frequence whereby merozoites initiate parasitaemias might contribute to the explanation of this long term phenomenon.

MacDonald (78) indicated that there is a discrepancy in malaria endemic areas, between the inoculation rate assessed by entomological methods and that estimated from the infant parasite rate. The former can be up to hundred times higher. It is tentative to suggest a relation with anti-sporozoite responses and interference of EEF-development. Garnham (79) has also referred to MacDonald, but he considered this discrepancy due to various causes, including a diminished exposure, natural racial immunity, the presence of protective traits like hemoglobin S, a milk diet and passive immunity inherited from the mother. Elsewhere (2), Garnham points to the fact that many of the sporozoites transmitted by mosquitoes are non-viable, and though he does not believe in the effectiveness of anti-sporozoite responses, he does not exclude the possibility that large numbers of such non-viable sporozoites, constantly introduced into man, provoke an immune response! This he considers to be one of the many factors which might contribute to the mildness of the desease in the indigenous population of holoendemic localities.

It is reiterated that, in the human system, only non-protective anti-sporozoite antibodies are demonstrable. Even so, the study of anti-sporozoite responses in people living in endemic areas deserves serious consideration, and attempts to potentiate such responses are, in our opinion, indicated.

The successful development and application of sporozoite vaccines seems dependent on the understanding of pre-erythrocytic development and the induction of immune responses against these stages in the natural hosts.

5.7 Suggestions for further research in animal models

5.7.1 *Understanding of the pre-erythrocytic cycle of a primary infection and the mechanisms of innate resistance*

General routes of sporozoites through the circulation might be studied by ligation, extirpation and transplantation of organs, especially the liver (80) and the spleen. The places or cells that are finally reached by sporozoites, and early development into EEF might be revealed by labeling and autoradiography (81), by electron microscopy, and by fluorescence in cryostat sections from different organs. It seems tempting to search for lurking sporozoites or cryptic tissue stages in the intestinal tract, since this is the predilection place for sporozoites of other *Coccidiida*. Their discovery anywhere might explain recrudescences in primate malaria and the origin of stages, indicated as chronic EEF in tree-rats (82, 83).

Genetic variation of the host species might influence susceptibility, as possibly expressed by different interaction of macrophages and liver parenchymal cells with sporozoites. Referring to the studies of Miller (84) on special merozoite receptors on erythrocytes, it should be examined if cell membranes of parenchymal cells contain special receptors for sporozoites. Another fact in the innate resistance of unsusceptible hosts might be the insufficiency of parenchymal cells to support EEF development. Requirement of certain chemical compounds should be studied (i.e. the beneficial effect of methionine has been described (12).

Innate macrophage-mediated resistance might be studied using anti-macrophage serum or phagocytosis-inhibiting chemical agents; the macrophage barrier in the liver might be bypassed by exposing parenchymal cells directly to infection through the injection of sporozoites into the bile duct (85). Infection of sucklings that have received a transfer of macrophages from syngeneic adult donors is also worthy of examination. By these approaches the mechanism of increasing resistance with age might also be revealed (85).

5.7.2 *Analysis of the protective anti-sporozoite immune response (antibody-mediated reactions)*

In addition to CSP and SNA reactions other serological methods should be applied and developed. Fluorescent antibody techniques, using sporozoites or cryostat sections of infected liver might be helpful to study IgM and IgG immunoglobulins, and their cross-reactivity with erythrocytic stages.

Anti-sporozoite responses in rodents, treated with suppressive or causal prophylactic drugs, could be studied. It should also be assessed if transplacental transmission of specific anti-sporozoite antibodies occurs, which may protect sucklings against infection. The role of various antibodies and classes and subclasses of these immunoglobulins should be studied further in relation to protection and if possible, the *in vivo* effects of antibody activated phagocytosis should be quantified.
Such techniques might be applied later on in epidemiological studies, to detect anti-sporozoite responses in humans, living in areas of various endemicities.

5.7.3 *Cell-mediated reactions*

Participation of thymus dependent cells in antibody production should be elaborated (38). The use of athymic mice, (neonatal) thymectomy, antithymocyte serum and immunosuppressuve drugs are recommended, including subsequent reconstitution with cells of normal sygeneic donors. Specific stimulation of thymus dependent lymphocytes might be elucidated by transfer of enriched populations of T-cells, of thymocytes or thoracic duct cells from immunized donors into syngeneic recipients, and sporozoite-infectivity assessed *in vivo*. *In vitro* stimulation could be measured in a lymphocyte transformation test, using purified sporozoites as antigen (39). Simultaneously the possible cooperation of antibodies with cell-mediated mechanisms could be studied. The potentials of macrophages in sporozoite and blood stage-immunized hosts, in terms of T-cell-mediated activation is worth consideration. Moreover, a supposed role of macrophages in processing the sporozoite antigens and presenting them to immunocompetent cells should be investigated.
The cellular responses to sporozoites might also be studied in avian malaria, since birds have a lymphoid organ in which antibody producing B-cells are processed (the bursa of Fabricius). The immune mechanisms may be studied by neonatal bursectomy and thymectomy.
Moreover, the site of intracellular multiplication of EEF in avian plasmodia are the macrophages. In other systems, like *Listeria*, *Leishmania* and *Toxoplasma*, T-cells play a role in the activation of macrophages and the subsequent killing of intracellular parasites. It is worth investigating if such mechanisms are also active in birds immunized with sporozoites.

5.7.4 *Differences between the protective responses to irradiated and viable sporozoites*

The timing of induction, boosting and duration of protective and non-protective humoral responses, to both irradiated and viable sporozoites should be studied further. The contribution of the cellular reactions at rupture of parasitized liver parenchymal cells to the anti-sporozoite response is unknown.
A qualitative and quantitative description of these infiltrations before and during immunization with viable sporozoites is worth performing.
The immunogenic properties of degenerate, non-viable sporozoites might give information on the contribution of these stages to the immune response, in comparison with infective and with attenuated sporozoites.
Some of these suggestions were already put forward as recommended research subjects in a joint review by IAEA and WHO (86), and several points are presently included in the research program at the Institute of Medical Parasitology of the Nijmegen University.

References

1. Garnham, P.C.C. and Bray, R.S. (1956). The influence of immunity upon the stages (including late exo-erythrocytic schizonts) of mammalian malaria parasites. Rev.Bras,Malar., *8*, 152-160.
2. Garnham, P.C.C. (1966). Immunity against the different stages of malaria parasites. Bull.Soc.Path.exot., *59*, 549-557.
3. Yoeli, M., Upmanis, R.S., Vanderberg, J., Most, H. (1966). Life cycle and patterns of development of *Plasmodium berghei* in normal and experimental hosts. Mil.Med., *131*, 900-914.
4. Wéry, M. and R.Killick-Kendrick (1967). Differences in the numbers of tissue schizonts of *Plasmodium berghei yoelii* in the livers of individual mice, and a comparison of two routes of inoculation of the sporozoites. Trans.Roy.Soc.trop.Med.Hyg., *61*, 447-448.
5. Wéry, M. (1968). Studies on the sporogony of rodent malaria parasites. Ann. Soc.belge.Méd.trop., *48*, 1-137.
6. Fink, E. (1974). Assessment of causal prophylactic activity in *Plasmodium berghei yoelii* and its value for the development of new antimalarial drugs. Bull.Wld Hlth Org. *50*, 213-222.
7. Vanderberg, J.P., R.S.Nussenzweig, H.Most (1968). Further studies on the *Plasmodium berghei - Anopheles stephensi* - rodent system of mammalian malaria. J.Parasit., *54*, 1009-1016.
8. Vanderberg, J.P. (1973). Inactivity of rodent malaria anti-sporozoite antibodies against exoerythrocytic forms. Amer.J.Trop.Med.Hyg., *22*, 573-577.

9. Bafort, J.M. (1971). The biology of rodent malaria with particular reference to *Plasmodium vinckei vinckei* Rodhain 1952. Ann.Soc.belge Méd.Trop., *51*, 1-204.

10. Most, H., R.Hermann and E.Schoenfeld (1967). Chemotherapy of sporozoite-induced *Plasmodium berghei* infections with selected antimalarial agents. Amer.J.Trop.Med.Hyg., *16*, 572-575.

11. Most, H. and W.A. Montuori (1975). Rodent systems (*Plasmodium berghei - Anopheles stephensi*) for screening compounds for potential causal prophylaxis. Amer.J.Trop.Med.Hyg., *24*, 179.

12. Dunn, M.A., T.C.Quinn, H.A.Terwedow (1972). Pre-erythrocytic rodent malaria, *Plasmodium berghei* - prevention of development in the ethionine fatty liver. Amer.J.Trop.Med.Hyg., *21*, 288-292.

13. Nussenzweig, R.S. R.Herman, J.Vanderberg, M.Yoeli, H.Most (1966). Studies on sporozoite-induced infections of rodent malaria. III. The course of sporozoite-induced *Plasmodium berghei* in different hosts. Amer.J.Trop.Med.Hyg., *15*, 684-689.

14. Gregory, K.G. and W.Peters (1970). The chemotherapy of rodent malaria. IX. Causal prophylaxis, part 1: A method for demonstrating drug action on exo-erythrocytic stages. Ann.Trop.Med.Par., *64*, 15-24.

15. Fink, E. (1970). Die quantitative Bestimmung der Wirkung exogener und endogener Faktoren and Sporozoiten und Gewebsformen der Kleintiermalaria (*Plasmodium cat'emerium, P.berghei yoelii*). Zeitschr. Tropenmed.Par., *21*, 357-372.

16. Fink, E. (1974). Assessment of causal prophylactic activity in *Plasmodium berghei yoelii* and its value for the development of new antimalarial drugs. Bull.Wld.Hlth.Org., *50*, 213-222.

17. Fink, E. and E.Schicha (1969). Influence of synthetic insect TC medium on the survival of malaria sporozoites in vitro. (*Plasmodium berghei yoelii* and *P.cathemerium*) Z.Parasitenk., *32*, 93-94.

18. Vanderberg, J. (1974). Studies on the motility of *Plasmodium* sporozoites. J.Protozool., *21*, 527-537.

19. Yoeli, M., H.Most and G.Boné (1965). The natural history of *Plasmodium berghei* in the field and under experimental conditions. Ann.Soc. belge Méd.trop., *45*, 267-274.

20. Fink, E. (1972). Kausalprophylaktische Wirkung von Standard-Malaria mitteln bei der Nagetiermalaria *(Plasmodium berghei yoelii)*.Z.Tropenmed. Parasit., *23*, 35-47.

21. Greenwood, B.M., J.H.L. Playfair and G.Torrigiani (1971). Immuno-suppression in murine malaria. I. General characters. Clin.exp.Immunol., *8*, 467-478.

22. Graham, H.A., L.A.Stauber, N.C.Palczuk and W.D.Barnes (1973). Immunity to exo-erythrocytic forms of malaria. I. Course of infection of *Plasmodium fallax* in turkeys. Exp.Parasit., *34*, 364-371.

23. Holtz, G., W.Mantel and W.Buck (1973). The inhibition of antigen-antibody reactions by chloroquine and its mechanism of action. Z.Imm.Forsch., *146*, 145-157.

24. Houba, V. and M.Adam (1963). Beinflussing von immunologischen Vorgangen durch Resochin. Zenralbl.Bakteriol.Abt.I.Orig., *189*, 326-334.

25. Panayi, G.S., W.A. Neill, J.J.R. Duthie, J.N. McCormick (1973). Action of chloroquine phosphate in rheumatoid arthritis. I. Immunosuppressive effect. Ann.Rheum.Dis., *32*, 316-318.

26. Rivera-Ortiz, C. and R.S.Nussenzweig (1972). Increased phagocytic activity of the reticulo-endothelial system during immunization with X-irradiated sporozoites of *Plasmodium berghei* in mice. Proc.Helm. Soc.Wash., *39*, 497-506.

27. Vanderberg, J.P., R.S.Nussenzweig, H.Most, C.G.Orton (1968). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. II. Effects of radiation on sporozoites. J.Parasit., *54*, 1175-1180.

28. Spitalny, G.L. (1973). Immunological aspects of sporozoite-induced resistance in rodent malaria. Ph.D. thesis New York University.

29. Warren, M. and P.C.C.Garnham (1968). The effect of X-irradiation of sporozoites of *Plasmodium cynomolgi* as seen by abnormal development in the liver. Trans.R.Soc.Trop.Med.Hyg., *62*, 460.

30. Warren, M. and P.C.C.Garnham (1970). *Plasmodium cynomolgi:* X-irradiation and development of exo-erythrocytic schizonts in Macaca mulatta. Exp.Parasit., *28*, 551-556.

31. Garnham, P.C.C. (1972). Comments on plasmodial ultramicroscopy. Proc.Helm. Soc.Wash., *39*, 194-197.

32. Spitalny, G.L. and R.S.Nussenzweig (1973). *Plasmodium berghei*. Relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. Exp.Parasit., *33*, 168-178.

33. Nussenzweig, R., J.Vanderberg, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. Mil.Med., *134*, 1176-1182.

34. Yoeli, M. (1966). Patterns of immunity and resistance in rodent malaria infections. Bull.Soc.Path.exot., *59*, 593-605.

35. Nussenzweig, R.S., J.Vanderberg, G.L.Spitalny, C.I.O. Rivera, C.Orton, H.Most (1972). Sporozoite-induced immunity in mammalian malaria. A review. Amer.J.Trop.Med.Hyg., *21*, 772-728.

36. Nussenzweig, R.S., J.Vanderberg, Y.Sanabria, H.Most (1972). *Plasmodium berghei* accelerated clearance of sporozoites from blood as part of immune-mechanism in mice. Exp.Parasit., *31*, 88-97.

37. Vincke, I.H. and J.Bafort (1968). Méthodes de standardisation de l'inoculum de sporozoites de *Plasmodium berghei*. Ann.Soc.belge Méd.trop., *48*, 181-194.

38. Spitalny, G.L., J.P.Verhave, J.H.E.Th.Meuwissen, R.S.Nussenzweig (1975). T-cell dependence of sporozoite-induced immunity in rodent malaria. Submitted for publication in Nature.

39. Krettli, A., D.H.Chen, R.S.Nussenzweig (1973). Immunogenicity and infectivity of sporozoites of mammalian malaria isolated by density-gradient centrifugation. J.Protozool., *20*, 662-665.

40. Verhave, J.P. and J.H.E.Th.Meuwissen (1974). Studies on cellular immunity in sporozoite induced malaria. Third Int.Congr.Paras., *2*, 1116-1117.

41. Alger, N.E., Harant, J.A., Willis, L.C., Jorgensen, G.M. (1972). Sporozoite and normal salivary gland induced immunity in malaria. Nature *238*, 341.

42. Russel, P.F. and B.N.Mohan (1942). The immunization of fowls against mosquito-borne *Plasmodium gallinaceum* by injection of serum and of inactivated homologous sporozoites. J.Exp.Med., *76*, 477-495.

43. Verhave, J.P. and J.H.E.Th.Meuwissen (1974). Inhibition of exo-erythrocytic form development following reinoculation with sporozoites of P.berghei. Proc.Third Int.Congr.Parasit., *3*, 1243-1244.

44. Jahiel, R.I., J.Vilček, R.Nussenzweig, J.Vanderberg (1968). Interferon inducers protect mice against *Plasmodium berghei* malaria. Science *161*, 802-804.

45. Jahiel, R.I., R.S.Nussenzweig, J.Vanderberg, J.Vilček (1968). Anti-malarial effect of interferon inducers at different stages of development of *Plasmodium berghei* in the mouse. Nature *220*, 710-711.

46. Jahiel, R.I., J.Vilček, R.S.Nussenzweig (1970). Exogenous interferon protects mice against *Plasmodium berghei* malaria. Nature *227*, 1350-1351.

47. Jahiel, R.I., R.S.Nussenzweig, J.Vilček, J.Vanderberg (1969). Protective effect of interferon inducers on *Plasmodium berghei* malaria. Amer.J.Trop.Med.Hyg., *18*, 823-835.

48. Herman, R., T.Shiroishi, C.E.Bachler (1973). Viral interference with exo-erythrocytic forms of malaria (*Plasmodium gallinaceum*) in ovo. J.Inf.Dis., *128*, 148-155.

49. Huang, K.Y., W.W.Schultz, F.B.Gordon (1968). Interferon induced by *Plasmodium berghei*. Science *162*, 123.

50. Kiger, N. (1974) De chalonen. Image Roche, no. 58, 2-7 (in Dutch).

51. Yoeli, M. and H.Most (1965). The pre-erythrocytic development of *Plasmodium berghei*. Nature *205*, 715-716.

52. Yoeli, M. (1965). Studies on *Plasmodium berghei* in nature and under experimental conditions. Trans.Roy.Soc.Trop.Med.Hyg., *59*, 255.

53. Yoeli, M. and Most, H. (1965). Studies on sporozoite-induced infections of rodent malaria. I. The pre-erythrocytic tissue stage of *Plasmodium berghei*. Amer.J.Trop.Med.Hyg., *14*, 700-714.

54. Shortt, H.E. and P.C.C.Garnham (1948). The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax*. Trans.R.Soc.trop.Med. Hyg., *41*, 785-795.

55. Lupascu, G., P.Constantinescu, E.Negulici, P.C.C.Garnham, R.S.Bray, R.Killick-Kendrick, P.G.Shute, M.Maryon (1967). The late primary exo-erythrocytic stages of *Plasmodium malariae*. Trans.R.Soc.trop. Med.Hyg., *61*, 482-489.

56. Schultz, W.W., K.Y.Huang, F.B.Gordon (1968). Role of interferon in experimental mouse malaria. Nature *220*, 709-710.

57. Rytel, M.W., H.D.Rose, R.D. Stewart (1973). Absence of circulating interferon in patients with malaria and with american trypanosomiasis. Proc.Soc.Exp.Biol.Med., *144*, 122-123.

58. Ingram, R.L., L.G.Otken, J.R.Jumper (1961). Stained erythrocytic stages with antisera prepared against sporozoites. Proc.Soc.exp.Biol. Med., *106*, 52.

59. Ingram, R.L. and R.K.Carver (1963). Malaria parasites: fluorescent antibody technique for tissue stage study. Science *139*, 405-406.

60. Sodeman, W.A. and G.H.Jeffery (1964). Immunofluorescent staining of sporozoites of *Plasmodium gallinaceum*. J.Parasit., *50*, 477-478.

61. Krotoski, W.A., J.R.Jumper, W.E.Collins (1973). Immunofluorescent staining of exo-erythrocytic schizonts of simian plasmodia in fixed tissue. Amer.J.Trop.Med.Hyg., *22*, 159-162.

62. Krotoski, W.A., W.E.Collins, J.R.Jumper (1973). Detection of early erythrocytic schizonts of *Plasmodium cynomolgi* by immunofluorescence. Amer.J.Trop.Med.Hyg., *22*, 443-451.

63. Vanderberg, J.P., R.S.Nussenzweig, Y.Sanabria, R.Nawrot, H.Most (1972). Stage specificity of anti-sporozoite antibodies in rodent malaria and its relationship to protective immunity. Proc.Helm. Soc.Wash., *39*, 514-525.

64. Houba, V., P.H.Lambert, A.Voller, M.A.O.Soyanwo (1975). Clinical and experimental investigation of immune complexes in malaria in press.

65. Jerusalem, C. (1969). Active immunization against malaria (*Plasmodium berghei*) III. Enhanced immunity caused by blockade of the RES macrophages during controlled low parasitemia. Z.Tropenmed.Par., *20*, 81-88.

66. Cantrel, W. and E.E.Elko (1970). *Plasmodium berghei:* phagocytic hyperactivity of infected rats. Exp.Parasit., *28*, 291-297.

67. Biozzi, G., C.Stiffel, D.Mouton, C.Decreusefond, A.Corradetti, F.Verolini, A.Bucci, A.Ventura (1970). Effect de la vaccination par *Plasmodium berghei* irradié sur l'activité phagocytaire du système réticuloendothélial au cours de l'infection du rat par ce plasmodium. Bull.Wld.Hlth.Org., *42*, 163-168.

68. Greenwood, B.M., J.C.Brown, D.G.DeJesus, E.J.Holborow (1971). Immunosuppression in murine malaria. II. The effect of reticulo-endothelial and germinal center function. Clin.Exp.Immunol., *9*, 345-354.

69. Criswell, B.S., W.T.Butter, R.D.Rossen, V.Knight (1971). Murine malaria: the role of humoral factors and macrophages in destruction of parasitized erythrocytes. J.Immunol., *107*, 212-221.

70. Loose, L.D., J.A.Cook, N.R.Di Luzio (1972). Malaria immunosuppression, a macrophage mediated defect. Proc.Helm.Soc.Wash., *39*, 484-491.

71. Verhave, J.P., J.H.E.Th.Meuwissen, J.H.M.Bex (1973). Cell mediated immunity in plasmodial infections. Ninth Int.Congr.Trop.Med.Mal., *2*, 194-195.

72. Golenser, J., J.P.Verhave, D.T.Spira, J.H.E.Th.Meuwissen. Specific lymphocyte transformation in murine malaria. Submitted for publication.

73. North, R.J. (1974). T-cell-dependent macrophage activation in cell-mediated anti-Listeria immunity. In: Activation of macrophages (eds. W.H.Wagner and H.Hahn). Excerpta Medica, Amsterdam.

74. Mauel, J., R.Behin, Biroum-Noerjasin, J.J.Doyle (1974). Survival and death of Leishmania in macrophages. In: Parasites in the immunized host: mechanisms of survival. CIBA Found.Symp., *25*, Elsevier-Excerpta Medica, Amsterdam.

75. Maegraith, B. (1968). Liver involvement in acute mammalian malaria with special reference to *Plasmodium knowlesi* malaria. Adv.Parasitology (ed.B.Dawes) *6*, 189.

76. Vanderberg, J., R.Nussenzweig, H.Most (1969). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. Mil.Med., *134*, 1183-1190.

77. Nussenzweig, R.S., J.Vanderberg, H.Most, C.Orton (1970). Immunity in simian malaria induced by irradiated sporozoites. J.Parasit., *56*, II, 252.

78. MacDonald, G. (1957). The epidemiology and control of malaria. Oxford Univ.Press, London.

79. Garnham, P.C.C. (1970). Primate malaria. In: Immunity to Parasitic animals. *2* (eds G.L.Jackson, R.Herman, I.Singer) Appleton-Century-Grofts, New York.

80. Hess, F., C.Jerusalem, M.N. v.d.Heyde (1972). Advantages of auxiliary liver homotransplantations in rats. Arch.Surg., *104*, 76-80.

81. Jacobs, R.L., L.H.Miller, L.C.Koontz (1974). Labeling of sporozoites of *Plasmodium berghei* with tritiated purines. J.Parasit., *60*, 340-343.

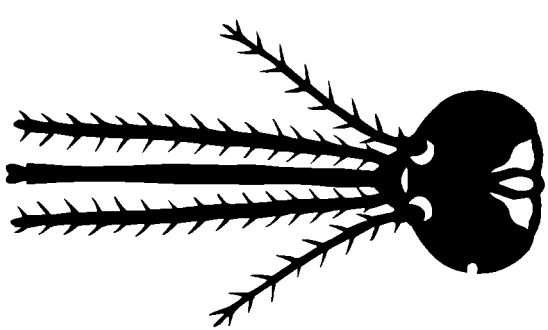
82. Landau, I. (1973). Diversité des méchanismes assurant la pérennité de l'infection chez les sporozoaires coccidiomorphes. Mem.Mus.Nation. His.Nat. (Nouv.Sér.) A, *77*, 1-62.

83. Yoeli, M. (1974). Slow maturing primary exo-erythrocytic schizonts of *Plasmodium berghei* in an experimentally infected tree-rat (*Thamnomys surdaster*). Trans.R.Soc.Trop.Med.Hyg., *68*, 302-305.

84. Miller, L.H. (1975). Erythrocytic receptors for (*Plasmodium knowlesi*) malaria. Science, in press.

85. Allison, A.C. (1974). Interactions of antibodies, complement components and various types in immunity against viruses and pyogenic bacteria. Transplant.Rev. *19*, 3-55.

86. IAEA & WHO consultants (1972). Use of radiation in the preparation of malaria vaccines. WHO/MAL/72.782 (mimeographed).



abortive infection:	unsuccessful development inside parasitized cell.
agglutination:	clumping of particulate ag in the presence of ab.
antibody (ab):	protein formed in response to ag and reacting specifically with it.
antigen (ag):	substance capable of stimulating ab-formation and able to react specifically with it.
attenuation:	loss of multiplitation potentials (e.g. after irradiation).
booster:	supplementary dose of ag to increase the immunological response.
causal prophylaxis:	prevention of plasmodial pre-erythrocytic development.
challenge:	test of immunity by exposure to infective, non-attenuated ag after specific immunization.
CSP:	circum-sporozoite precipitation.
EEF:	exo-erythrocytic form(s).
epidemiology:	complex of factors determining the incidence of malaria in the field.
exo-erythrocytic:	plasmodial stage developing inside liver parenchymal cell (mammalian).
immune:	state of being protected against infection.
immunization:	repeated administration of ag, to make the animal immune.
infectivity:	parasite-host inter-relationship, permitting invasion and intracellular multiplication.
interferon:	protein produced by cells after uptake of virus or protozoon suppressing growth of same or different organisms in other cells.
Kupffer cell:	fixed macrophage in the liver.
lymphocyte:	either antibody producing, (B) or thymus-dependent (T) small cell in circulation or lymphoid organs.
macrophage:	mononuclear phagocyte.
merozoite:	product of plasmodial segmentation of a tissue (EEF) or erythrocytic schizont.
oocyst:	fertilized plasmodial cell after encystment on mosquito stomach,and producing sporozoites.
parasitaemia:	condition in which erythrocytic stages are present in the blood.
phagocytes:	circulating or tissue cells, capable of engulfing and destructing foreign material.
pre-erythrocytic:	plasmodial stages from inoculation with sporozoites to liberation of merozoites from the liver.
prepatent period:	time lapse between inoculation of sporozoites or erythrocytic stages and the detectable onset of parasitaemia.
SNA:	sporozoite neutralization activity.
sporozoite:	plasmodial stage produced in oocyst and occurring in salivary glands of mosquito; transferred to vertebrate host while biting.
trophozoite:	erythrocytic form in its early stage of development.
vaccine:	suspension of dead or living (attenuated) organisms, injected to establish resistance to the disease.
viability:	condition of the parasite, being intact, mobile, healthy, and supposedly capable of multiplication.

S U M M A R Y

Information on immunogenicity of plasmodial sporozoites comes mainly from work with attenuated parasites. In this study we were able to demonstrate that viable sporozoites of *Plasmodium berghei* cause also an immune response in mice and rats, which may last for months. The effectiveness of this response increases with the number of sporozoite-inoculations and may cause prevention of exo-erythrocytic development on challenge. The response becomes completely protective and parasitaemias don't develop.

The mechanism of this response is not clear. Anti-sporozoite antibodies are demonstrable, and activity of the mononuclear phagocyte system may also be involved. The role of these phenomena in protection is not established, but it was found that thymocytes are required for the generation of a protective response.

The density of exo-erythrocytic forms (EEF) in the liver is taken as a measure of the number of infective sporozoites escaping natural and acquired mechanisms of resistance. Animals are kept on chloroquine during immunization, to ensure that the immune responses to parasitaemia don't interfere with anti-sporozoite immunity. We found that sporozoites inoculated during the acute stage of parasitaemia give rise to considerably fewer EEF. The density of EEF is also considerably reduced in animals that are immune to erythrocytic stages. This effect is probably non-specific.

Another type of interference of EEF development becomes evident when a second inoculation of sporozoites is given within the 48 hours of EEF maturation after the first inoculation. This reduction of development of second set EEF probably has a non-immunological background.

The findings of this study may be useful for an insight into unexplained epidemiological phenomena, and the investigation of the possibility of potentiating any naturally occurring anti-sporozoite response is suggested. Moreover, the development and application of sporozoite vaccines is dependent upon the understanding of anti-sporozoite responses. We are only at the beginning of the exploration of the host's reactions to the presence of pre-erythrocytic stages of the malaria parasite.

In de gecompliceerde levenscyclus van de malaria-parasiet is alleen het stadium in de rode bloedcellen pathogeen. Onderzoek naar de immunogeniciteit heeft zich dan ook voornamelijk op deze vormen van de parasiet gericht. Andere stadia leken geen immunologisch verweer op te roepen; met name sporozoieten die overgebracht worden door muggen, werden beschouwd als niet immunogeen, omdat ze ofwel te kort in de circulatie zouden blijven, ofwel in te kleine aantallen toegediend zouden worden. In hoofdstuk I hebben we de beschikbare literatuur gegevens gerangschikt.
Door het werk van Nussenzweig, die bestraalde sporozoieten inspoot, bleek dat er wel een duidelijk verweer wordt opgebouwd tegen dit stadium, waarbij de dieren na herhaald contact met deze verzwakte sporozoieten immuun worden. Ze zijn beschermd tegen infectie met infectieuze sporozoieten.
Onze interesse ging in de richting van een meer natuurlijke benadering. We hebben onderzocht hoe de gastheer (rat) reageert op herhaald contact met infectieuze sporozoieten (*Plasmodium berghei*). Om dat te meten hebben wij een tel-methode ontwikkeld, waarmee relatieve dichtheden van parasieten in de lever werden bepaald. In the parenchym cellen van de lever ontwikkelen zich exo-erythrocytaire vormen (EEF) uit sporozoieten. Na veelvuldige deling produceren deze EEF de erythrocytaire stadia, waarmee de parasitaemia begint. In ons onderzoek hebben we die erythrocytaire stadia tijdens de immunizatieprocedure onderdrukt met een anti-malaria middel dat niet op de preerythrocytaire stadia werkt (chloroquine).
Hoofdstuk II beschrijft de techniek en methoden die werden toegepast.
Hoofdstuk III is gewijd aan een statistische verantwoording van de telmethode en we hebben aangegeven op welke wijzen deze methode toepasbaar is in allerlei onderzoek naar de pre-erythrocytaire parasiet-gastheer verhouding.
In sektie 4.1 van hoofdstuk IV, hebben we de experimenten beschreven waarbij de gastheer meermalen sporozoieten kreeg toegediend. We vonden dat de dieren hierdoor beschermd konden worden tegen hernieuwde infecties met sporozoieten. Minder intensief contact met sporozoieten leidt wel tot aanzienlijke vermindering van het aantal EEF in de lever, maar niet altijd tot preventie van latere EEF-ontwikkeling. Het effect blijft maanden lang meetbaar.
In sektie 4.2 is het verschijnsel beschreven dat een tweede dosis sporo-

zoieten, toegediend binnen 48 uur na een eerste dosis, resulteert in lagere parasieten-dichtheden dan in dieren voor welke die tweede dosis de eerste was. Een poging om aan te tonen dat interferon, geproduceerd na de eerste infectie, hiervan de oorzaak is, had geen duidelijk positief resultaat.
In sectie 4.3 staan de experimenten beschreven die betrekking hebben op de invloed van parasitaemieën op de dichtheid van EEF na inoculatie met sporozoieten. Ook hier werd een duidelijke remming aangetoond.
In sectie 4.4 zijn enkele losse experimenten beschreven, die waren opgezet om het werkingsmechanisme van de beschermende immuun response te bestuderen. Verschillende typen van anti-sporozoieten-antistoffen blijken aanwezig in immune dieren, noch Chloroquine behandeling, noch parasitaemieën lukten daarop een duidelijk effect.
In de discussie (hoofdstuk V) is ingegaan op de betekenis van deze bevindingen. Het is door dit onderzoek duidelijk geworden, dat sporozoieten niet verzwakt behoeven te worden om een immuun response te veroorzaken. Verrassend waren de vondsten dat pre-erythrocytaire vormen en erythrocytaire vormen kunnen interfereren met de infectiositeit van sporozoieten.
Het is onjuist om resultaten in een knaagdier model van toepassing te verklaren op het humane model. Toch hebben we gemeend te moeten overwegen wat een anti-sporozoieten verweer kan betekenen voor mensen die regelmatig door geinfecteerde muggen gestoken worden, en voor de epidemiologie van malaria. Het lijkt de moeite waard om onderzoek te beginnen naar dit specifieke verweer bij mensen in endemische gebieden.
Tenslotte is aangegeven welke lijnen van onderzoek in het dier model nu voor de hand liggen.

D A N K W O O R D

Nu dit proefschrift gereed is, gaat in de eerste plaats mijn dank uit naar het Bestuur van de Faculteit der Geneeskunde, voor de vooruitziende blik bij de toestemming tot de bouw van een entomologisch laboratorium, waarin onderzoek zou worden gedaan, dat niet direct van Nederlands belang is.

Verder wil ik graag bedanken:
Janny Heeren, door wier ijver en nauwgezetheid de resultaten er staan, zoals ze er staan.

De andere medewerkers van het Instituut voor Medische Parasitologie, met name Ank, voor de hulp bij de interferon bepalingen; Jan, Bernadette en Ditsy, voor hun aandeel in het opzetten en onderhouden van de muggenkweek; Joep en Pieter, voor hun collegialiteit en vriendschap.
Door jullie belangstelling en geduld waren de dagen steeds waard om te werken en te leven.

De heer H. Peters en de biotechnische staf, voor hun vakkundige en steeds bereidwillige hulp bij de proeven en het verzorgen van de dieren.

Ir. Th.M. de Boo, voor het inzicht en de samenwerking bij het opstellen van een statistische verantwoording van dit werk.

De heren A.T.A. Reynen en C.P. Nicolasen, voor het vervaardigen van de illustraties.

Els Salemink-Brinkman en mevrouw E.S. Haan-Busé, voor het geduld en de accuratesse bij het tijpen van de vele voorlopige versies en het uiteindelijke manuscript.

I am greatly indebted to
Drs L. Schnur (Liverpool) and M.L. Weiss-Oppé (Nijmegen), for correcting the English text and their valuable remarks.

Dr G.L. Spitalny (New York), for our regular discussions on anti-sporozoite immunity and the help in performing some serological tests.

The members of the International Collaborative Research Program on Malaria Immunity and Immunopathology (Ibadan, Colombo, Jerusalem, Nijmegen), for the stimulating discussions. It is encouraging to share the task of "scientific replication".

Voorts wil ik in dit dankwoord betrekken
Dr. E.A. Meuleman, aan wie ik door haar energieke en kritische begeleiding mijn blijvende belangstelling voor parasiet-gastheer verhoudingen toeschrijf. Het onderwijs in de medische parasitologie, zoals ik dat van haar leerde, blijft voor mij een boeiende opdracht.
Met haar, ook de andere docenten van de Vrije Universiteit, Amsterdam, van wie ik onderricht in de biologie ontving.

Dr. B.H. Rep, Universiteit van Amsterdam, aan wie ik ook een deel van mijn wetenschappelijke vorming in de parasitologie dank.

De jaargenoten, ruwe wormen, met jullie voortdurende vriendschap, je liefdevolle en creatieve aanwezigheid. Onze band zal nooit vergaan.

Onze ouders, van wie we zoveel toegenegenheid genieten en die ons leven rijk maken.

Bovenal dank ik Hem, die mij inspireert tot navolging.

L E V E N S L O O P

Geboren 31 december 1942 te Bussum, als oudste zoon van Johanna Wilhelmina van Ravenzwaay en Johannes Verhave, zoon van Pieter, zoon van Jacobus, zoon van Thomas Hermanus, zoon van Adrianus, zoon van Jacobus, zoon van Jacob, zoon van Carel, zoon van Philip Verhave.
Christelijk Lyceum (HBS-B) te Hoogeveen, 1955 - 1961.
Dienstplicht, 1961 - 1963.
Studie biologie aan de Vrije Universiteit te Amsterdam, 1963 - 1971.
Wetenschappelijk medewerker aan de Katholieke Universiteit, op het Instituut voor Medische Parasitologie, sinds 1971.
Echtgenoot van Catharina Johanna Wilhelmina van Duijn, en vader van Wilhelmina Hadewij en Jacoba Catharijne.

Ingaande october 1975 tijdelijk verbonden aan het Naval Medical Research Institute te Bethesda, U.S.A., in het kader van een postdoctoral research associateship.

S T E L L I N G E N

I.

Protectieve immuniteit tegen sporozoieten lijkt bepaald te worden door opsoniserende antistoffen uit de IgG klasse, welke zonder de hulp van thymus-afhankelijke lymfocyten niet worden aangemaakt.

Brown (1974), Antigenic variation and immunity to malaria. In "Parasites in the immunized host, mechanisms of survival". CIBA Found.Symp. 25

II.

Het is waarschijnlijk dat infectieuze sporozoieten minder bijdragen tot het sporozoiet-specifieke verweer dan niet-infectieuze sporozoieten; de antigenen die vrij komen na voltooiing van de intracellulaire ontwikkeling in de lever kunnen hierbij echter wel van belang zijn.

Dit proefschrift.

III.

Er zijn aanwijzingen dat lever parenchym cellen de capaciteit tot endocytose hebben; dit maakt het aannemelijk dat ze actief betrokken zijn bij het "binnendringen" van sporozoieten.

Kief et al. (1974), Endocytotic functions of Kupffer and liver cells. In "Activation of macrophages". (Eds. Wagner & Hahn)

IV.

Immunodepressie tijdens het acute stadium van de malaria-parasitaemie begunstigt de vermeerdering van andere microörganismen, maar niet de ontwikkeling van homologe sporozoieten.

Dit proefschrift.

V.

Bij sporozoieten-infecties van naakte, thymusloze muizen dient rekening te worden gehouden met verminderde aantallen exo-erythrocytaire vormen, door de levernecrose, die deze dieren vaak vertonen.

VI.

De mate waarin een geschikt gebleken diersoort wordt geexploiteerd ten behoeve van bio-medisch onderzoek, creëert een markt waarvoor het natuurlijk reservoir niet toereikend is. Zo dienen ten behoeve van malaria onderzoek bestaande alternatieven voor de in het wild gevangen dourocouli's uitgewerkt en gefinancierd te worden.

VII.

Bij de bestrijding van overbrengers van malaria en andere ziekteverwekkers dringt zich de noodzaak op, toepassing van persistente insecticiden te integreren met biologische en genetische methoden. Voor evaluatie van de verschillende benaderingswijzen verdient het oecologisch onderzoek een hogere prioriteit.

WHO Techn. Rep. 561 (1975), Ecology and control of vectors in public health.
Wetenschap en Samenleving 75/2

VIII.

Voor inzicht in de transmissie van parasieten door geleedpotigen is kennis van huid- en lymfeklierreacties op de orale secretie van belang; daarbij doet het minder ter zake of de betreffende antigenen uit het speeksel of van concomitante microörganismen afkomstig zijn.

IX.

De suggestie dat *Acanthamoeba polyphaga* chronische cornea zweren kan veroorzaken, steunt niet op onderzoek, waarin wordt aangetoond dat aan de postulaten van Koch is voldaan. Het is dus niet terecht reeds te spreken over een "Amoebic infection of the eye".

Nagington et al.(1974) Lancet 2,1537.

X.

Premunitie speelt geen rol van betekenis in het verweer tegen experimentele herinfectie van de hond met mijnworm-larven.

XI.

De associatie van *Pneumocystis* infecties bij de mens met virale infecties in de longen, en de recente bevindingen van een dergelijk samengaan in rattelongen, maakt waarschijnlijk dat dit microörganisme een saprofytische levenswijze heeft en tot opportunistische infecties kan leiden.

XII.

Malaria onderzoek door de Amerikanen, tijdens de afgelopen oorlog in Z.O.-Azië, weerspiegelde allerminst de goede bedoeling van de interventie voor de inheemse bevolking.

XIII.

Voor inperking van de zilvermeeuwen-populatie in het Nederlandse Waddengebied, zo die al zou moeten plaats vinden, is het rapen van eieren geen effectief middel.

XIV.

Het advies van de Rijksdienst voor de Monumentenzorg om bij restauratie van de 14^{e} eeuwse kerk te Heumen alle 19^{e} eeuwse, gietijzeren ramen te handhaven, is op historische en estetische gronden aanvechtbaar; met name in het schip zouden ze naar oude vorm hersteld dienen te worden.

XV.

De aanspraken van thans in leven zijnde leden van de familie Verhave op het familiewapen van het geslacht Verhaven zijn ongegrond.

XVI.

De Duif in het blazoen van de Katholieke Universiteit herinnert aan rijke beloften. De tijd is rijp om te woekeren met charismatische talenten.

Malden, augustus 1975.

Jan Peter Verhave.