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A.N. VERMEULEN, W.F.G. ROEFFEN and J.H.E.T. MEUWISSEN, *Department of Medical Parasitology, University of Nijmegen**Plasmodium berghei: purification of immunologically active proteins of sporozoites*

Immunization with purified sporozoite proteins has not been attempted yet by other investigators. One of the reasons is the lack of a good purification method for the significant proteins. We describe here a method to isolate and purify two immunologically active proteins of *Plasmodium berghei* sporozoites. These proteins, Sp110 and Sp53, bind to antibodies from rabbits and rats, immunized by bites of infected mosquitoes (Vermeulen *et al.* (1982) *Exp. Parasitol.* 53, 199-208.), as well as monoclonal antibodies of hybridoma 3D11, kindly provided by Prof. R. S. Nussenzweig (Vermeulen *et al.* *Mol. Biochem. Parasitol.* in press.) Antibodies of the 3D11 line have shown to neutralize the infectivity of *P. berghei* sporozoites as described by Potocnjak *et al.* (1980, *J. Exp. Med.* 151, 1504-1513).

Sporozoites have been purified according to Vermeulen *et al.* (1982, *Exp. Parasitol.* 53, 199-208). Sp110 and Sp53 were extracted from these sporozoites using 10mM Tris-0.5mM MgCl₂-0.5% Triton X100 (pH 8.0). The soluble fraction was chromatographed on a 90×1.5 cm SephacrylS300 column using 0.2M Tris-5mM MgCl₂-0.5% Triton X100 (pH 8.0) as eluting buffer (Vermeulen *et al.* *Mol. Biochem. Parasitol.* in press.) The fractions containing the two proteins were pooled and run through an immuno-affinity column, consisting of 3D11 antibodies coupled to Sepharose 4B. After washing away the non-bound material Sp110 and Sp53 were eluted using 0.05M citric acid-0.5M NaCl-0.5% Triton X100 (pH 2.6). As determined with ¹²⁵I-labelled proteins nearly 100% of Sp110 and Sp53 could be released from the affinity column in this way. Coomassie Brilliant Blue staining of an SDS-PAGE pattern of purified proteins from 2×10⁷ sporozoites showed a positive staining of Sp53.

This procedure gives a pure and concentrated protein fraction, which can be used in immunization experiments. It should be stated, however, that the quantity of material necessary to induce protection *in vivo* might show to be the limiting factor.

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Infection of rat-hepatocytes with sporozoites of Plasmodium berghei and the role of Kupffer cells therein. Ultrastructural evidence is presented that the normal route of entry of sporozoites into the hepatic parenchymal cells *in vivo* is via the Kupffer cells. Observations were made in livers of Brown Norway rats fixed 15 minutes to 10 hours after inoculation. Many intact sporozoites were seen inside endocytotic vacuoles of Kupffer cells and some of these moved into pseudopods that extended from the Kupffer cells into the space of Disse. They left there and started to penetrate an adjacent hepatocyte. The sporozoites interiorized therein by invagination of the cell membrane and a parasitophorous vacuole was created. Sporozoites which were not able to leave Kupffer cells were degraded in secondary lysosomes later on.

Despite extensive searches the transformation of sporozoites has never been described before. We present

here the first ultrastructural observations on the natural transformation of intrahepatocytic sporozoites into exoerythrocytic forms *in vivo*. Sporozoite-derived trophozoites were detected in parasitophorous vacuoles 4 and 10 hours after inoculation. The identity of early sporozoite-derived cells was readily confirmed by the presence of sub-pellicular microtubules, but in comparison with sporozoites their form and appearance had changed markedly. The area around the nucleus was enlarged and rounded and at some places the triple pellicle membranes were reduced to a single one. The 10 hour form bore no resemblance to the sporozoite anymore; it gained the same electron-lucent appearance as the mature exoerythrocytic schizont. The inner pellicle membranes and microtubules of the sporozoite cytoskeleton were lost during this early differentiation and except some endoplasmic reticulum no organelles were yet to be seen. However, membrane-whorls in the parasitophorous vacuole were still present. Pili of parasite cytoplasm projected into the vacuole and made contact with its membrane.

These observations further complete the description of the life cycle of mammalian malaria parasites.

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Brain edema in clinical (P. falciparum) and experimental (P. berghei) malaria

Eighty-five autopsy specimens of South Vietnamese patients (aged 18 to 56 years) who died of cerebral manifestations such as coma and convulsions of *P. falciparum* malaria were investigated histologically.

For comparison the brain of mice suffering from experimental *P. berghei* malaria was studied ultrastructurally as well as histo- and cytochemically. The brain tissue of 71 patients exhibited typical disseminated petechial hemorrhages. In none of these cases, however, was the phagocytosis by (glial) macrophages of the erythrocytic extravasate observed, suggesting the rupture of vessels to be a late (probably agonal) complication. In 14 patients the hemorrhagic diathesis was notably absent although the patients had suffered from convulsions and from comatous states up to two weeks. In these cases the prominent histopathological finding was brain oedema and scattered hypoxaemic shrinkage and necrosis of nerve cells.

Experimental malaria (*P. berghei*) reflects many features of pernicious malaria tropica in man. As judged from the distribution of an intravenously injected tracer the initial malfunction was found to be comparable to the "no-reflow" and the "skimming" phenomenon.

Blocking of microcirculation may occur at the level of a) postcapillary venules (through hemorrhological sludging), b) capillaries (compression by swelling of glial cells, and c) arterioles (due to spasms).

Histochemically the break-down of the blood-brain-barrier is associated with a distinct decrease of alkaline phosphatase, ATP-ase and 5-nucleotidase of the vascular endothelium. It remains open to question whether the abnormal leakage and subsequent brain oedema results from electrolytic imbalance, excess of histamine, and/or activation of complement via the alternative pathway secondary to endotoxaemia.

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Topography of the brain lesion in experimental (P. berghei) malaria

Histopathological brain lesions characteristic of cerebral malaria in both man (*P. falciparum*) and mice (*P. berghei*) appear not to be randomly distributed throughout the cerebrum. To exclude accidental findings in the mouse *P. berghei* model systematic studies were carried out to determine the relationship between the type and the localisation of the lesion. Histopathology: As stated for pernicious *P. falciparum* (see Jerusalem *et al.*, this symposium) the petechial haemorrhage is not the sole morphologic lesion. In addition to severe brain oedema microthrombosis, sludging of mononuclear cells, arteriolar spasms, scattered disturbance of microcirculation, and the occasional proliferation of gliocytes were the prevailing morphological changes. Topographic distribution: A pronounced perivascular oedema (with compression of capillaries) and ischemic demyelination were particularly frequent in the nucleus caudatus putamen, while the adjacent regions (radiatio corporis callosi, claustrum, hippocampus and fimbria hippocampi) were the sites of predilection of the petechial haemorrhage. These regions are drained by the thalamostriate vein. Arteriolar spasms were particularly frequent in branches of the supracallosal artery. The proliferation of gliocytes was almost restricted to the fasc. med. prosencephali and to the subependymal zone of the lateral

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"Paludisme blanchi", pathogenesis and cause of death

The French term "paludisme blanchi" is used to characterize a particular complication (10 to 15 per cent) of cerebral (*P. falciparum*) malaria in South Vietnam. Although the parasites have been cleared from the circulation by specific therapy, the cerebral symptoms of coma and occasional convulsions persist and the patients die of cardiac or respiratory failure, and/or hyperthermia 3 to 18 days after the elimination of the parasites ("bleached out").

We studied 25 autopsy specimens of paludisme blanchi. Six of these were excluded because of severe complications (important for differential diagnosis!) such as persistent renal failure and anuria (n=3), septicemia secondary to corticosteroid treatment (n=2) and one assumed hepatic coma because of an underlying fulminant viral (B) hepatitis.

In the case of paludisme blanchi the main histopathological cerebral changes were 1) persisting, pronounced brain oedema, occasionally resulting in compression of capillaries and metarterioles, and in inflow congesting of the ophthalmic and vorticosose veins, 2) incomplete resolution of occluding thrombi but attempts of mononuclear macrophages to phagocytose a perivascular haemorrhage, 3) probably fixed spasms of small arteries and arterioles and 4) focal accumulation of lymphocytes mainly in the perivascular space. The lesions mentioned in 1 to 3 result in progressive ischaemic necrosis of nerve cells, demyelination, and finally in the interruption of the vital functions of the brain. The picture of paludisme blanchi can therefore be explained with a primary *P. falciparum*-induced cerebral lesion which is too severe and too extended to regress spontaneously after the eradication of parasites. For this reason, in addition to specific therapy medication to stimulate the cerebral blood flow, anticonvulsants, tranquillizers and perhaps antihistamines should be started as early as possible in the case of incipient pernicious *P. falciparum* malaria.

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Effect of immunosuppression on *P. berghei* infections in mice

In mice, the untreated *Plasmodium berghei* infection is always lethal. The mortality pattern after a standardized infection (10⁶ PE mouse) is strain specific. The proportion of mice dying early in the second week of infection varies from 100% (C3H StZ mice) to 0% (Balb c mice). Moreover, this early mortality was absent in T-cell deficient mice in contrast to the euthymic parent strain (C57B1 mice). Although the cause of death is unknown, and may be complex, this result suggested that T-cell dependent reactions could contribute to early mortality, and that immunosuppression could be beneficial to the host, at least temporarily. Subsequently it was found that splenectomy could prevent early mortality, even when performed shortly before expected mortality. Next, spleen irradiation, treatment with corticoids, treatment with anti-T-cell serum or treatment with immune serum all appeared to be able to prevent early mortality. Different immunosuppressive treatments were not equally effective during infection and in different strains. Treatment with anti T-cell serum remained effective until day 7 of infection; spleen irradiation became increasingly ineffective after day 6 of infection; corticoid treatment became ineffective when started after day 5 of infection. Splenectomy, or spleen irradiation, or treatment with anti T-cell serum or corticoids, were all effective in C57B1, B101 P and Balb c mice, but hardly effective or ineffective in C3H StZ mice, whereas immune serum was effective in all strains tested. These results indicate that in certain strains T-cell dependent reactions are generated in the spleen that contribute to early mortality, whereas in other strains early mortality is caused by factors independent of such immune reactions. Parasite-host interaction consists of two types of reactions, host-anti-parasite reactions trying to eliminate the parasite, and

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parasite-anti-host reactions trying to prevent parasite elimination. (Early) mortality may depend on failure of host-anti-parasite reactions, and/or effective parasite-anti-host reactions. This in turn may explain strain specificities observed.

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The role of complement of the phagocytosis and destruction of Plasmodium berghei infected erythrocytes in vitro

Microorganisms can be lysed by complement either directly (alternative pathway) or with the help of antibodies (classical pathway). Moreover, the complement product C3b promotes contact of target cells with cells carrying C3b receptors on their surface (like macrophages) and thus facilitates phagocytosis. We investigated whether the complement system plays a role in the destruction of parasitized erythrocytes (PE) and the uptake of these cells by adherent peritoneal exudate cells (APEC) *in vitro*. Therefore we incubated APEC with radioactively labelled PE in the presence or absence of complement. Addition of fresh mouse serum to the culture system enhanced the association of radioactive material with the macrophages when compared to inactivated mouse serum. Essentially the same results were achieved when guinea pig serum was used as a source of complement. The amount of radioactivity associated with the macrophages might be the result of adherence of the PE to and/or uptake of these cells by the phagocytes. Indeed we found adherence of PE to the macrophages when fresh serum was used. Inactivation of the serum inhibited this adherence. We investigated also whether PE were lysed in the presence of complement. Addition of fresh serum to a suspension of parasitized erythrocytes resulted in a release of free parasites into the culture medium. Furthermore, free parasites were phagocytized more readily than intraerythrocytic parasites. From these results we conclude that complement can lyse at least some PE resulting in the generation of free parasites and that the complement product C3b can coat PE. Both activities result in facilitated uptake of parasites by macrophages. These mechanisms could be useful in the defense of the host against the parasite.

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Clearance of modified erythrocytes in the study of the effector mechanism against Plasmodium berghei

Measurement of clearance of parasitized erythrocytes (PE) marked with Iritium-methionine (^3H -PE) from blood to organs is supposed to reflect the dynamic activity of the immune mechanism.

Clearance of ^3H -PE from blood, their distribution to immunocompetent organs, measurement of the fraction ^3H -PE bound in the liver during cannulation *in situ*, and results obtained in splenectomized hosts, could be interpreted in the light of specific or non-specific responses to modified erythrocytes of spleen and liver.

Strictly speaking every PE is a modified erythrocyte. A routine bloodsmear shows all maturation stages of the parasite suggesting that modification could remain undetectable in the sense of not being directly phagocytized by the cells of the mononuclear phagocyte system (MPS), not even in the early phase of the disease.

Treatment of the PE during a short period of time (1-2 min.) with a low concentration of glutaraldehyde (GPE) (0.01%) resulted in sequestration of PE in the spleen, while prolonged treatment (15-30 min.) or higher concentration (0.1%) resulted in PE being bound mostly in the liver. In a splenectomized non-immune host, slightly modified GPE remained in the bloodstream; but clearance of these GPE continued enough at a lower level in the immune host after splenectomy. Other studies have shown that PE treated at 48-52°C and P released from PE by NH_4Cl treatment are cleared from the blood very fast, and the organ distribution shows that they are bound mainly by the MPS of the liver. Since the spleen plays an important role in malarial immunity the above results might suggest that slightly modified PE which are sequestered in the spleen can contribute to development of immunity.

Strongly modified PE however (possibly also the merozoites and "free" parasites) are regarded as foreign particles being removed very effectively by the liver MPS in a non-specific way. This might explain the difficulty failure to immunize with parasite homogenates.

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The effect of splenectomy and irradiation on pathology and early mortality in Plasmodium berghei malaria
Splenectomy before, or during infection, and even shortly before expected death, prevented early mortality in part of the *P. berghei*-mouse strain combinations (C57B1/Rij, -/67, -/10, Balb/c, B10LP), whereas it was hardly effective or ineffective in others (Swiss, C3H/StZ). Moreover, splenectomy before infection restricted or prevented liver pathology, thymus involution and even prevented an otherwise complete loss of immune reactivity. Splenectomy restricted liver pathology during infection independent of its effect on early mortality in all strains tested. Since comparable observations were made in T-cell deficient mice, it is believed that T-cell and spleen dependent reactions contribute to the morbidity and early mortality of this infection in some parasite-mouse combinations.

Next, it was investigated whether irradiation of the spleen instead of splenectomy could prevent early mortality too, and whether changes in serum aspartate transaminase associated with liver pathology could be used as a marker for changes in the morbidity of the infection. The results showed that irradiation of the spleen like splenectomy could prevent early mortality. The spleen dependent reaction was very sensitive to irradiation, because 300 rad sufficed to eliminate this process at least temporarily. Unlike splenectomy the beneficial effect of spleen irradiation was only observed until day 6 of infection, after which it gradually decreased. Changes in serum aspartate transaminase could not be used as a marker for the life saving effect of neither splenectomy nor spleen irradiation. Serum aspartate levels remained low when splenectomy or spleen irradiation was performed before the second day after infection, but gradually rose to control values or even became higher the later treatment was given.

This may indicate that a radiation sensitive process contributing to liver pathology evolves in the spleen, the evolution being stopped by splenectomy or irradiation. The radiation sensitivity and T-cell dependency of the process may indicate that an increasing number of T-cells becomes involved in a radiation insensitive process e.g. macrophage activation, which contributes to liver pathology in the course of the infection. The results also indicate that early mortality is not primarily caused by liver pathology.

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Total and unbound (biologically active) plasma corticosterone in Plasmodium berghei malaria
A remarkable proportion of mice, immunized to *P. berghei*, suffered from recrudescence during pregnancy. This loss of immunity might be related to the increase of plasma corticosterone during pregnancy, acting as an immunosuppressive serum factor. Several arguments support this statement. The rise of plasma corticosterone and the recrudescences occurred in the same period of pregnancy, adrenalectomy before pregnancy delayed increase of plasma corticosterone, and substantially lowered the recrudescence rate. Furthermore, the concentration of total plasma corticosterone in mice with recrudescence was higher as compared to that in mice that remained immune. Plasma corticosterone, not bound to corticosterone binding globulin, is thought to be the biological active fraction of the hormone. No increase of unbound plasma corticosterone was found in normal pregnancy, but during malaria recrudescence this fraction was elevated, and remained high until death or recovery.

During primary infection in non-pregnant mice the total plasma corticosterone hardly increased whereas unbound hormone increased significantly when infection became patent. In non-immune pregnant mice the effect of a primary infection on plasma corticosterone levels is more complicated, and especially the rate of increase of the concentration total plasma corticosterone depended on which day of pregnancy the animals were infected.

In summary, the concentration unbound plasma corticosterone is elevated in all forms of patent malaria, in primary infection in non-pregnant and pregnant animals and in malaria recrudescence in immune pregnant mice. This increase of unbound hormone could be ascribed to a decrease of corticosterone binding globulin production as a result of disturbed liver function during a malaria infection. Elevation of the total plasma corticosterone concentration above normal is only found during malaria in pregnancy, both during recrudescence and during a primary infection, the latter being dependent on the day of infection. These results emphasize the question whether the pregnancy associated increase in plasma corticosterone is functional in suppressing maternal immunity leading to malaria recrudescence, and the excessive increase is a consequence of a patent infection during pregnancy.

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Transmission blocking immunity induced in mice by vaccination with gametes of Plasmodium berghei
In recent years the experimental vaccination with plasmodial microgametes emerged as an alternative and unconventional way of interrupting the parasite's life cycle. Anti-gamete antibodies could be raised in healthy animals; the immunization does not affect the course of parasitaemia and the development of gametocytes. Only when mosquitoes take a bloodmeal, the emerged gametes come into contact with the antibodies in the plasma. This interaction prevents the fertilization and zygote formation in the midgut, and thereby brings about a transmission blockade.

We have used both micro- and macrogametes of *P. berghei* to immunize Balb/c mice. Blood of infected mice was exposed to ambient temperature and air, and both types of gametes were harvested and separated from other parasites and erythrocytes.

Results can be evaluated properly if the parasitaemia induced by the challenge infection has the same course in vaccinated and control mice. Two parameters were used to evaluate the transmission and its blockade in *Anopheles stephensi* mosquitoes: the mean probability of infecting a mosquito and the median of oocyst numbers in positive mosquitoes. Results were such that in comparison with controls vaccinated mice invariably infected a lower percentage of mosquitoes and those mosquitoes that still became infected had lower numbers of oocysts. We found that high numbers of microgametes (2×10^7) did not give much better results than lower numbers (2×10^6) and that five inoculations induced not a better blockade than three. However, the intravenous route and live microgametes caused a blocking factor that was highly effective, as compared with the intramuscular route and formalin fixation. In the former cases transmission blockade was nearly complete. Vaccination with fixed and live macrogametes along the intramuscular route was also possible. In preliminary experiments significant reductions were achieved, both in the percentage of infected mosquitoes and in the numbers of oocysts in positive mosquitoes. Further purification of macrogametes and their morphological differentiation are presently carried out for future vaccinations.

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Transmission of Plasmodium berghei from different mouse strains and the occurrence of a natural transmission blockade

The early mortality of Swiss mice infected with *P. berghei* prompted us to choose another strain, for the production of high numbers of gametocytes. C57b1/Rij mice were reported to survive much longer. We confirmed this by finding a 50% cumulative mortality at day 22, versus day 7 for Swiss mice.

It has been reported by us and several other workers, that the transmission of parasites from various rodents to mosquitoes drops after the first rise in parasitaemia. We found a rapid decrease after day 4-5 in the Swiss mouse - *Anopheles stephensi* model, and almost no transmission after day 8. The decrease was revealed both for the probability of infecting mosquitoes (percentage of oocyst carriers) and for the mean number of oocysts per positive mosquito. Blockade occurred despite the mounting numbers of male and female gametocytes in the surviving mice. The phenomenon has never been explained properly.

In C57b1/Rij mice the peak values during the course of parasitaemia and gametocytaemia were somewhat lower than in Swiss mice but of similar pattern. Mosquitoes fed daily on these mice continued to be infected at about 80-90% until day 15 and decreased slowly thereafter until day 25. Mean oocyst numbers fluctuated from day to day and peaks were found on alternate days.

It was proposed by Mendis and Targett (1980) that natural transmission blockade is caused by a serum factor. As splenectomy suppresses both IgG formation and delays the occurrence of natural transmission blockade, the factor may be an antibody. IgG production in C57b1/Rij is easily disturbed by immunosuppressive measures and probably also by parasitaemia; this may explain the lack of natural transmission blockade in these mice. Otherwise the blockade and the acute pathology in other mouse strains and rats might be explained by some macrophage derived factors in the serum, and these might have influenced the sporogony in the mosquito.

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Circadian rhythm in the genesis and maturation of Plasmodium berghei gametocytes

Hawking has repeatedly pointed to a phenomenon of synchronous maturation of gametocytes of various plasmodial species. He found exflagellation occurring at different rates during the 24 hour daily cycle. Others have found elevated numbers of gametocytes during the activity period of the host and a decrease when at rest. The day-night fluctuation or, in more general terms, the host's circadian rhythm seems to influence the gametocytogenesis, irrespective of lack or absence of synchronicity of the asexual cycle.

As we needed large numbers of mature gametocytes for vaccination purposes, we have explored the possibility of exploiting the circadian rhythm of host mice, infected with *P. berghei*. We hypothesized that mosquitoes would become infected optimally, when fed at midnight, since Hawking had found peak exflagellations in *P. yoelii* at that period. By exposing *Anopheles stephensi* every four hours to Balb/c mice, we found indeed high numbers of oocysts in the midnight group. For convenience mice were put on an inverted light-darkness scheme for two weeks and they were subsequently infected with parasitized erythrocytes, together with normal controls. Blood was taken and mosquitoes were fed at noon on days 3-5 after inoculation.

At that time the mice on the inverted scheme were in their "midnight"-activity period and the normal mice were in their daily rest period. No consistent differences were found in numbers of gametocytes and of exflagellations, and in percentages of infected mosquitoes fed on day-mice and night-mice. But the mean and median numbers of oocysts were always higher in mosquitoes that had fed on "inverted mice".

There are three main problems in quantification: a) the morphological differentiation between young and mature macrogametocytes is difficult and this may have veiled growth waves, b) counting of gametocytes in smears is very unreliable, c) mosquitoes feeding on one animal at one time show broad ranges of oocyst numbers, depending on the blood volume they ingest. For expression of oocyst numbers both mean and median numbers are poor parameters.

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Gametes of Plasmodium falciparum: the use of antisera and monoclonal antibodies to characterize stage-specific antigens

The application of a gamete derived vaccine in order to control malaria transmission has gained more and more attention. This is partially due to successful experiments in animal models (Carter *et al.* (1979) Exp. Parasitol., 47, 1-15; Mendis and Targett (1979), Nature, 277, 389-391, and Vo van Nhan, paper at this meeting.), partially to the development of automated cultivation systems by which it is possible to obtain sufficient quantities of mature *Plasmodium falciparum* gametocytes (Ponnudurai *et al.* (1982) Trans. R. Soc. Trop. Med. Hyg., 76, in press.).

In this presentation we report preliminary results on the antigenic characterization of *P. falciparum* female gametes. Mature gametocytes were allowed to activate and exflagellate for 30 min. at 26°C. Macrogametes zygotes were purified using a biphasic Metrizamide gradient (Vermeulen *et al.* submitted for publication). The resulting suspension containing the female gametes was practically free of any other developmental stage and only contaminated with some uninfected red cells. About 37% of the number of macrogametocytes present in the culture could be recovered as macrogametes. Using this material as immunogen in Balb/c mice monoclonal antibodies were developed according to the procedure of Köhler and Milstein (1975, Nature, 256, 495-497). Two hybridoma lines could be established, 7F3 and 7F11, which produced gamete specific antibodies. 7F11 reacted also with microgametes as determined using an IFA test. Both lines showed to react with surface components of macrogametes in the IFA test using freshly isolated, viable macrogametes as antigen. Macrogametes were surface-labelled using the lactoperoxidase-catalyzed ¹²⁵Iodination. A 200kD protein was recognized by antibodies from mice immunized with purified macrogametes, but not by 7F3 or 7F11 antibodies. Further investigations will be focussed on the production of more specific hybridoma lines as well as on the improvement of the techniques to immunoprecipitate monoclonal-recognized antigens.

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Control of malaria by immunization, a mathematical assessment of expected short-term effects of a gamete vaccin

A large amount of research is directed towards the development of gamete vaccins. Therefore epidemiological questions with respect to the impact of control measures based on such vaccins will become increasingly important. It is clear that the effects will depend on a lot of factors e.g. the effectiveness of the vaccin (the transmission blocking capability), the vaccination design, the local endemic characteristics such as the gametocyte rate, the vectorial capacity and the mosquito resp. human susceptibility to infection.

In order to obtain a first impression of the short-term effects of a hypothetical application of a gamete vaccin a sensitivity analysis has been performed by varying the above mentioned factors over a wide range. Control by vaccination seems relatively most effective in malarious foci with a low or moderate vectorial capacity (≤ 10) and a low effective gametocyte rate (≤ 0.01). The relative effect, i.e. the incidence of malaria infections relative to the incidence in case of no vaccination, is under these conditions approximately independent of the actual value of the vectorial capacity and the gametocyte rate.

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Preliminary data on the selection for refractoriness in the Anopheles stephensi/Plasmodium falciparum system

The availability of infectious *P. falciparum* parasites enabled a start to be made for the selection of refractoriness in *A. stephensi* mosquitoes. The first experiments involved a study of the effect of age of the female mosquito on its susceptibility to infection by *P. falciparum*. Mosquitoes of 4, 7, 8, 10, 11 and 17 days old were submitted to infection by allowing them to feed through membranes on infected blood from cultures that contained both male and female gametocytes. The percentage of mosquitoes which were infected dropped from 93% for 4 day old mosquitoes to 25% for 17 day old mosquitoes, indicating a strong negative correlation. This same relationship was also found for the average number of oocysts per infected midgut. The average dropped from 22.3 ± 10.6 in 4 day old mosquitoes to 1.6 ± 3.9 in 17 day old ones. It was concluded that for the future selection work all mosquitoes should be 4 days old.

Using a specially composed *A. stephensi* strain showing a large amount of heterogeneity (Nij-strain) selection experiments were begun. Five days after feeding on infected blood, females were placed in individual cages and were allowed to lay eggs. On the 6th day the mosquitoes were dissected and the numbers of oocysts recorded. Eggs laid by females having low oocyst numbers and those having high numbers were retained. These two populations are then reared and when available in sufficient quantities, will be tested again for their susceptibility. In the future, populations from different geographical origin will be examined and selected for refractoriness to infection with *P. falciparum*.

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The functional cell biology of malarial parasites: a discussion of some current problems

Despite new and intensive studies on the molecular genetics of the malarial parasite *Plasmodium*, our understanding of genome organisation throughout the complex cell cycle is poor and isolated reports are often dramatically contradictory. Only two basic facts are proven: the asexual merozoite is haploid, and there is a sexual process during which two nucleated gametes fuse and produce a diploid zygote. Attempts to estimate the chromosome number in *Plasmodium* have been singularly unsuccessful. Initially complete organelles were misidentified as chromosomes and later electron microscopy revealed that the schizont nuclei were syncytial polyploid structures within which lie the minute (1.5 μ m. long) spindles. E.M. also showed that the chromosomes failed to condense individually at any time in the parasite cell cycle. To date the only estimates of chromosome number (10) are based upon kinetochore counts in serial E.M. sections but even this study has had to assume a 1:1 kinetochore: chromosome ratio. It is suggested that meiosis is an immediate one step post zygotic division without the intervention of a "4C" state, this makes chiasma formation and the possibility of intrachromosomal recombination less likely.

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Most estimates of the DNA content of the malarial genome have been obtained by biochemical techniques, their results differ by a factor of 38 times! Realistic values must be obtained to allow a rational foundation for current molecular biology studies. Dr. Overdulve stressed the inherent errors in biochemical methods: determination of cell number, cell integrity and homogeneity of the cell population etc. He indicated that microcytophotometric techniques are now sensitive enough to estimate the DNA content of a single gamete of *Toxoplasma* (at 0.1 p.g./cell), and to indicate that as little as 4% of this could reliably be calculated as being the mitochondrial genome. Preliminary cytophotometric estimates on *Plasmodium berghei* suggest a genome size of 2.7×10^7 base pairs (0.025 p.g./cell) a result which concurs with the biochemical estimates. In contrast biochemical estimates for different lines of *P. falciparum* maintained in culture vary from 1×10^7 to 3.8×10^8 base pairs/cell. Although it was felt this error was too great to be explained by the presence or absence of repetitive DNA sequences (see below) it was questioned whether sustained asexual replication *in vitro* (analogous to repeated laboratory blood passage) would result in such losses of parasite DNA. It was generally agreed that more, and recent isolates should be examined to determine the genome size with greater certainty.

The role of the repetitive DNA (18% of total) recently detected in lines of *P. berghei* which have infective gametocytes, but reduced (3% of total) in those lines with non-infective gametocytes was questioned. It was noted that the macrogametocyte is perhaps the only stage of growth to have a defined nucleolus. The possible subcellular localization of the repetitive DNA (size 400-5000 base pairs, with a repeat frequency of some 60 times) within the nucleolus was discussed. Interestingly the related parasite *Toxoplasma* is reported as having 15% more DNA in the macrogametocyte compared to the microgametocyte. *Toxoplasma* gametocytes may be distinguished by the presence of a nucleolus in the female cell only.

It was concluded that more precise studies on the parasite genome are urgently needed. Such studies should be capable of distinguishing the different stages of parasite development and examining the genome in each separately. Further such studies ought to be applied to numerous different isolates of the human parasite *P. falciparum*. Such molecular approaches must be related to some understanding of the chromosomal organisation of the parasite throughout its cell cycle, and it is perhaps in this area that results will be the most difficult to obtain. A balanced search for such basic knowledge would provide a sound basis for future studies on the detailed molecular organisation of the parasite genome.

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Immunopathology in malaria

The interaction between parasite and host is functional when both parasite and host survive. In naturally occurring combinations, evolution has selected the combinations which were able to mount this balance, whereas in experimental laboratory models this is usually not the case. As an example *P. berghei* gives a low grade chronic infection in its natural host *Thamnomys* but is rapidly fatal in laboratory mice. The parasite and its products being foreign to the host trigger defence reactions which try to eliminate the parasite. To prevent this the parasite can hide, change its make-up, or try to stop the antiparasitic immune reactions. Interference with antiparasitic immune reactions may be attained by poisoning the system (parasitotoxin) or by triggering immune reactions that, being part of the network, are able to suppress development of effector immune reactions. In balanced, naturally occurring systems this may lead to premunition. In laboratory models and even within a given model e.g. *P. berghei* in mice the outcome depends on the strength of the reactions being induced. In the uniformly lethal *P. berghei* infection in mice the parasite dependent antihost reactions are stronger than the host-protective immune reactions. Nevertheless, in some *P. berghei*-mouse strain combinations (e.g. C57Bl, B10LP, Balb c) a wide range of immunosuppressive measures administered before or during infection prevent early mortality and associated pathology. Mice normally dying early in the second week of infection (10^5 PE on day 0) with severe liver pathology, involution of lymphatic tissue (spleen, thymus) associated with anergy, severe anemia, and at least in some cases cerebral pathology, survive for periods up to five weeks when appropriate immunosuppression is given before or during infection. Early mortality and some associated pathology can be prevented a.o. by treatment with immune serum, and an antiserum against T-cells, by splenectomy, and is absent in T-cell deficient mice. These mice, despite the immunosuppressive treatment, and having comparable or only slightly reduced parasitemias, do not become anergic in the course of the infection. These results suggest, that parasite dependent, T-cell dependent, spleen dependent immune reactions contribute to early mortality, and some associated pathology. In combination with sensitivity of these reactions to spleen irradiation

this might indicate that T-cell reactions in the spleen are involved. Since splenectomy is still effective when other immunosuppressive measures become ineffective, the spleen dependent reaction actually contributing to morbidity and early mortality, though probably generated by interaction with T-cells, is not mediated by T-cells. The spleen is an ideal place for a direct contact between parasites or their products and T-cells, and thus for specific and unspecific stimulation of T-cells, producing lymphokines that a.o. stimulate macrophages. Activated macrophages or their products may be more directly involved in pathological processes. In the models described here the parasite induced immunological injury is dominant in the parasite-host interaction during infection. Immunosuppression, though it probably also prevents development of protective immune reactions, suppresses this dominant reaction, which is temporarily beneficial to the host. If sufficient specific immunosuppression of the immunological injury reactions can be achieved, it might be possible to turn a lethal infection into a transient infection.

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Development of vaccines against malaria

Fundamental studies on parasitic biology and parasite-host interaction are essential for a better understanding of immunity against malaria and the eventual induction of antimalaria immunity by vaccines. Anti-merozoite, anti-sporozoite and/or anti-sexual stage vaccines might contribute to check parasite multiplication in the vertebrate host or the vector. This was discussed in more detail along with the role of monoclonal antibodies and the biological engineering for vaccine development.

Monoclonal antibodies have been prepared against merozoites and asexual blood stages, sporozoites, microgametes, macrogametes, zygotes and ookinetes. Some of these provide passive protection or block transmission. They are especially used for the isolation, characterization and identification of antigenic polypeptides and their epitopes. Moreover they are applied in molecular biological studies. In this respect various approaches are available to achieve the production of defined antigens.

Translation of stage specific *P. falciparum* RNA and identification of the *in vitro* synthesized antigenic polypeptides by screening with specific (monoclonal) antibodies in order to identify and isolate antigen-encoding-mRNA. This RNA can be used for cloning (by the production of copy DNA) in *E. coli* and as a hybridization probe of a "shotgun" clone bank. Once positive clones have been identified the genes of interest can be characterized and manipulated into appropriate host-vector systems that will permit expression of the desired protein(s). In Nijmegen this line of research has proceeded to the point of expression of *P. falciparum* gametocyte RNA in a rabbit reticulocyte cell-free protein synthesizing system.

Gamete specific (monoclonal) antibodies may also be useful as a direct primary screen to identify the required bacterial clones from a "shotgun" clone bank. At present, such a *P. falciparum* DNA library, containing $\pm 50,000$ recombinants, has been prepared. Colonies that express gamete specific antigens or their determinants have not yet been detected.

These are the first steps towards the development of antimalaria vaccines. In the future we will learn: which of such vaccines, based on components of sporozoites, asexual, sexual parasitic stages or a mixture of these, are the most useful; to what extent they will be effective when applied to populations continuously exposed to the risk of infection; how well they can contribute as single method to malaria control; or - more likely - whether vaccination will have to be combined with other control methods. Preliminary results obtained from experimental mathematical models indicate that at its best the effect of vaccination on the intensity of malaria transmission correlates with the percentage of successful vaccinations in the populations.