

IMMUNISATION AGAINST GAMETES AND  
ASEXUAL ERYTHROCYTIC STAGES OF  
RODENT MALARIA PARASITES

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by

Kamini Mendis, M.B.,B.S., M.Sc.

London School of Hygiene and  
Tropical Medicine

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To Nirosha, Nalaka  
and my parents



ABSTRACTIMMUNISATION AGAINST GAMETES AND ASEJUAL ERYTHROCYTIC  
STAGES OF RODENT MALARIA PARASITES

by

Kamini Mendis

It was possible to block the transmission of an infection of a rodent malaria parasite Plasmodium yoelii nigeriensis to Anopheles stephensi mosquitoes by immunising mice with a vaccine containing formalin-fixed gametes. A single dose of the gamete vaccine containing  $2 \times 10^6$  male gametes given intravenously was effective in blocking completely the transmission of a blood induced challenge infection. The vaccine was also effective when given intramuscularly and immunity was found to last at least six months.

Transmission blocking immunity was found to reside in a serum factor, probably antibody, and to be directed against extracellular gametes, acting on them in the gut of the mosquito while gametocytes in the circulation of the vertebrate host remained unaffected.

A limited study involving experimental vaccination with formalin-fixed erythrocytic parasites was also undertaken. A crude erythrocytic stage vaccine protected mice against the asexual blood stages of a challenge infection and protection was found to be enhanced by

killed Bordetella pertussis organisms used as an adjuvant.

The gamete vaccine afforded partial protection against the disease. Immunisation with asexual parasites alone showed that this protection was due to the presence of asexual forms as contaminants and that anti-gamete immunity is stage specific.

Factors affecting the infectiousness of gametocytes in a natural infection were also investigated. It was found that mice elaborate anti-gamete immunity in response to an infection which renders it non-infectious to mosquitoes after about the fifth day. In addition, gametocytes display a pattern of altered viability during the course of an infection where they lose the capacity to exflagellate. The possibility of this being either a manifestation of an intrinsic cycle of gametocyte development or immunity mediated by non-specific non-antibody factors that affect gametocytes within red cells is discussed.

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INTRODUCTION

The health of nearly a third of the world's population is today threatened by malaria; the disease is prevalent throughout the tropical and subtropical areas with an estimated incidence of 150 million cases per annum. In Africa alone it causes a mortality of 1 million mainly among children, and a considerable morbidity, which together impose severe constraints on the economic development of the Third World which it mainly afflicts (World Health Organisation (WHO), 1974, 1978).

Eradication measures, based on vector control using insecticides, and chemotherapy have been in the past successful in eliminating malaria from parts of Europe, Asia and North America. Integrated programmes based on these measures and formally endorsed by the WHO have been able to keep the disease in abeyance in Asia but have been incapable of affecting the malaria situation in much of Africa. Besides, the recent resurgence of the disease in South East Asia and Central America gives cause for grave alarm. These failures are largely attributable to the lack of financial resources and organisational structure demanded by long-term eradication programmes.

In addition, serious technical problems threaten even the existing anti-malarial measures. Spreading multiple resistance of vectors to insecticides and altered vector behaviour which render them less vulnerable to insecticides have both been reported (WHO, 1976, 1978) and blunt a principal weapon used against the disease. Further, resistance of Plasmodium falciparum to chloroquine, hitherto the most reliable anti-malarial drug, has been reported in 20 countries of South East Asia and South America (WHO, 1974) and more recently in Africa (Kean, 1979; Stille, 1979). These imply that efforts to curb the advance of malaria based on existing anti-malarial measures would encounter serious difficulties, and alternative methods of malaria control would have to be sought. Vaccination against malaria has been an obvious alternative choice.

## CHAPTER 1

### IMMUNITY TO MALARIA AND VACCINATION AGAINST THE DISEASE:

#### A SHORT REVIEW

##### IMMUNITY TO MALARIA

The acquisition of immunity to malaria can be best appreciated from studies carried out in areas of stable malaria where the disease is endemic and transmission occurs perennially at a high level. The newborn upto the first few months of life develop only mild infections, this being largely due to passive protection by antibody acquired from their mothers. Thereafter, upto about 5 years of life children are extremely vulnerable to the disease, showing high parasitaemias and suffering the highest rates of morbidity and mortality. Then follows a gradual acquisition of anti-parasitic immunity directed against asexual blood stages of the parasite, which leads to an alleviation of clinical symptoms and lowering of parasite densities (see McGregor, 1965, 1972), this immunity is non-sterile. Adolescents and adults manifest very low numbers of circulating parasites despite continuous sporozoite challenge, which is clearly necessary for immunity to be maintained at a high level. In areas of unstable malaria where transmission is seasonal and erratic, whole populations rather than the young children alone are susceptible to the disease and epidemics may occur. Clearly then, acquiring immunity to malaria is a slow process.

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The role of humoral immunity in acquired resistance to malaria is now well established. Infection in man leads to a significant increase in the levels of serum immunoglobulins (Rowe et al., 1968) and their rates of synthesis (Cohen and Butcher, 1971). However, absorption experiments have shown that only a small part of the increased immunoglobulins is parasite specific (Cohen and Butcher, 1971) and probably only a fraction of this is protective. Nevertheless, passive transfer of adult immunoglobulins to young African children resulted in a striking reduction in the parasitaemia and a clinical cure (Cohen and McGregor, 1963). Indications from this study and experiments on rodent malaria (Diggs and Osler, 1975) were that antibody acted either on mature schizonts or on extracellular merozoites. In vitro studies using P.knowlesi in immune rhesus serum confirmed that they act primarily by blocking merozoite invasion of red cells (Cohen et al., 1969). These merozoite inhibitory antibodies are species specific, both IgG and IgM, and agglutinate extracellular merozoites in vitro; the reaction is complement independent.

Until recently, naturally acquired immunity to malaria was considered to be directed only against the asexual erythrocytic stages of the parasite, but there is evidence now to show that immunity against sporozoites also exists. Rats maintained on chloroquine to suppress the development of erythrocytic stages, and repeatedly

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inoculated with non-attenuated P. berghei sporozoites subsequently develop fewer exo-erythrocytic forms in the liver (Verhave, 1975; Beaudoin et al., 1975). More recently it was demonstrated that antibodies to sporozoites occur in the serum of more than 90% of the adults living in an area of hyperendemic malaria (Nadin et al., 1979). Anti-sporozoite antibodies are also produced in rodents immunised with attenuated sporozoites (Vanderberg et al., 1970) as well as after the injection of viable sporozoites (Spitalny and Nussenzweig, 1973), and have been detected by the circumsporozoite precipitation (CSP) reaction (Vanderberg et al., 1969) and sporozoite neutralising (SN) activity (Nussenzweig et al., 1977). Their correlation with protection has been frequent but not invariable (Spitalny and Nussenzweig, 1973).

Relatively less is known about naturally acquired immunity to sexual stages of the parasite. Reports on the occurrence, in semi-immune individuals, of antibodies that react with gametocytes have been very few (Voller and Bray, 1962; Smalley and Sinden, 1977; Carter et al., unpublished). These studies were carried out on human populations exposed to endemic P. falciparum malaria, and functional transmission blocking immunity was not found to occur to a significant extent even in adults, in the single study in which this was investigated (Carter et al., in press). However, fragmentary but convincing evidence to the contrary also exists (McCarthy et al., 1978;

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Sinden et al., 1978) and these are supported by studies on P.gallinaceum in chickens, where transmission blocking immunity was found to be elaborated in response to an infection (Carter et al., 1979b). The subject is reviewed and discussed at length in the following chapter.

There is much evidence now to suggest that acquired immunity to malaria depends on cell mediated immune responses as well as on humoral immunity, though the exact role of cells is not clear. Initially, the adoptive transfer of spleen cells from immune animals conferred a significantly greater degree of protection than passive transfer of immune serum (Philips, 1970), and subsequently, changes in both numbers and function of T-cells during murine malaria infections have been demonstrated (Jayawardena et al., 1975). Since then many investigations have established the involvement of T-cells in immunity to malaria (reviewed by Brown, 1976). There is evidence to suggest that T-cells may possess other functions in mediating immunity independent of their role in antibody formation. This has been convincingly shown by Roberts and Weidanz (1979) who reported that B cell deficient mice that had been immunised with a drug controlled infection of P.yoelii, were resistant to re-infection. Cellular changes, particularly in the macrophage-monocyte series, have been reported during murine malaria infections (Lelchuk et al., 1979). It has been suggested

that sensitised T-cells, (associated with delayed hypersensitivity), by elaborating soluble factors mediate these changes (Cottrell et al., 1978; Playfair et al., 1979).

The synergistic effect of cells and antibody is well established (see Cohen, 1979) and there is evidence that cell mediated effector mechanisms may act in the absence of antibody; yet a convincing demonstration of cell mediated cytotoxicity has not so far been made. Coleman et al., (1975) obtained indications of a cytotoxic effect in P.berghei malaria in mice, which they considered involved both lymphocytes and antibody, but this has not been confirmed.

Humoral anti-sporozoite immune responses as well as protection against sporozoite challenge have also been shown to be thymus dependent. Thymectomised, sporozoite immunised mice fail to become protected against challenge, their sera fail to develop sporozoite neutralising antibody, and even a minimal circumsporozoite precipitation reactivity can only be detected in the sera of some of these animals (Spitalny et al., 1977).

Resistance to malaria can also be induced by agents such as BCG that apparently stimulate immunity non-specifically (see Playfair, 1979). A soluble non-antibody factor secreted by macrophages has been postulated as having a non-specific cytotoxic action on parasites within red cells (Clark et al., 1975).

Genetically determined characteristics govern the susceptibility of certain hosts to species of Plasmodium. The mechanisms of this innate resistance have been reviewed recently by Miller and Carter (1976), and have not been included here. Because this account is only a broad outline of the present knowledge on immunity to malaria rather than a comprehensive review, reference needs to be made to the more detailed reviews of Brown (1976), Playfair (1978) and Cohen (1979).



## VACCINATION AGAINST MALARIA

Several stages in the life cycle of the parasite that could be vulnerable to host immunity have been recognised and exploited as targets for active immunisation, namely, sporozoites, asexual erythrocytic stages and more recently gametes.

### Sporozoite vaccination

It was initially demonstrated by Mulligan et al., (1941) that attenuated sporozoites induce an effective immune response to malarial infection. This mode of vaccination has since been extensively investigated by Nussenzweig and her co-workers, mainly in rodent malarias, and has led to limited vaccination trials in man (reviewed by Nussenzweig, 1977). A strong immunity is produced in mice by 3 or more intravenous inoculations of irradiated sporozoites (Nussenzweig et al., 1969a); protection is maintained for about 2 months and declines progressively thereafter. Mature sporozoites obtained from mosquito salivary glands were found to be more immunogenic than those obtained from oocysts in mosquito midguts which were ineffective (Vanderberg et al., 1972). Protection induced by sporozoite vaccination is stage specific (Nussenzweig et al., 1969b) but not species specific, protecting mice against heterologous as well as homologous challenge. As mentioned previously, immunised animals

develop CSP and SN antibodies which frequently but not always correlate with protection (Vanderberg et al., 1969; Spitalny and Nussenzweig, 1973).

Sporozoite vaccination against the simian malaria P.cynomolgi, has produced less encouraging results (Collins and Contacos, 1972). Attempts to vaccinate against humans have similarly given mixed results; following repeated exposure over several weeks to infected irradiated mosquitoes, some of the volunteers showed a strong immunity to homologous but not to heterologous challenge (Rieckmann et al., 1974; Clyde, 1975).

#### Vaccination with asexual blood stages

A variety of methods of immunisation has been used against the asexual blood stages of the parasite including repeated long-lasting infection with viable parasites, and injection of attenuated and killed parasites and parasite fractions.

That active infection immunises against most malarias has been shown experimentally. Infections controlled with chemotherapy have resulted in good immunity against P.falciparum infections in Aotus monkeys (Voller and Richards, 1970). Relatively avirulent strains of parasites have been used to immunise successfully against virulent mutants of the same species (Yoelli and Hargreaves, 1974). Repeated passage of parasites through tissue cultures (Weiss and Giusti, 1966) and withholding essential growth factors for the parasite (such as para-aminobenzoic acid),

from the host's diet (Jerusalem, 1966) have been used to attenuate the virulence of parasites; infection with such parasites protected mice against virulent challenge.

The use of live attenuated parasites in vaccination, in the form of irradiated parasitised erythrocytes has also been investigated. Against rodent malarias and blood stages of P.falciparum a considerable degree of protection has been obtained (Corradetti et al., 1966; Wellde et al., 1972), though results have been more variable in primate malarias (Corradetti, 1974).

Killed vaccines have been prepared from schizont-infected red cells, parasite fractions, lysed parasitised blood and merozoites. Schizonts of P.knowlesi or schizont infected red cells when given with Freund's complete adjuvant (FCA) has given protection, though immunity was largely variant specific (Targett and Fulton, 1965; Brown et al., 1970). Extracts of P.knowlesi when used with FCA resulted in some protection (Schenkel et al., 1973; Simpson et al., 1974) though it was accompanied by an unexplained anaemia. A very effective immunity against P.yoelii and to a lesser extent against P.berghei has been obtained by using formalin-fixed saponin-lysed parasitised red cells with killed Bordetella pertussis as an adjuvant (Playfair et al., 1977b).

Extracellular merozoites have given good protection when used as a vaccine (Reviewed by Cohen et al., 1977;

Cohen, 1979). Merozoites of P.knowlesi isolated from in vitro cultures (Mitchell et al., 1975) when given intramuscularly with FCA immunise rhesus monkeys effectively against a subsequent challenge which would have otherwise been lethal (Mitchell et al., 1974, 1975). Protection is species specific because the immunised animals remain susceptible to P.cynomolgi. Vaccinated monkeys develop patent blood infections on challenge, which are of low parasitaemias and of short duration, to be followed by a sterilising immunity that lasts upto about a year. When immunised animals were challenged with sporozoites, they developed patent blood infections suggesting that immunity may not affect the development of pre-erythrocytic stages (Richards et al., 1977). The use of adjuvants other than FCA has failed to produce effective immunity against P.knowlesi malaria (Cohen, 1979).

Vaccination of douroucouli monkeys with P.falciparum merozoites (Mitchell et al., 1977) and schizonts containing mature merozoites (Siddiqui et al., 1977) in FCA has produced effective immunity against blood stages of P.falciparum. Similar results have also been obtained by vaccinating douroucouli monkeys with mature schizonts of P.falciparum using a muramyl dipeptide derivative as an adjuvant which appears to produce less adverse reactions than FCA (Siddiqui et al., 1978).

Of these experimental vaccines, the sporozoite and merozoite vaccines appear to be most promising, and critical reviews of these are available (Richards, 1977; Miller, 1977; Mitchell, 1977). As is widely acknowledged, many obstacles have to be overcome before any of these vaccines can be developed for human use. Sporozoite vaccines have been evaluated in many experimental systems and even to a limited extent in man. On account of its stage specificity, vaccination must be absolutely effective because partial immunity would afford little or no protection to the host. Also, to date successful vaccination requires the use of viable sporozoites, and anti-sporozoite immunity is of short duration. In addition, mass production of sporozoites through in vitro cultures appears to be difficult. The recent achievement of the technique of producing large amounts of monospecific sporozoite antibody from hybrid cells would greatly facilitate the eventual characterisation of protective antigen (Yoshida et al., 1980).

Merozoite vaccines have been studied only in a single experimental system but they have the advantage of using killed parasites which permit storage. Immunity induced by merozoite vaccines also lasts longer than anti-sporozoite immunity. The continuous in vitro culture of erythrocytic parasites by Trager and Jensen

(1976) has improved considerably the chances of producing antigen on a large scale for merozoite and other asexual blood stage vaccines. However, a serious drawback in the development of a merozoite vaccine is the requirement of FCA which is unacceptable for human use.

Therefore it seems imperative for the development of an anti-malarial vaccine for human use that research should continue both to improve existing experimental vaccines as well as to investigate alternative methods of vaccination. One such attempt has been to induce immunity against sexual stages of the parasite to block transmission of the disease.

CHAPTER 2BIOLOGICAL ASPECTS OF TRANSMISSION

Male and female gametes of Plasmodium and their precursors the respective gametocytes, act as both mediators of sexual reproduction and vehicles of malaria transmission from man to mosquito, thus linking inextricably two processes vital for parasite propagation and survival. Yet, despite earlier work (Garnham, 1931, 1966; Field and Shute, 1956) biological aspects of gametocytes remain poorly understood. The paucity of information on the subject is partly attributable to difficulties encountered in studying the developmental process, for two main reasons. Firstly, in P.falciparum, where gametocytes are easily recognised from other blood stages, they develop in deep relatively inaccessible tissues. Secondly, where development is less cryptic, such as in murine malarias, experimental infections tend to be asynchronous making their identification difficult. However, recent times have witnessed a surge of new information germane to the subject. The achievement of successful continuous in vitro cultivation of asexual blood stages (Trager and Jensen, 1976) some of which differentiate into gametocytes in culture (Carter and Beach, 1977; Jensen, 1979) and the ability to grow gametocytes in short term cultures (Row, 1928; Mitchell

et al., 1976; Smalley, 1976; Philips et al., 1978) have greatly facilitated the study of gametocyte biology. Recent reports on successful experimental vaccination with sexual stages to block transmission (Gwadz, 1976; Carter and Chen, 1976; Mendis and Targett, 1979) might also have led to a revival of interests in the sexual phase of development. The account that follows is an assessment of knowledge presently available on gametocyte and gamete biology viewed from a purely functional standpoint of transmission.

#### Gametocytogenesis

That gametocytes can be formed directly from asexual erythrocytic stages is now supported by a multitude of evidence. After many years of blood passages strains of rodent malaria that were producing only asexual parasites, when subjected to low temperature treatment have been shown to reinitiate gametocyte production (Bafort, 1965; Vincke and Scheepers-Biva, 1965). By using cloning techniques Walliker et al. (1973) demonstrated that individual asexual parasites of rodent malaria can give rise to a complete infection leading to infective gametocytes, thus establishing that asexual parasites possess a complete genome that can lead to either sexual or asexual development. Finally, gametocytes of P.falciparum have been shown to arise continuously within the in vitro culture system of Trager and Jensen (1976) over several



consecutive cycles of development (Carter and Beach, 1977; Vanderberg et al., 1977; Jensen, 1979). Studies carried out on rodent malaria parasites have shown that merozoites arising directly from hepatic schizonts also, can, without an intervening erythrocytic cycle develop into gametocytes (Killick-kendrick and Warren, 1968; Landau et al., 1979). However, in view of their non-cyclic development, the potential of hepatic schizonts in producing gametocytes would be obviously limited.

The point at which an asexual parasite gets committed to develop into a gametocyte is not known though gametocyte differentiation has been morphologically traced as far back as ring stages. Smalley (1976) studying gametocytogenesis of P.falciparum in vitro showed conclusively that very young gametocytes as newly formed rings invaded the peripheral circulation along with young asexual rings that come from the same population of schizonts. Based on electron microscopic studies of P.berghei, Ladda (1969) and Kilby and Silverman (1971) concluded that the earliest recognisable distinctive ultrastructural features of gametocytes were found in early trophozoites and thereby assumed that differentiation occurred at this stage. However, that genetic commitment may occur at a very early stage is suggested by the results of Klimes et al., (1972)

on the sexual differentiation of Eimeria tenella in which merozoites or even preceding schizonts were already determined as to the sex of the gametocyte that will be produced upon reinvasion.

Early in vivo studies on P.falciparum (Thomson, 1914; Miller, 1958) seemed to suggest that gametocytes are not produced immediately the blood infection begins, rather that there is a definite point in the infection where gametocytogenesis starts for the first time. Smalley (1976) in his studies on gametocytogenesis in culture reaffirmed this and proposed that there was a trigger for the start of the process, and that once it has begun in a persisting asexual infection then the production of gametocytes becomes an integral part of the life cycle. Such a mechanism for the onset of gametocytogenesis has been contemplated by previous authors (Thomson, 1914; Miller, 1958) but the nature of this trigger still remains a complete mystery. Studying the course of natural infections of P.falciparum in adult humans, Miller (1958) found a positive correlation between increased parasitaemia and gametocytogenesis but presented evidence to suggest that the determining factor for gametocyte production was the onset of clinical malaria rather than a mere increase in the number of asexual parasites. Others (Thomson, 1914; Bishop, 1955) speculated further to implicate

host immunity as a factor mediating the switch to gametocyte production, but evidence that the process can now be spontaneously induced in an in vitro culture system indicates that factors other than host immunity are in operation. The time of onset of gametocytogenesis may also show a species variation; in P. yoelii there is evidence that gametocyte production commences simultaneously with the blood infection (Killick-Kendrick and Warren, 1968; Landau et al., 1979).

The ability to produce gametocytes is undoubtedly an intrinsic property of the parasite; it is known that strains of Plasmodium differ in their ability to produce gametocytes under similar conditions (Garnham, 1966). There is also little doubt that this inherent ability is subject to environmental influences. A strain of the parasite may produce gametocytes when inoculated into one host species but not in another (Thomson and Huff, 1944; McGhee, 1951) or produce more gametocytes when the host environment is modified (McLeod and Brown, 1976). A strong environmental influence is also suggested by the observation that the ability to produce gametocytes can be lost by continuous syringe passage of asexual parasites (Boyd, 1945; Vincke et al., 1943) and that this can be revived by various treatments such as subjection to low temperatures (Bafort, 1965) and chemotherapeutic agents (Peters, 1970). As has been stated by Peters (1970) genes responsible for directing gametocytogenesis would be repressed in

agametocytaemic strains, and the action of some inducers associated with cold treatment or chloroquine must bring about derepression of these genes. It has also been shown that in a strain which was gradually losing its ability to produce gametocytes, it is possible to restore this ability completely by transmitting the parasite through the vector (Wery, 1968). It would be possible to include cyclical transmission as a factor capable of causing derepression of such genes, possibly by reassortment of the genetic make-up brought about by the act of fertilisation. There is evidence that gametocytogenesis can be induced in in vitro cultures by the addition of cyclic AMP (R.Carter, unpublished). Gametocytogenesis would then seem to be a manifestation of a genetic determinant the expression of which is controlled by a mechanism which is receptive to a number of external stimuli; it is yet a poorly understood process.

In falciparum malaria, very young gametocytes recede into internal organs to complete their development and reappear in the peripheral circulation as morphologically mature gametocytes (Thomson, 1914). It has long been suspected that the process of development takes about 10 days (Thomson, 1914; Garnham, 1966) and this has been confirmed recently by in vitro studies (Smalley, 1976) though there have been a few reports to the contrary (Miller, 1958; Mitchell et al., 1976). Simian and

murine malariae differ from P.falciparum in that the time taken for their development appears to be much shorter, though accurate estimates of the period of development are lacking. Based on a study designed primarily to demonstrate asexual and sexual circadian rhythms, Hawking et al., (1972) proposed that the time required for development of a male gametocyte from merozoite to exflagellation in P.chabaudi was presumably 44 hours and in P.yoelii 45 hours but admitted that in both cases it could have been 24 hours less. In a similar study on simian and avian malaria parasites (Hawking et al., 1968) it was conjectured that the period of development was a few hours more than a single asexual cycle. In one rodent malaria parasite P.berghei yoelii (P.yoelii yoelii) it has been shown that the period of development from an exo-erythrocytic merozoite to a functionally mature gametocyte is about 22 hours (Killick-Kendrick, 1968).

Early morphological studies on differentiation of P.falciparum gametocytes were based on spleen or bone marrow aspirations, autopsy material and on cases where young gametocytes were found in peripheral blood (Thomson and Robertson, 1935; Field and Shute, 1956). Descriptive classifications based on light microscopic studies have been proposed for avian and simian Plasmodia (Hawking et al., 1968) and several studies on the fine structure of

mature gametocytes have been published (Smith et al., 1969; Kass et al., 1971; Miller, 1972; Sinden et al., 1978) and reviewed by Sinden (1977), but the transition of a newly invaded merozoite into a mature male or female gametocyte still awaits ultrastructural scrutiny. In gametocytes of P.falciparum there is known to be an interval of 2 to 4 days between the development of morphological maturity and the attainment of functional maturity (Jeffery and Eyles, 1955) but whether the same applies to other Plasmodia is not known.

#### Gametogenesis

In order that their purpose be realised functionally mature gametocytes need to be taken up by an appropriate mosquito vector, and in the lumen of its midgut male and female gametocytes transform almost immediately into respective gametes. Gametogenesis in the male, a process which appears to be more dramatic than in the female has been recognised for as long as the malaria parasite itself has been (Laveran, 1880) and many aspects of the process have been studied (reviewed by Sinden, 1977). Based on studies on P.yoelii nigeriensis (Sinden and Croll, 1975) and P.falciparum (Sinden et al., 1978) with the aid of light and electron microscopy and videotape analysis, the sequence of events and morphological changes that take place during gamete formation have been described in detail. Microgametogenesis commences with a phase of 'maturation' (subsequently

termed activation) whereby the male gametocyte may emerge from the host erythrocyte. This is followed by exflagellation during which phase microgametes emerge from the parent gametocyte, and escape from it during the final phase of microgametogenesis. The gametes then disperse. The ultrastructural events of gametogenesis have also been studied in P. berghei (Garnham et al., 1967). Cytological changes include the formation of kinetosome-axoneme complexes with inclusion of the developed axonemes within the flagellum of the microgametes. Concurrently the microgametocyte undergoes 3 mitotic divisions with the nuclear material segregating into 8 distinctive microgamete nuclei.

Macrogametogenesis is a relatively inert process; apart from the fact that emergence from the host erythrocyte occurs it was observed that the female gametocyte undergoes nuclear changes in which a single pole of an intranuclear spindle was detected in P. falciparum (Sinden et al., 1973).

The physiology of microgametogenesis has also been extensively studied. The mechanism whereby functionally mature gametocytes remain quiescent in the circulation of the vertebrate host and get activated soon after they reach the mosquito midgut, or when a drop of gametocyte carrying blood is exposed to air, captured the attention of early malariologists. Ross in 1897 disputed the then popular belief that a drop in

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temperature caused as a result of leaving the vertebrate host was the prime factor in inducing gametogenesis. Evidence available at present indicates that temperature can be a limiting factor in the induction of gametogenesis in that above 30°C gametogenesis does not occur (Sinden and Croll, 1975). Machoux and Chorine (1932) working on Haemoproteus and Chorine (1933) with P. falci-  
parum were the first to demonstrate that exflagellation was inhibited in the vertebrate host by normal levels of CO<sub>2</sub> in the blood, and they suggested that exflagellation was induced by the escape of CO<sub>2</sub> from the blood and the associated rise in pH. Bishop and McConnachie (1956, 1960) confirmed that the loss of CO<sub>2</sub> from the blood stimulated exflagellation but disagreed on the role of the pH. They also showed that exflagellation does not occur in the absence of Na<sup>+</sup>, Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> ions. The most recent work on the subject (Carter and Nijhout, 1977; Nijhout and Carter, 1978) has shown conclusively that in P. gallinaceum emergence and exflagellation of gametocytes in vitro occur independently of the CO<sub>2</sub> tension but are rigidly correlated with the pH of the external medium. The authors proposed that what stimulates gametogenesis on exposure to air was a decrease in the CO<sub>2</sub> tension causing the pH to rise, CO<sub>2</sub> acting indirectly. They found that the pH range to which gametogenesis was sensitive was 7.7-8.0, which also

corresponded to that found in the midguts of Aedes aegypti mosquitoes after a blood meal (Bishop and McConnachie, 1956). Carter and Nijhout (1977) confirmed the necessity for  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{HCO}_3^-$  ions and also found that glucose deprivation impedes exflagellation irreversibly. With this information they were able to prepare a 'suspended animation' solution consisting of a tris-buffered NaCl solution with added glucose at a pH of 7.4 in which gametogenesis was reversibly suppressed, thus enabling greater manipulation of functionally mature gametocytes in vitro. It seems clear that gametogenesis can be induced by simple environmental stimuli but the possibility cannot be excluded that mosquitoes possess vector specific synergistic or inhibitory substances capable of controlling gametogenesis in vivo independently of the factors elucidated in vitro (Micks et al., 1948).

Following microgametogenesis the microgametes disperse and some fertilise a macrogamete. The cytology and kinetics of fertilisation has been described (Sinden and Croll, 1975).

#### Infectiousness to the vector

It has long been considered that infectiousness of malaria to susceptible mosquitoes is determined by the interaction of many factors, pertaining to both the

host and the parasite. Those that were considered to be important at the time were cited by Boyd (1949), subsequently reviewed and updated by Vanderberg (1977) and discussed in terms of their work by Smalley and Sinden (1977). These factors include gametocyte density, their maturity, longevity and sex ratio and finally any immune response made by the host that would affect gametocytes or their infectivity.

The numerical density of gametocytes in the circulation of the vertebrate host generally appear to bear a positive relationship to the percentage of susceptible mosquitoes infected as well as the number of oocysts produced (Shah et al., 1934; Knowles and Basu, 1943; Eyles, 1951; Draper, 1953) though exceptions to this are also numerous. On investigating the minimum gametocyte count in the blood or the threshold for infecting mosquitoes, variable and some remarkably low values have been found. Some workers found that gametocyte counts of approximately  $50-100/\text{mm}^3$  are required (James, 1931; Boyd and Kitchen, 1937; Collins, 1962) but counts below  $10/\text{mm}^3$  or even gametocyte densities too low to be detected by techniques used are known to have produced infections in mosquitoes (Young et al., 1948; Muirhead-Thomson and Mercier, 1952; Jeffery and Eyles, 1955). Equally common and more significant is the fact that high and presumably adequate gametocyte

densities do not invariably guarantee mosquito infections (James, 1931; Muirhead-Thomson, 1954; Jeffery and Eyles, 1955). Clearly then, not all gametocytes are infective.

Maturity of gametocytes, first cited by Boyd (1949) as a factor determining infectiousness, refers to functional maturity, a concept endorsed by the finding that P.falciparum gametocytes only become infective to mosquitoes 2 to 4 days after they attain morphological maturity (Jeffery and Eyles, 1955). At any one time during a wave of gametocytaemia, before its decline, a proportion of the gametocytes will be functionally immature. Hawking and his co-workers (1968) provided evidence in support of a hypothesis that there exists a circadian rhythm in the maturation of gametocytes of malaria parasites. They contended that the gametocytes of murine and primate malaria parasites (Hawking et al., 1968, 1972) are mature and infective to mosquitoes for only 6 to 8 hours and proposed that the 24, 48 and 72 hour cycles in the development of asexual erythrocytic stages was a purposeful adaptation to produce short-lived mature gametocytes coinciding with the time of maximum mosquito biting activity. Hawking et al., (1971) extended this theory to P.falciparum though subsequently Bray and his co-workers (1976) found no evidence for a circadian rhythm in the infectivity of P.falciparum

Garnham and Powers (1974) in a study of P.cynomolgi found that a circadian rhythm seemed to occur in some but not all cases. Parallel studies in other malarias have not been undertaken. In view of strong evidence to the contrary, the theory propounded by Hawking et al. needs further investigation before it can be accepted.

In a very recent study of P.yoelii in mice, Landau et al. (1979) recognised 4 different morphological types of gametocytes during the course of their development, of which only the first 2 types were infective to mosquitoes. Based on the time of appearance of these gametocyte stages they concluded that gametocytes had a periodicity of about 2 days, but that they were likely to be infective only for about 12 hours.

Smalley and Sinden (1977) showed that the life-span of P.falciparum gametocytes is about 20 days, and contrary to predictions of Hawking et al. (1971) that microgametocyte viability is preserved throughout, with the same gametocyte population infective to mosquitoes for at least 11 to 12 days.

A disturbance of the optimum sex ratio may conceivably affect infectiousness. In P.falciparum, female gametocytes are known to outnumber males by values which range from 5 and 20 to 1 (Shute and Maryon, 1951) to 4 to 1 (Smalley and Sinden, 1977) though a preponderance of males have also been observed (Hawking et al., 1971). A physiological disturbance in the sex

ratio has been reported at the beginning of gametocytogenesis in P.falciparum where female gametocytes appeared in the peripheral blood before males and during the decline of a gametocytaemia when males were removed faster (Shute and Maryon, 1951). However, Smalley and Sinden (1977) found that the sex ratio remained constant during the post-peak decline of a gametocyte wave.

The view that during the course of a malaria infection, its infectivity to mosquitoes declines is widely held and supported (Jeffery and Eyles, 1955). Host immunity has often been implicated in this phenomenon though few direct studies exist to substantiate it. In individuals exposed to endemic malaria, gametocyte densities in the peripheral blood declines with age. Early workers (Thomson, 1935; Christophers, 1924; Wilson, 1936) found that in regions where P.falciparum was endemic, children showed a much higher gametocyte count than adults whereas in epidemic areas counts were high in both adults and children. These data were earlier interpreted as evidence that with development of immunity P.falciparum gradually loses its ability to produce gametocytes (Garnham, 1931; Hackett, 1941; Bishop, 1955). This view was dispelled with recognition of the fact that the decline in gametocyte numbers was comparable to that of the asexual stages and merely resulted from it. Viewed in the light of recent evidence

that gametocyte production occurs in in vitro cultures in the absence of host immunity, it paved the way for the currently held view which denies host immunity any direct role in effecting a lowered rate of gametocyte production.

There is also ample evidence for the ability of gametocytes to evade destruction by the hosts immune system, possibly by virtue of residing within red cells. P.falciparum gametocytes are known to survive in the peripheral circulation for upto 2 to 3 weeks (Smalley and Sinden, 1977) even at a time when asexual stages are being vigorously destroyed by the hosts immune reaction (Jeffery and Eyles, 1955). Further, Cohen et al., (1961) and Edozien et al., (1962) demonstrated that immunoglobulin taken from hyperimmune individuals exposed to endemic malaria, when inoculated into heavily infected children rapidly cleared asexual parasites from the circulation but failed to affect gametocyte densities. Such evidence also exists for avian, rodent and simian malarias. In these species, when the infectivity of gametocytes was suppressed by artificial immunisation, gametocytes in the circulation remained completely unaffected as shown by the fact that when washed and resuspended in non-immune serum they exflagellated normally and those that were fed to mosquitoes proved to be infectious (Gwadz, 1976; Carter and Chen, 1976; Mendis and Targett, unpublished; Gwadz and Green, 1979).

Though host immunity appears not to affect gametocyte production or longevity, it is possible to manipulate the hosts immune system to block infectiousness of gametocytes by artificial immunisation, in avian, rodent and simian malarias (Gwadz, 1976; Carter and Chen, 1976; Mendis and Targett, 1979; Gwadz and Green, 1979). In these cases humoral immunity has been shown to be directed against extracellular gametes, acting on them in the gut of the vector. It thus remains to be seen whether anti-gamete immunity of this nature is elaborated by the host during the course of a natural infection.

If there is a decline in infectiousness of a P.falciparum infection in the face of an increasing gametocyte count it would strongly support the contention that immunity can affect infectiousness, but such striking evidence has not been found. Instead, there is evidence that particularly during the latter part of an infection, presumably adequate levels of gametocytes fail to infect mosquitoes or often produce infection intensities below that expected from such gametocyte counts. One such study (Jeffery and Eyles, 1955) relates the infectivity to mosquitoes to the gametocyte density and duration of infection. It showed that there was a decline of infectivity with time, in that the proportion of lots of mosquitoes and the percentage of mosquitoes infected during the initial continuous parasitaemia was very much higher than during the terminal intermittent



period. This, not unexpectedly, was accompanied by a decline in gametocyte densities but they found that infectivity during the terminal period was actually biased to a higher figure because feedings were done only when gametocytes were present. In many of these instances detectable and presumably adequate gametocyte densities produced low infections or none at all implying that a decline of infectivity was not attributable solely to lowered gametocyte numbers.

More supporting evidence comes from a recent study (McCarthy et al., 1978) in which a semi-immune volunteer was exposed to homologous and heterologous challenge with strains of P. falciparum. On both occasions blood infections developed and gametocytes were produced in numbers ranging from 160 to 480/mm<sup>3</sup> that were well above the threshold for mosquito infections. Yet they failed to infect mosquitoes whereas in non-immune volunteers the same strain produced mosquito infections even at lower gametocyte densities. The authors exclude the possibility that this was due to a functional immaturity of the gametocytes because mosquito feedings were done on the 4th to the 7th day of gametocytaemia. In view of the fact that during the volunteer's primary experience with one of the challenge strains the infection was highly infective to mosquitoes, they reinforce the contention that transmission blocking immunity may be acquired during the course of a natural infection.

The observation by Sinden et al., (1978), reliably based on videotape recordings, that the behaviour of male gametes during exflagellation in vitro of microgametocytes obtained from some infected adults in the Gambia was akin to that seen in experimental animals immunised with gamete vaccines (Gwadz, 1976), viz., of there being an arrest of movement just before or soon after their escape from the parent gametocyte, is also very suggestive of the occurrence of anti-gamete immunity in populations exposed to hyperendemic malaria. Further, it has been demonstrated that chickens, after the peak of asexual infection with P.gallinaceum, elaborate anti-gamete antibodies related to transmission blocking immunity (Carter et al., 1979b). Such is the evidence supporting the contention that a natural infection or repeated infections of malaria may induce transmission blocking immunity in the host.

If this was of wide occurrence and the ensuing immunity was significant, it would be expected that in areas of stable endemic malaria, the main reservoir of infection would be young children and immigrants; which is in fact a popular and long held belief. The only study that was designed to investigate this on the basis of demographic information and transmission studies carried out in Liberia (Muirhead-Thomson, 1957) provides evidence that the population above 15 years of age

represented about a quarter of the total human reservoir of infection. Though it shows that with the acquisition of immunity reduction in transmission can be achieved, it casts doubts as to whether this could greatly affect the dynamics of transmission. Besides, this reduction in transmission could have merely been a consequence of lowered gametocyte densities in which case host immunity can only be credited for its indirect role, and not due to the elaboration of anti-gamete antibodies. This in fact was the conclusion drawn by Carter et al., (in press) from a very recent study in which they set out to determine the prevalence, nature and significance in transmission, of antibodies against sexual stages of P.falciparum in a human population exposed to endemic malaria. Previous studies have, using fluorescent antibody (FA) techniques, demonstrated the presence of antibodies that react with gametocytes of P.falciparum in sera of semi-immune individuals (Voller and Bray, 1962; Smalley and Sinden, 1977). These antibodies have neither been consistently detected in such individuals nor been correlated with functional transmission blocking immunity.

in their study, Carter et al., (in press) surveyed more than 100 sera using FA techniques against air-dried parasites. They found high antibody titres against

asexual parasites which increased with the age of the population but very low or absent titres against sexual parasites. Titres against asexual and sexual parasites differed widely in individual sera, but titres against gametocytes and gametes were closely related. All sera, mainly of adults over the age of 20 years, (when potentially infective gametocytes were washed and resuspended in them and fed to mosquitoes) led to a moderate reduction in mean oocyst densities, but no correlation was found between FA titres and the degree of suppression of infectivity. The absolute oocyst counts in mosquitoes fed on both control and test sera were very low and caution needs to be exercised in their interpretation, but the authors also reported that none of the sera tested were observed to have an effect on the motility of viable gametes, in complete contrast to the results of Sinden et al., (1978), implying that anti-gamete immunity was hardly detectable in the sera tested.

Though the evidence available is very limited and conflicting, it seems almost certain that in areas of stable endemic malaria adults show a lowered transmission rate due largely to reduced gametocyte densities, yet there is also a possibility that some degree of anti-gamete immunity is acquired during the course of repeated infections. However, it is not clear whether such immunity is acquired to an extent or degree that would affect the dynamics of transmission in a population.

Evidence for a cellular mechanism of immunity against sexual stages also exists. Sinden and Smalley (1976) demonstrated phagocytosis by blood monocytes of sexual stages of P.falciparum in vitro, and to a much lesser extent in vivo in the blood meal within the mosquito gut. They concluded that conditions in the mosquito gut are not conducive to the phagocytic activity of leucocytes, and suggested that this is unlikely to prejudice the successful transmission of the parasite in heavy infections.

The strategy of any parasite oriented control measure aimed at reducing the transmission of malaria could profitably be based on a clear understanding of the biological basis of transmission.

CHAPTER 3IMMUNISATION AGAINST SEXUAL STAGESINTRODUCTION

Commencement of the sexual phase of a Plasmodium life cycle is marked by the development of gametocytes which reside within circulating erythrocytes of the vertebrate host. When ingested by an appropriate mosquito with its blood meal, functionally mature male and female gametocytes emerge from their host erythrocytes in the lumen of its midgut to develop into extracellular gametes. Motile spermlike male gametes and non-motile female gametes undergo fertilisation to produce ookinetes (zygotes), and their development in the mosquito results in the formation of oocysts and then sporozoites which are ultimately capable of infecting a vertebrate host. Transmission of the parasite from a vertebrate to an invertebrate host is therefore achieved through its sexual stages. An account of the biology of these stages is presented in chapter 2.

A course of action that would hamper the sexual development of the parasite should have the obvious effect of curtailing its transmission from vertebrate host to vector, and could be used to restrict spread of the disease. Manipulation of the hosts immune system to

achieve such an end forms the basis of gamete vaccination.

That host factors can interfere with the capacity of gametocytes to produce infections in mosquitoes was first noted by Byles (1952). He found that P.gallinaceum gametocytes past their peak infectivity could have a significant degree of their infectivity restored by washing and resuspending in serum from healthy uninfected chickens. Huff et al. (1958) followed up this observation by immunising birds with formalinised parasites of P.fallax and P.gallinaceum and challenging them with a homologous infection. It was found that though gametocytaemias attained during the course of a challenge infection were comparable between vaccinated and control birds, gametocytes from vaccinated birds were less infective to mosquitoes.

More recently, the work of Gwadz (1976), who for the first time demonstrated the immunological nature of the underlying mechanism, revived interest in this approach to vaccination and emphasised its potential as a method for the control of human malaria. By vaccinating chickens with repeated intravenous doses of formalinised or x-irradiated erythrocytes infected with P.gallinaceum, he was able to reduce transmission of a subsequent challenge infection to mosquitoes by 95 to 98%. Carter and Chen (1976) improved on the effectiveness of

vaccination by using partially purified x-irradiated male and female gametes. It was found that both vaccines had little effect on the course of asexual parasitaemia, and that gametocyte production continued unabated during the course of a challenge infection, be it blood or sporozoite induced (Gwadz, 1976; Carter and Chen, 1976; Carter *et al.*, 1979a).

An understanding of the nature of transmission blocking immunity was based on the observation that gametocytes in blood drawn from immunised birds exflagellated normally *in vitro* but that microgametes thus formed were immobilised soon after, in contrast to the sustained mobility of male gametes seen in blood from non-immune birds (Gwadz, 1976). It was suggested that transmission blocking immunity was mediated by the direct interaction of serum factors with malarial gametes in the mosquito midgut. Such an interaction could prevent fertilisation from occurring and thus sterilise the infection in the mosquito. Gamete immobilising properties that reside in the serum have been found to be associated with antibody, largely confined to the IgG fraction, and the gamete immobilising reactions are complement independent (Gwadz, 1976). Carter *et al.*, (1979b) recognised two distinct serum mediated anti-gamete reactions *in vitro*, surface fixation (SF) which was closely correlated with transmission blocking



immunity in vivo, and gamete agglutination which was found to occur even in the absence of transmission blocking immunity. Both reactions were found to be associated with the immunoglobulin fraction of the serum.

In vitro studies have indicated that the action of immune serum is restricted to the period between gamete formation and fertilisation, and that it does not affect the development of the zygote if fertilisation is allowed to take place (Carter et al., 1979b).

More recently, experimental vaccination with gametes has been extended to P.knowlesi infections in rhesus monkeys (Gwadz and Green, 1978). By immunising monkeys with an antigen mixture of gametes and asexual parasites they were able to suppress the asexual parasitaemia of a challenge infection in addition to blocking its transmission, but the vaccine was fully effective only when combined with Freund's complete adjuvant (FCA).

Experimental gamete vaccination in an avian malaria model has been successful, providing encouraging results and valuable information on the nature of transmission blocking immunity. However, on account of the many basic differences that exist between avian and mammalian Plasmodia and immunological discrepancies in host systems, the value of these results is considerably limited in extrapolating them to human malarial. In an

attempt to extend gamete vaccination to a more appropriate mammalian experimental system (an almost essential pre-requisite before evaluating its potential as a human malaria vaccine), the simian model has proved to be not wholly successful owing to its need for FCA.

The value of rodent malarias in experimental immunological studies is widely acknowledged. The possibility of considerable immunological manipulation of the rodent hosts and the vast amount of available information on various immunocompetent cells and their interactions makes the rodent malarias particularly suitable for research on immunisation. Therefore, this study was undertaken with a view to extending gamete vaccination to a rodent malaria model, investigating the possibility of immunising mice against the transmission of P.yoelii to Anopheles stephensi mosquitoes.

## MATERIALS AND METHODS

### Experimental animals

7 to 9 week old inbred female BALB/c mice, each weighing 18 to 20 grammes were used in most immunisation experiments. 8 to 12 week old outbred female Theiler's Original (TO) mice were used in all pilot experiments, for cyclical transmission of the parasite, for a few preliminary immunisation studies and for raising blood stages of the parasite for preparation of vaccines. Mice were fed on a standard commercial diet.

Guinea pigs were used to provide blood meals for mosquitoes in maintaining colonies. All animals were obtained from commercial breeders and housed in the Animal Unit of the London School of Hygiene and Tropical Medicine.

### Rearing the vector

Anopheles stephensi served as the vector and the strain was obtained from the Ross Institute, London School of Hygiene and Tropical Medicine by courtesy of Dr. C. Curtis. The technique for rearing mosquitoes was adapted from many descriptions of methods used by other authors (Shute and Maryon, 1966; Gerberg et al., 1968; Thomson and Bell, 1968; Gerberg, 1970) and the method

used in the Ross Institute Insectories.

Different optimal temperatures required for rearing mosquitoes and for development of the parasite in the vector entailed the use of two temperature controlled rooms. Aquatic stages of the mosquito were reared at a temperature of 27-28°C and artificial lighting was employed to create a daily cycle of 12 hours of light during the day alternating with 12 hours of darkness. The adult mosquitoes used for both parasite development and maintenance of colonies were kept at 24-25°C with a relative humidity of 80-85%. In this room the light cycle was reversed so that darkness prevailed during the day; mosquitoes were fed at daytime and they being night feeders the reversed light cycle simulated natural feeding hours. This was found to facilitate an avid and consistent feeding behaviour in mosquitoes. Adult mosquitoes were housed in wire framed net cages and fed on a 5-10% glucose solution. Females were given 2 blood meals a week, provided by guinea pigs which were anaesthetised with Nembutal (Abbott Laboratories, Ltd.) and placed on the roof of the mosquito cage. Eggs were laid 2-3 days after a blood meal in small bowls of water lined with filter paper in order to prevent drying and desiccation of eggs that stick to the sides of the bowl. Just before the next blood meal the egg bowls were removed to the room where aquatic stages were reared and soon after the eggs hatched larvae were transferred into rearing bowls

These consisted of large plastic bowls 18 inches in diameter, containing tap water to a depth of 2 inches with some fresh grass and soil. Larvae were fed throughout on finely powdered cereal baby food (Robinsons) and the bowls were scanned daily for pupae. Using a suction pump pupae were transferred into small enamel bowls which were then placed within the adult cages. The bowls were each fitted with a cone shaped lid with an apical opening which enabled emerging adults to escape freely into the cage while ensuring that females in the cage had no access to the bowl for egg laying. The entire cycle of development from egg to adult took 14 to 18 days. When adult mosquitoes were required for experiments, bowls of pupae were transferred into appropriate cages thus ensuring minimal handling of adults. Removal of mosquitoes from cages when required was carried out with a battery operated suction apparatus.

#### History of the parasite

The parasite, Plasmodium voelii nigeriensis (strain N 67) was isolated in 1967 from a thicket rat Thamnomys rutilans in Nigeria (Killick-Kendrick et al., 1968). Early references to the parasite as Plasmodium berghei nigeriensis were based on previous classification and its present name was adopted when sub-species voelii acquired specific status at a taxonomic revision in 1974 (Killick-Kendrick, 1974). A detailed history of the parasite since its isolation to 1960 (Killick-Kendrick, 1970) and then onwards (see appendix I) indicates that

it has been maintained by cryopreservation and syringe passage in animals, regularly interrupted by cyclical transmission through the vector.

#### Maintainance of the parasite

The parasite was cryopreserved soon after cyclical transmission through mosquitoes and was maintained as stabilates (reference LUMP 1316, 1357 and 1382).

The technique for cryopreservation was based on a method described for the cryopreservation of trypanosomes (Lumsden et al., 1973). Mice with a parasitaemia of 15-20% were bled by cardiac puncture and the measured volume of heparinised blood was mixed with an appropriate quantity of glycerol to make a final concentration of 7.5%; the procedure was carried out in an ice bath. The suspension was transferred into capillary tubes which were heat sealed at both ends and cooled overnight at  $-70^{\circ}\text{C}$  in a deep freeze before being stored in liquid nitrogen.

#### Cyclical transmission of the parasite

Parasites were cyclically transmitted through the vector prior to stabilisation. The procedure which was found to be optimal for this experimental model was based on descriptions of the life cycle of the parasite (Killick-Kendrick, 1970, 1973) and related species (Wery, 1963) and previous work done on cyclical transmission of rodent malaria parasites (Killick-Kendrick, 1971).

Mice were infected with an intravenous inoculum of parasitised erythrocytes. 4-5 day old female mosquitoes were fed on the mice on day 2 or 3 of an infection, during which period infectivity was found to be highest. In order to facilitate feeding, mice were anaesthetised with Hypnorm (Jenssen Pharmaceutica, Beerse, Belgium) intraperitoneally and placed on the net roof of the mosquito cage. Fed mosquitoes were maintained on a 5-10% glucose solution at a relative humidity of 80-85% and a temperature of 24-25°C. 10-11 days later gland infections were confirmed by dissection of a sample and the mosquitoes were allowed to feed on uninfected mice, thus transmitting the infection.

#### Parasites as a source of antigen for vaccines

P. voelii preferentially invades reticulocytes (Killick-Kendrick, 1973) and phenylhydrazine is known to act as a haemolysis-inducing drug which indirectly increases reticulocytosis (Viens et al., 1971, Playfair et al., 1977a).

Phenylhydrazinium chloride (BDH Chemicals, Ltd.) was dissolved in distilled water immediately before use and injected subcutaneously into mice, 0.18 mg/gm body weight in 3 divided doses on alternate days. The day after the final dose mice were infected with  $2-3 \times 10^7$  parasitised red cells injected intraperitoneally. On

the third day of the infection parasitaemias of 70-80% with gametocytaemias of 0.5-1.5% were reached in all the mice.

Parasitological examination of blood slides: assessment of parasitaemia

Thin blood films were prepared from tail blood, air dried and fixed in methanol. They were stained with a solution of Giemsa (Hopkin and Williams, Ltd.) at a dilution of 1 in 10 in buffered distilled water (pH 7.2) for 30 minutes and washed momentarily in tap water before drying.

Stained blood films were examined at a magnification of x1000 in a Wild M20 microscope. 100 parasites or the number of parasites in 20 microscopic fields were counted which ever came first. In assessing gametocytaemias, 200 fields or 30 gametocytes (which ever came first) were counted. Both were expressed as a percentage of the total erythrocytes.

Preparation of inocula

Frozen stabilates were thawed (37°C water bath) and diluted in phosphate-buffered-saline (PBS, see appendix II) immediately prior to intraperitoneal injection into mice. A donor mouse which was thus infected from a stabilate, during the first 3 days of its patent infection, was taken as the source of parasites throughout



the course of this study. The red cell count of the donor mouse was ascertained using a haemocytometer and based on this and the parasitaemia of the donor mouse at the time, the volume of the inoculum was calculated. An appropriate quantity of blood was diluted in PBS prior to inoculation.

#### Preparation of the gamete vaccine

High parasitaemias were induced in mice as described earlier (plate 1) and the mice were bled by intracardiac puncture into 50 volumes of suspended animation (SA) solution (see appendix II) in order to wash the red cells free of any serum factors which might interfere with gametogenesis. The suspension was centrifuged at 500g for 10 minutes, the supernatant discarded and the red cells pipetted into a gamete releasing medium (see appendix II) in a volume ratio of 1 to 3. The pH of the suspension was readjusted to 8.0 with 1 molar NaOH and kept at room temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ). Gametogenesis was observed at regular intervals in a drop of the suspension under a phase contrast microscope (plate 2). In about 30 minutes when almost all the gametes were free, the suspension was centrifuged at 500g for 10 minutes. The supernatant contained male and female gametes and some 'free' asexual parasites but a large proportion of gametes were entrapped in the red cell column. In order to recover as many gametes as possible the supernatant

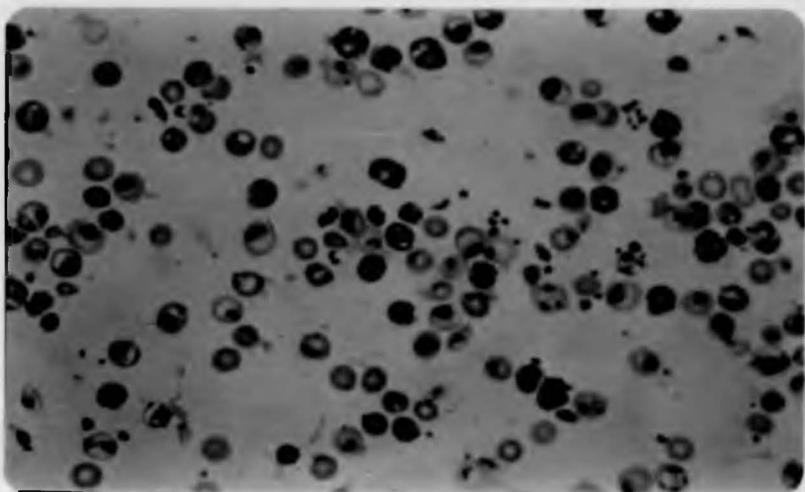


PLATE 1. Giemsa stained film of heavily infected mouse blood used for preparing vaccines. Note high proportion of gametocytes (magnification x 500).

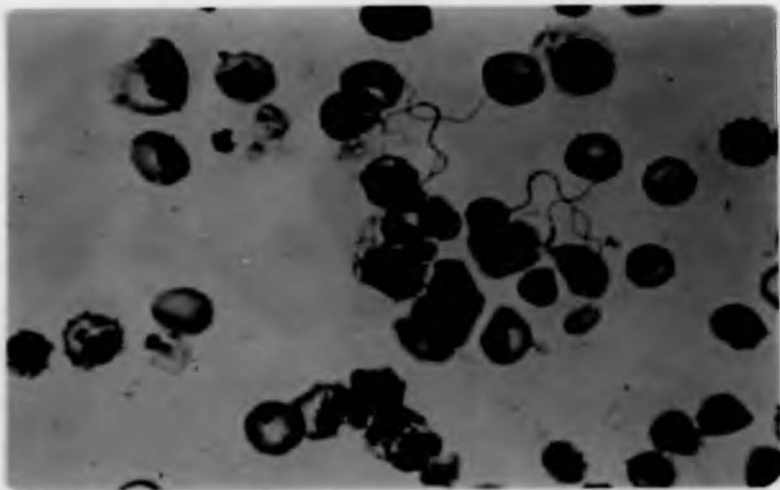


PLATE 2. Giemsa stained film of infected blood in which gametogenesis was induced in vitro. Note thread-like male gametes (magnification x 1000).

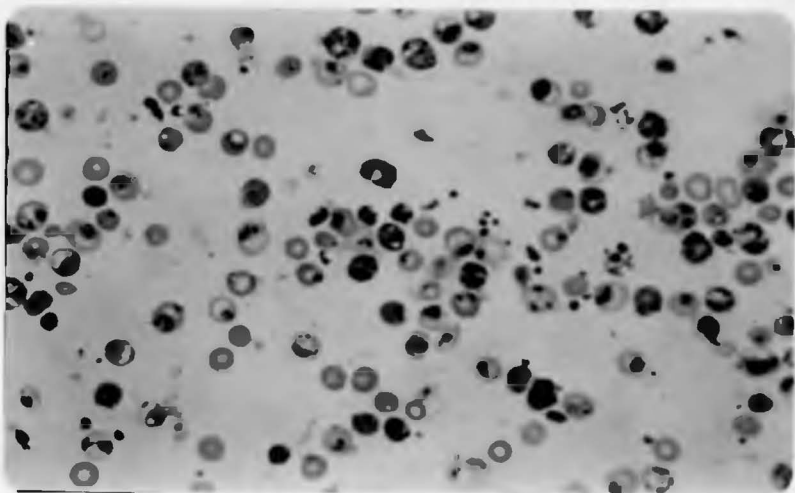


PLATE 1. Giemsa stained film of heavily infected mouse blood used for preparing vaccines. Note high proportion of gametocytes (magnification x 500).

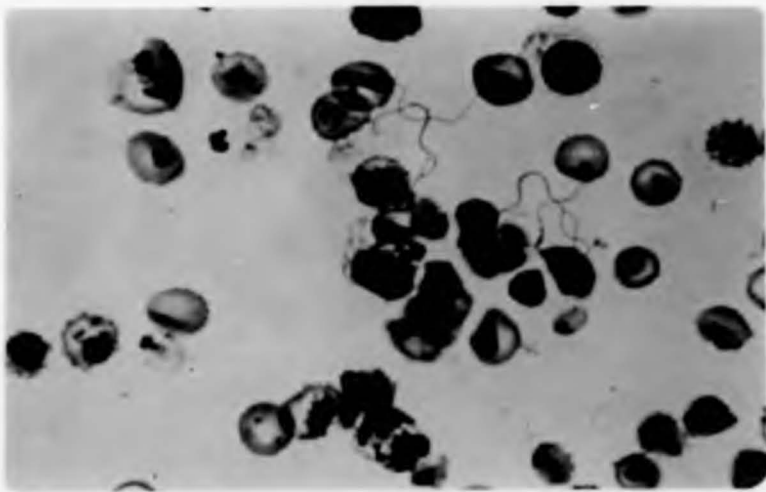


PLATE 2. Giemsa stained film of infected blood in which gametogenesis was induced in vitro. Note thread-like male gametes (magnification x 1000).

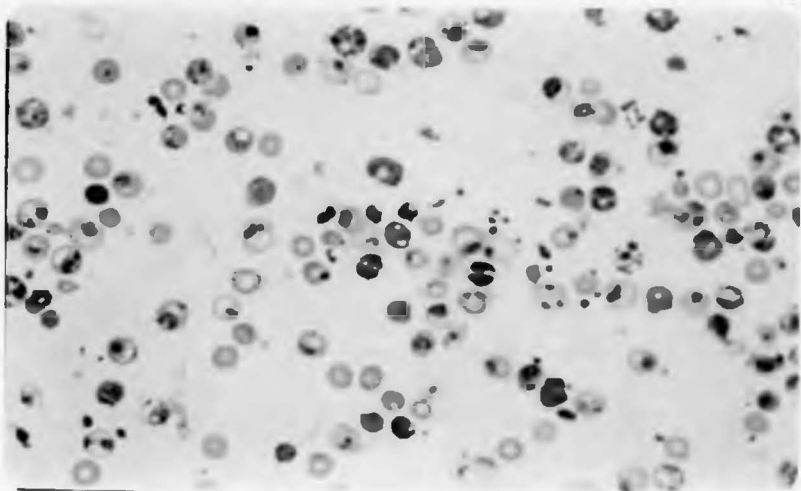


PLATE 1. Giemsa stained film of heavily infected mouse blood used for preparing vaccines. Note high proportion of gametocytes (magnification x 500).

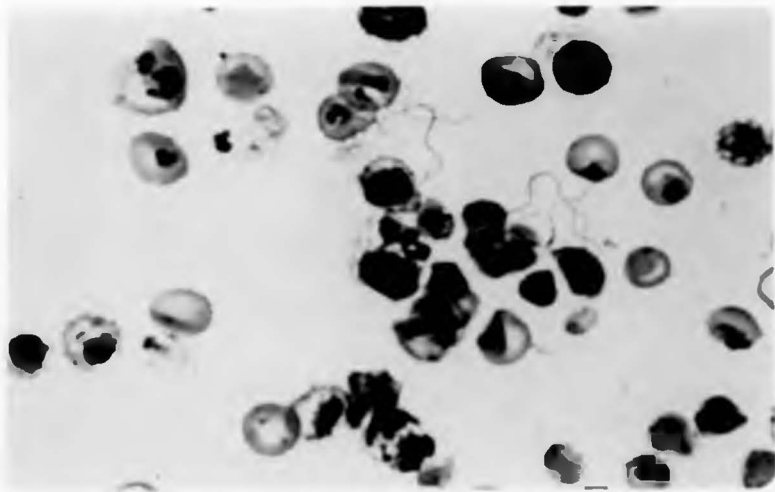


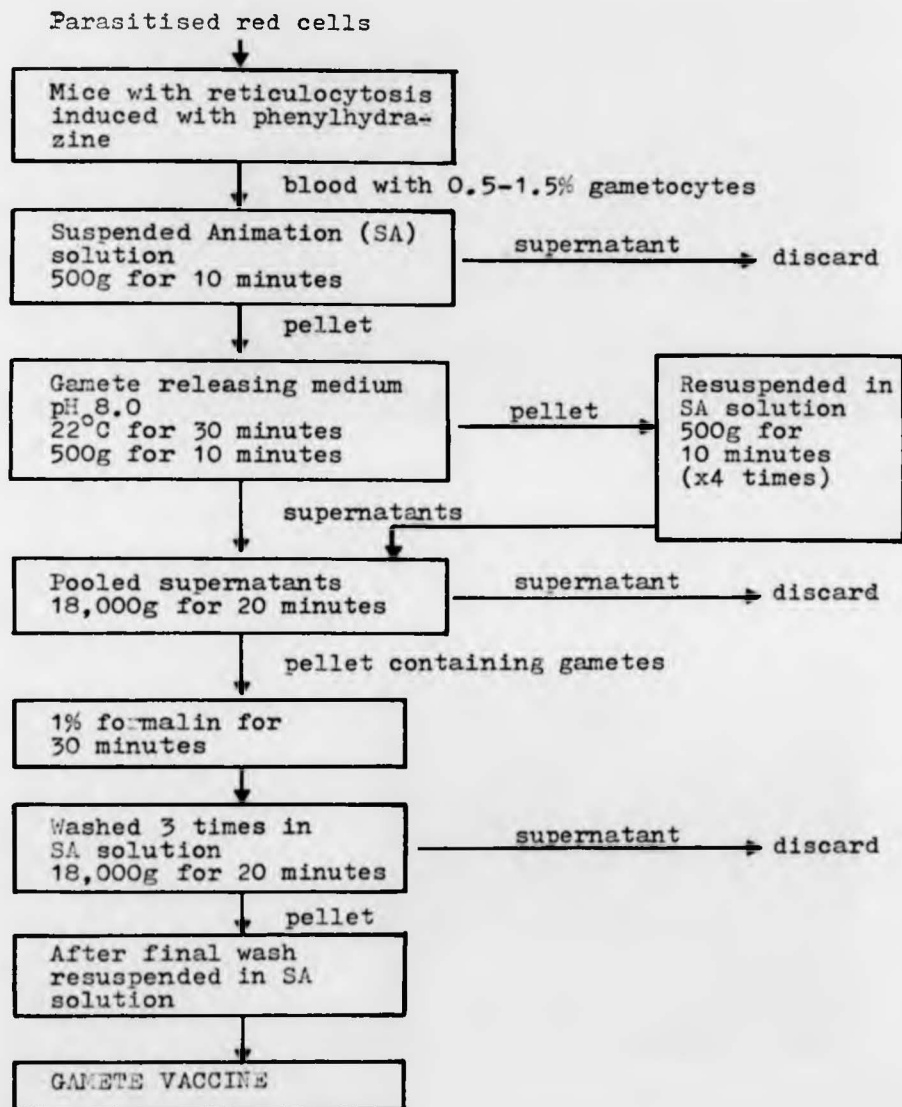
PLATE 2. Giemsa stained film of infected blood in which gametogenesis was induced in vitro. Note thread-like male gametes (magnification x 1000).

was withdrawn and the red cells were resuspended in SA solution and subjected to the same centrifugation procedure at least 4 times until the supernatant was relatively free of gametes. The pooled supernatants were centrifuged at 18,000g for 20 minutes and the pellet containing gametes was resuspended and fixed in 1.0% formalin for 30 minutes. The material was then washed 3 times in SA solution at 18,000g. After the final wash, counts were made in a haemocytometer using a phase contrast microscope and adjusted such that 0.2 ml contained the number of male gametes required for a single dose.

Figure 1 is a flow diagram showing the preparation of the gamete vaccine.

Giemsa stained thin films of the vaccine were examined under the light microscope and relative proportions of the components of the vaccine were estimated. This data was corroborated with counts done on a wet preparation of the vaccine in a haemocytometer under phase contrast optics. The vaccine contained in addition to male and female gametes, asexual parasites that were largely merozoites which under the light microscope appeared to be free of host red cells, occasional red cell 'ghosts' and cellular debris composed mainly of reticulocyte membranes. The presence of these elements was confirmed by electron microscopic studies (plates 3, 4 and 5). The number of male gametes in the vaccine was

FIGURE 1. Flow Diagram of Preparation of Gamete Vaccine



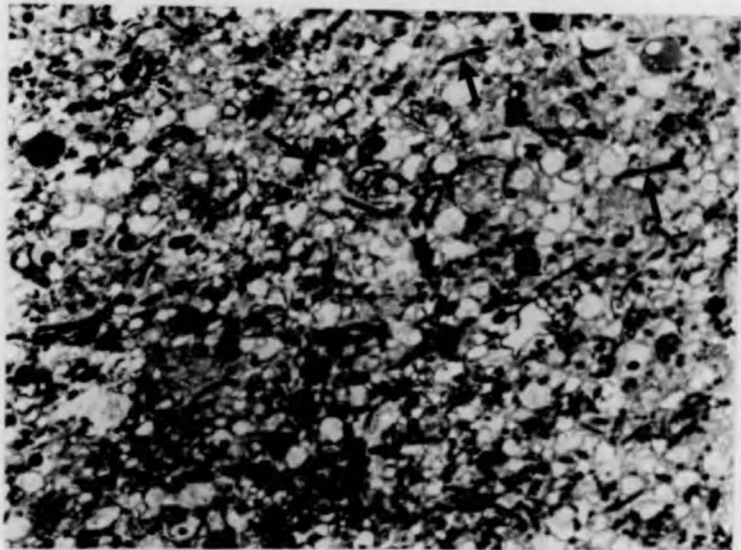


PLATE 3. Transmission electron micrograph of gamete vaccine showing numerous (←) flagella of male gametes (magnification x 3,600).

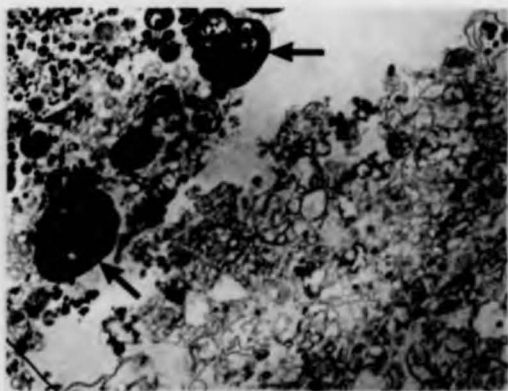


PLATE 4. Transmission electron micrograph of gamete vaccine. The large parasites (←) appear to be female gametes (magnification x 3,200).

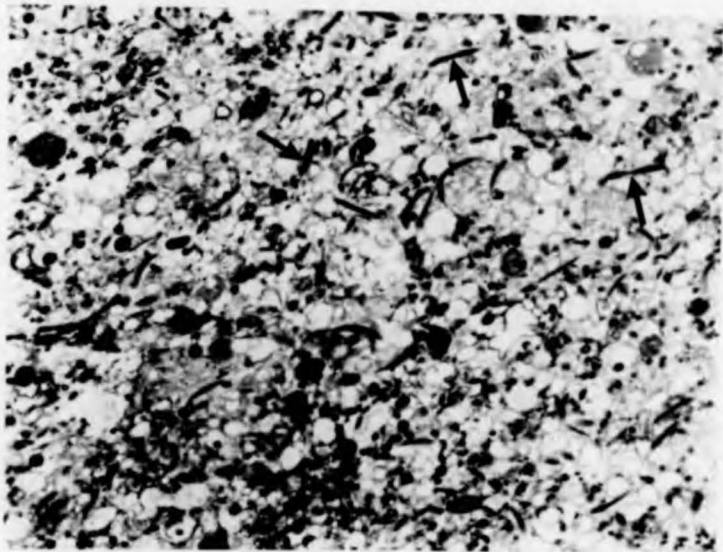


PLATE 3. Transmission electron micrograph of gamete vaccine showing numerous (←) flagella of male gametes (magnification x 3,600).

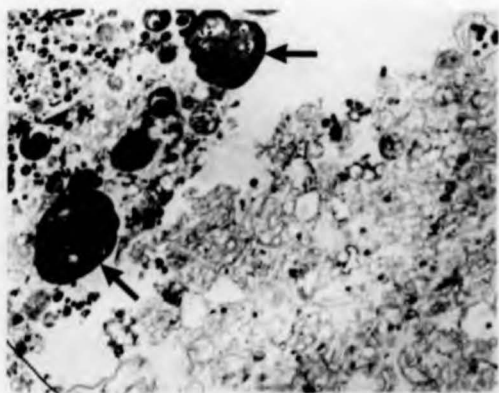


PLATE 4. Transmission electron micrograph of gamete vaccine. The large parasites (←) appear to be female gametes (magnification x 3,200).



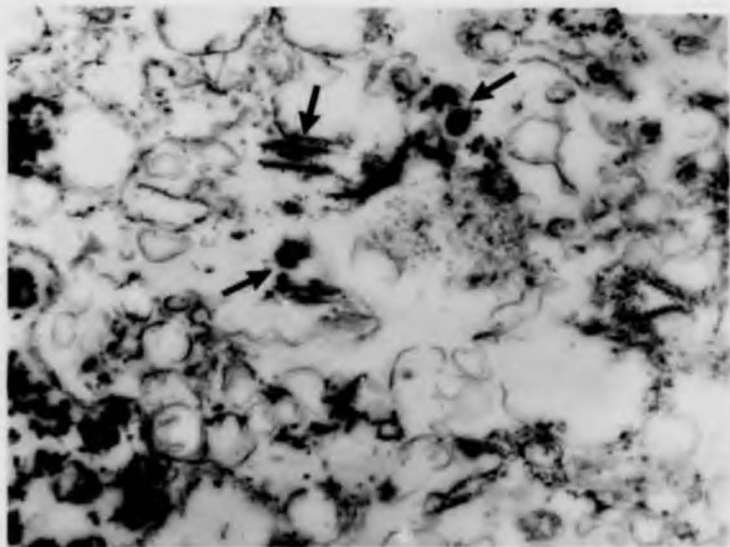


PLATE 5. Transmission electron micrograph of gamete vaccine showing many flagella of male gametes in longitudinal and transverse sections (←), surrounded by red cell membranes (magnification x 23,000).

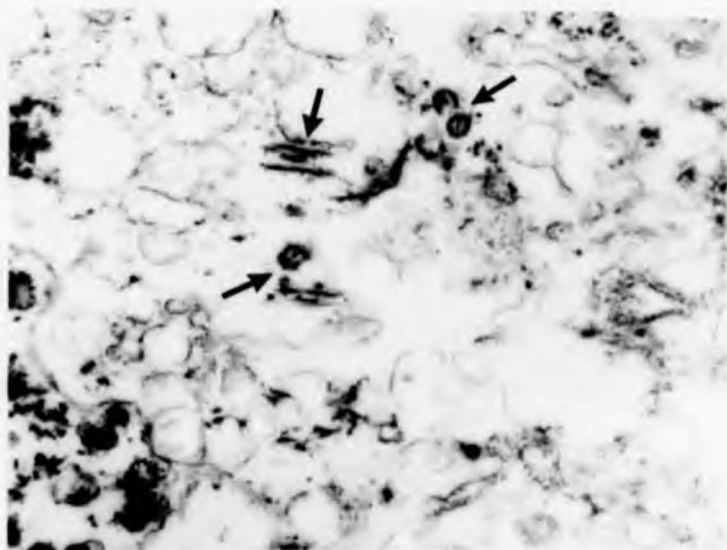


PLATE 5. Transmission electron micrograph of gamete vaccine showing many flagella of male gametes in longitudinal and transverse sections (←), surrounded by red cell membranes (magnification x 23,000).

taken as a measure of the immunising dose. A dose containing  $2 \times 10^7$  male gametes contained  $6-8 \times 10^6$  female gametes and  $10^5-10^6$  asexual erythrocytic parasites, and such a quantity was derived from approximately 5.0 ml of infected blood.

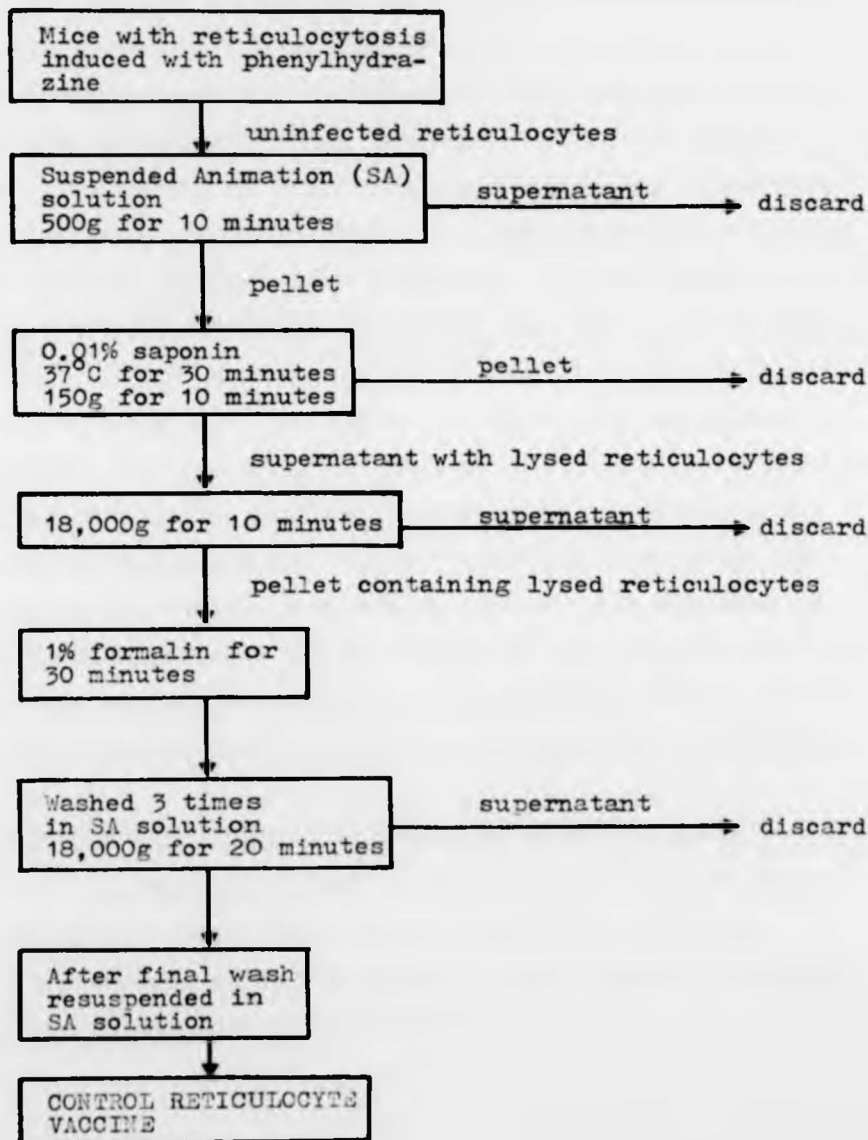
#### Preparation of the control reticulocyte vaccine

A reticulocytosis was induced in mice as described previously. 3 days after the final dose of phenylhydrazine mice were bled by cardiac puncture into 50 volumes of a SA solution and the suspension was centrifuged at 500g for 10 minutes. The supernatant was discarded and the washed red cells were suspended in 40 volumes of a 0.01% saponin solution and incubated at  $37^\circ\text{C}$  for 30 minutes in order to lyse the red cells. (Based on a method used by Spira and Zuckerman (1962) and Playfair et al., (1977a)). The suspension was then centrifuged at 150g for 10 minutes and the supernatant containing lysed reticulocytes was removed and centrifuged again at 18,000g for 10 minutes. The pellet was resuspended and fixed in 1.0% formalin for 30 minutes, washed 3 times in SA solution at 18,000g and resuspended in the same.

Figure 2 is a flow diagram showing the preparation of the control reticulocyte vaccine.

The purpose of using a control reticulocyte vaccine was to evaluate the effect of host cell components

FIGURE 2. Flow Diagram of Preparation of Control Reticulocyte Vaccine



in the experimental vaccines used. In the gamete vaccine and asexual stage vaccines which are referred to in the next chapter, the source of red cell membranes was the host erythrocytes from which gametes emerged and those which contained mature schizonts that ruptured spontaneously. It was therefore necessary to deviate from the procedure adopted for preparing vaccines and resort to artificial lysis of red cells. Ideally mice would have to be vaccinated with a quantity of reticulocyte membranes that was equal to that found in a dose of the gamete vaccine. Such a dose was arrived at by visual estimation of the volume of the pellet of lysed reticulocytes. A volume that was equal to that of a standard dose (containing  $2 \times 10^7$  male gametes) of the gamete vaccine, when both were spun under similar conditions was used. This estimation was an approximate one and did not make an allowance for the volume of gametes and asexual parasites in the gamete vaccine, and therefore the dose of the reticulocyte vaccine used was in excess of the ideal.

#### Challenge infection and assays of immunity in vivo

In each experiment vaccinated mice and an equal number of age matched control mice were challenged intravenously with a homologous blood induced infection of  $2 \times 10^5$  parasitised red cells.

The challenge infections were monitored by making thin blood films and ascertaining the parasitaemia and gametocytaemia daily. Immunity against asexual blood stages was assessed using the following parameters of a challenge infection.

1. Height of the parasitaemia.
2. Time taken for clearance of the infection.
3. Outcome of the infection.

To determine the effects of immunisation on the sexual development of the parasite, mosquitoes were fed on vaccinated and control mice, one cage per group. Feedings were carried out during the first 6 days of an infection in initial experiments and, on finding that infectivity of control mice was restricted to a period between the second and the fifth days of an infection, in subsequent experiments feedings were confined to this period. The technique of feeding mosquitoes has already been described. During feeding, mosquitoes could be observed to become engorged with blood. Any unfed females were removed and the remaining mosquitoes kept at 24-25°C with a relative humidity of 70-80%. Thereafter no further blood meals were given and the mosquitoes were maintained on a sugar solution. 7 days after feeding 10 mosquitoes from each cage were dissected and their midguts mounted in saline under a coverslip. Oocysts counts on each midgut were done microscopically at  $\times 100$  magnification, and served as an index of transmission.

#### In vitro assay of immunity

The presence of anti-gamete activity in the serum or plasma of immunised mice was demonstrated in vitro by observing microgametogenesis in whole blood or when gametocytes from days 2 or 3 of an infection were washed and resuspended in immune serum. Microgametogenesis was observed in wet preparations of blood using phase contrast optics, at a magnification of x1000.

#### Recombination of infected red cells and plasma or serum from different donors

The mouse donor of infected red cells was bled by cardiac puncture into 20 volumes of SA solution and the cells centrifuged at 500g for 10 minutes. The supernatant was discarded and an aliquot of red cells was immediately resuspended in 3 volumes of the appropriate plasma or serum. When the donor mouse could not be sacrificed for this purpose, tail blood was used in an identical manner as a source of red cells.

Plasma was obtained by bleeding the donor mouse similarly into heparin (Freeze dried preparation, Evans Medical, Liverpool.) at a concentration of 7 units per ml and centrifugation at 700g for 10 minutes. The supernatant which contained plasma was used fresh or stored at  $-20^{\circ}\text{C}$  and thawed before use.

To obtain serum, the donor mouse was bled by cardiac puncture and the blood allowed to clot at  $37^{\circ}\text{C}$  for

2 hours in a glass container. Serum was decanted, centrifuged at 1,200g for 10 minutes and decomplexed in a water bath at 56°C for 30 minutes. Serum was stored at -20°C and thawed before use.

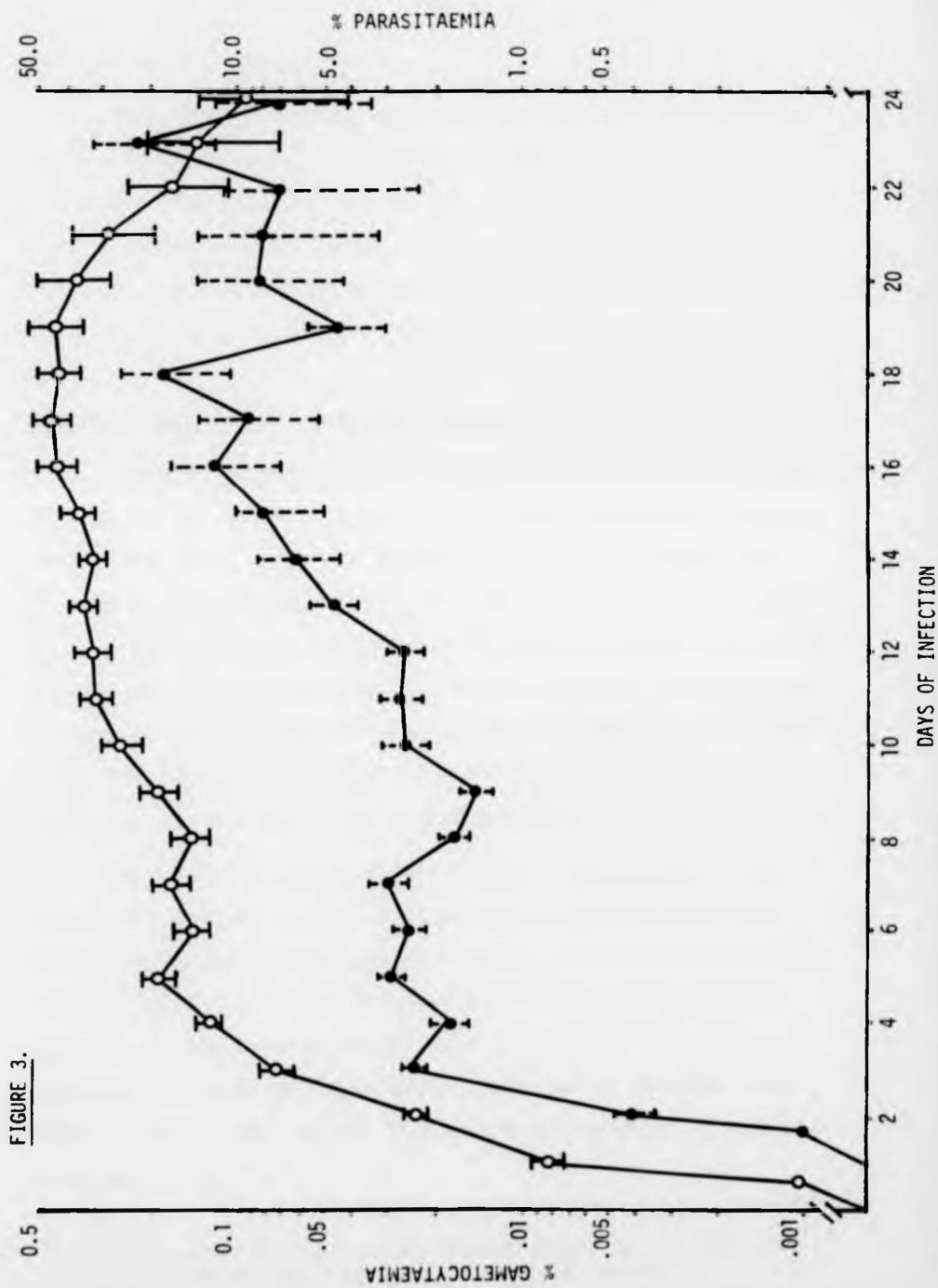


## RESULTS

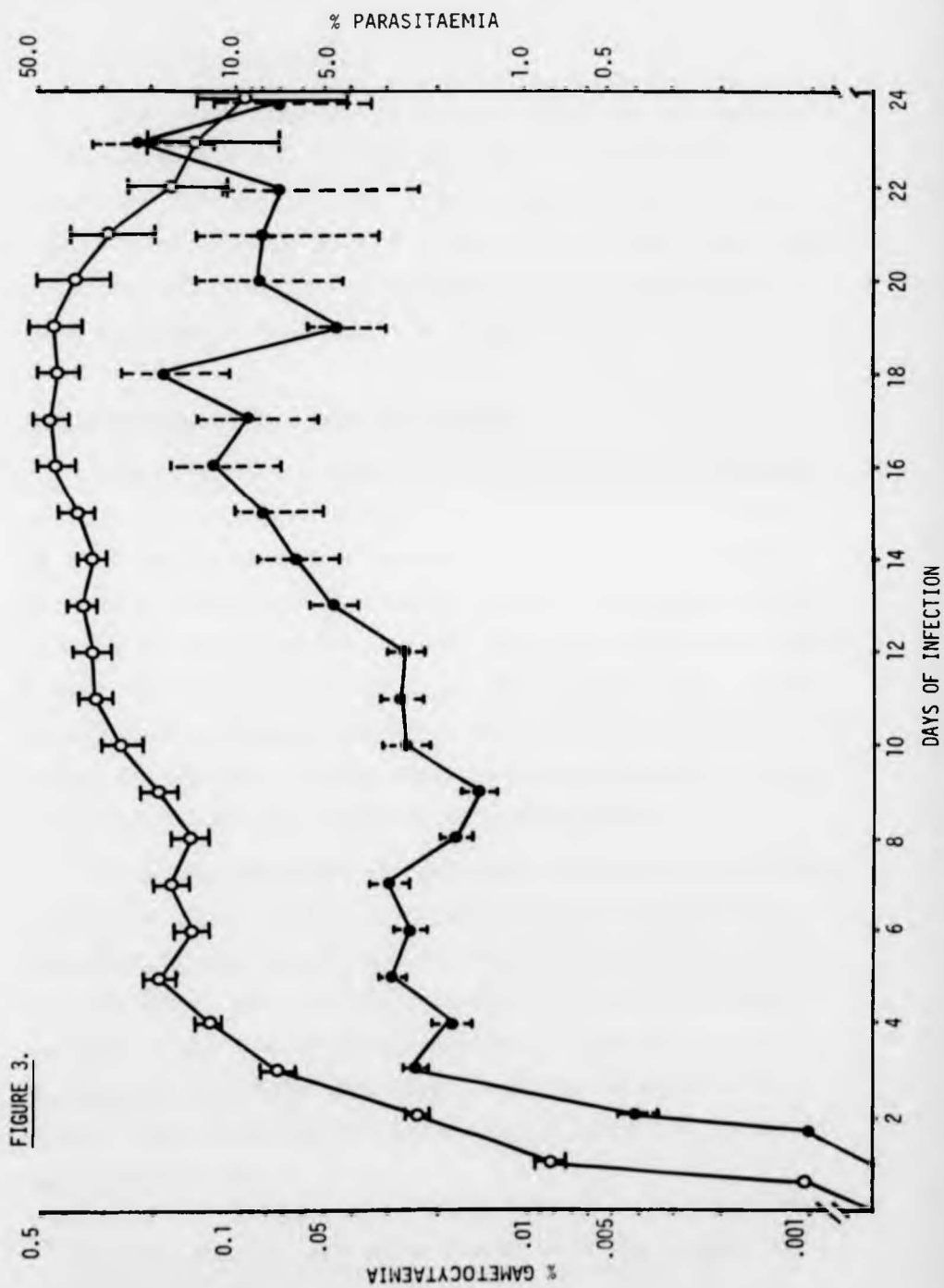
### The nature of an infection of *Plasmodium yoelii nigeriensis* in mice

The parasite causes a fatal infection in about 80% of mice, and in those that succumb to the infection, the parasitaemias continue to rise from the time of patency, usually interrupted by a lag phase between the 7th and the 10th day (Figure 3). Death occurs between 5th and 19th days, but commonly between the initial peak of parasitaemia at about 5th to the 8th day, with parasite counts reaching upto 45%, or later between 14th and 19th days with parasitaemias as high as 85%. In the small proportion of mice that survive, the infection takes a similar course except that the lag phase tends to be more pronounced, often manifested by a depression of the parasitaemia curve and followed by another transient depression at the end of the 2nd week. Parasitaemias of upto 85% were reached in some mice before the infection was cleared between days 20 and 30. The mice remained apparently aparasitaemic thereafter.

Detailed results of gametocytaemias and infectivity to mosquitoes of infections have been presented in chapter 5. Briefly, gametocyte counts tended to run parallel to the parasitaemia (Figure 3) and the highest values recorded during the course of an infection varied



**FIGURE 3.** The mean (○) parasitaemias and (●) gametocytaemias ( $\pm$  standard error of the mean) of 50 primary infections of *P.yoelii nigeriensis* in unvaccinated mice.



between 0.01% and 0.8%.

The total numbers of oocysts produced by feeding 10 mosquitoes a day during the first 5 days of an infection frequently lay in the range of 1000 to 3000, though occasionally higher values of upto 5000 have been obtained. Infectivity to mosquitoes were highest on days 2, 3 and 4 (see Table 8, page 130).

#### Immunisation with a gamete vaccine

Results\* were obtained from 3 separate experiments in which 9 mice were vaccinated; each received 3 doses of the freshly prepared gamete vaccine intravenously at weekly intervals. A single dose of the vaccine contained  $2 \times 10^7$  male gametes. A challenge infection was given 1 week after the final dose of the vaccine. Blood films were made on 3 occasions at 4 day intervals after vaccination in this and in all subsequent experiments. In no case did vaccination produce a parasitaemia.

The parasitaemia of challenge infections in vaccinated and control mice is represented graphically in Figure 4.1. The infection was fatal in all but 1 of the control mice. In 3 of the control mice death occurred on days 6 and 7 with parasitaemias of 20-30% and in 5 on days 19 and 20 with parasitaemias of 50-60%. The animal that survived the infection cleared its parasitaemia on day 17.

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\* Full results of all experiments from the graphs are shown (including standard errors of means) in appendix III.

FIGURE 4.1. Mean parasitaemias of challenge infections in (---○) unvaccinated mice and (—●) mice vaccinated with 3 intravenous doses of the gamete vaccine, each containing  $2 \times 10^7$  male gametes.

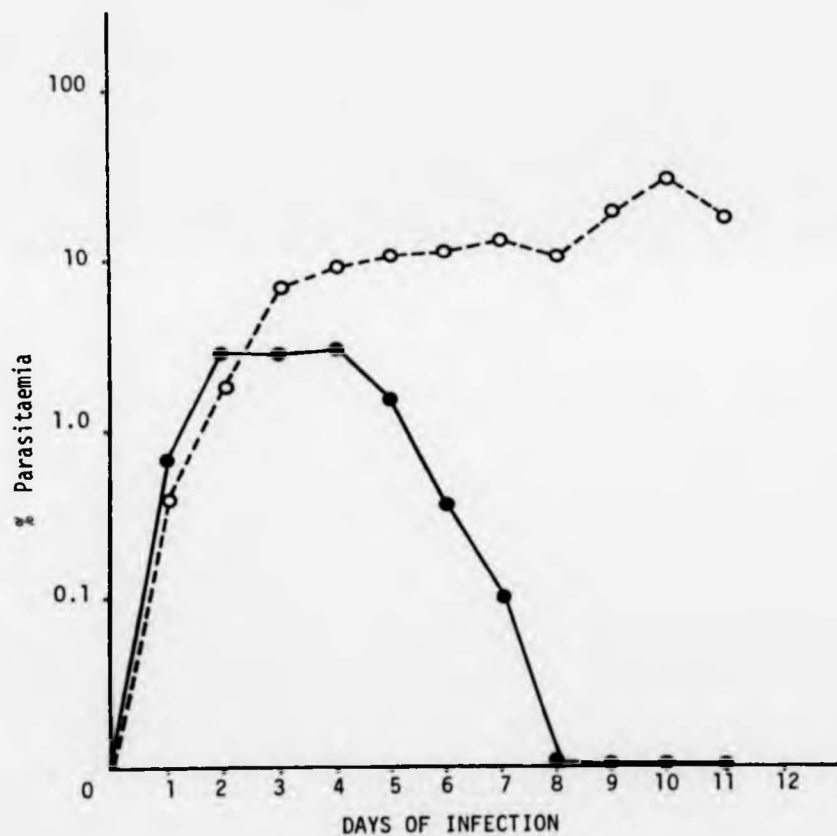
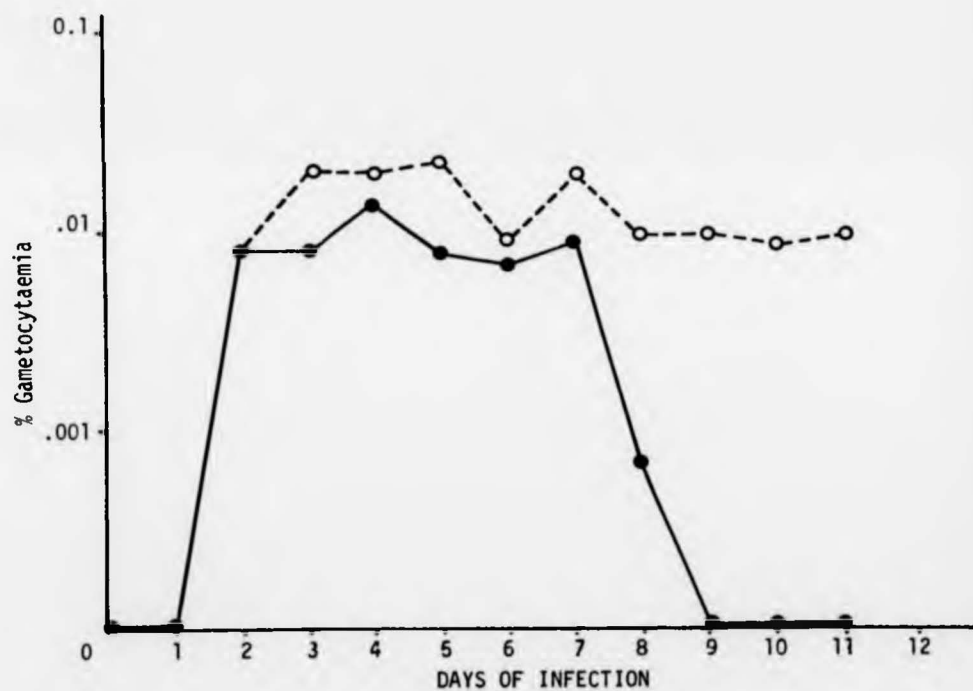


FIGURE 4.2. The mean gametocytaemias of challenge infections in (---○) unvaccinated mice and (—●) mice vaccinated with 3 intravenous doses of the gamete vaccine, each containing  $2 \times 10^7$  male gametes.



In contrast, the vaccinated mice were apparently aparasitaemic by day 12 with a mean clearance time of 8.8 days ( $\pm 1.7$  days)\*. The highest mean parasitaemia recorded in vaccinated mice was 3.1%, and was reached on day 4.

The mean gametocyte counts in vaccinated and control mice remained in close approximation during the first 7 days of the challenge infections (Figure 4.2). Mosquitoes fed on control mice had high oocyst counts which totalled to 2,016, 2,238 and 3,449 in 3 experiments, these being derived mainly from feeds taken on days 2, 3 and 4 after challenge. Mosquitoes fed on vaccinated mice showed no oocysts at all in their midguts throughout the infection (Table 1 and Plates 6 and 7).

Preliminary observations on the mechanisms of transmission blocking immunity

The processes of microgametogenesis and gamete dispersal were observed following suspension of infected erythrocytes in homologous and heterologous serum or plasma. The following descriptions of these processes in vitro have conformed to the nomenclature introduced by Sinden and Croll (1975) and Sinden *et al.*, (1976). Thus, microgametogenesis has been described under the

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\* Standard deviation



TABLE 1. Oocyst production in mosquitoes fed on unvaccinated mice and mice vaccinated with 3 doses of the gamete vaccine, each containing  $2 \times 10^7$  male gametes.

Day	Unvaccinated mice			Mean number of oocysts per mosquito gut	Vaccinated mice
	Numbers of oocysts / 10 mosquitoes				Number of oocysts per 10 mosquitoes
	Experiments				Experiments
	<u>1</u>	<u>2</u>	<u>3</u>		<u>1, 2 and 3</u>
1	0	0	0		0
2	114	1,638	2,162	130.4	0
3	496	498	1,287	76.0	0
4	1,406	40	0	48.2	0
5	0	62	0	2.0	0
6	0	0	0	0	0
Total counts	2,016	2,238	3,449		



PLATE 6. Uninfected midgut of a mosquito fed on immunised mice during a challenge infection (magnification x25).

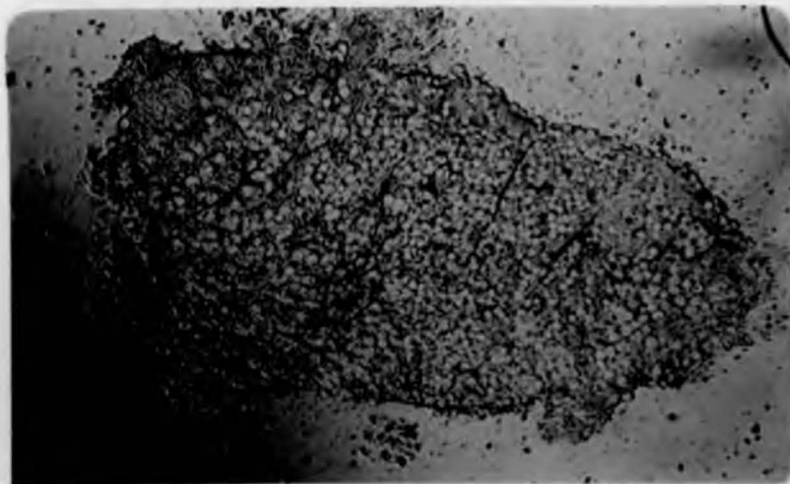


PLATE 7. Heavily infected midgut of a mosquito fed on unvaccinated control mice during a challenge infection. Note the numerous oocysts which appear as colourless circular areas on the gut (magnification x 63).



PLATE 6. Uninfected midgut of a mosquito fed on immunised mice during a challenge infection (magnification x25).

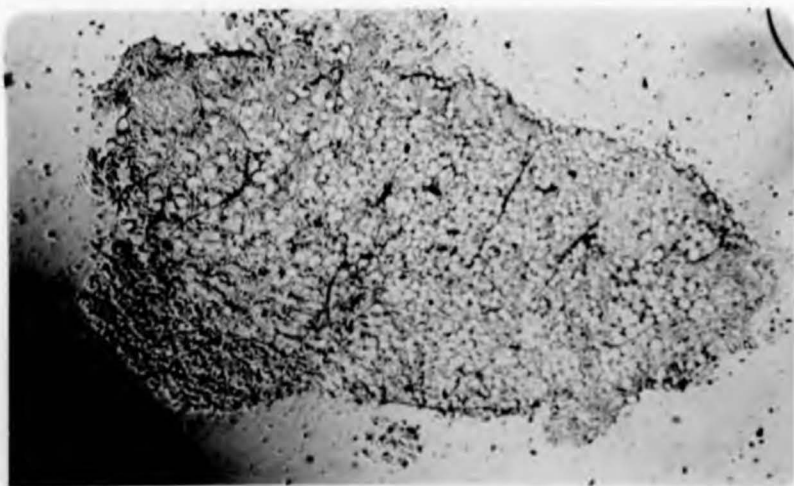


PLATE 7. Heavily infected midgut of a mosquito fed on unvaccinated control mice during a challenge infection. Note the numerous oocysts which appear as colourless circular areas on the gut (magnification x 63).



PLATE 6. Uninfected midgut of a mosquito fed on immunised mice during a challenge infection (magnification x25).

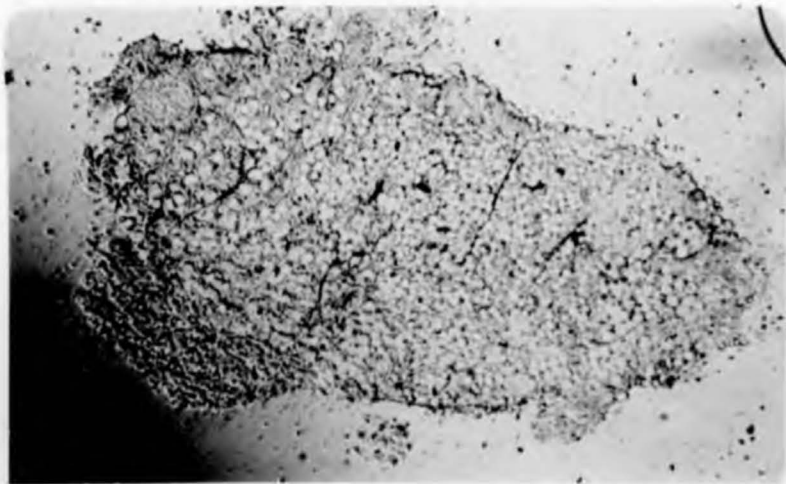


PLATE 7. Heavily infected midgut of a mosquito fed on unvaccinated control mice during a challenge infection. Note the numerous oocysts which appear as colourless circular areas on the gut (magnification x 63).

phases of activation, exflagellation and escape of male gametes, to be followed by microgamete dispersal. These terms pertaining to the different phases have been used sensu stricto.

Microgametogenesis and gamete dispersal in blood obtained from unvaccinated control mice

Gametocytes of days 2 and 3 of challenge infections in control mice, in whole blood or when washed and resuspended in their own serum underwent microgametogenesis and gamete dispersal normally as described below.

Activation - The first sign of gametogenesis appeared within 2 minutes as active movement of the pigment granules within the gametocytes, which formed rapidly changing patterns varying from seemingly random and widely dispersed movement to stranded or clustered appearances. At 6-7 minutes the movement of pigment granules suddenly ceased in most gametocytes.

Exflagellation - Gametocytes again became active almost immediately, each rotating rapidly about its own axis. This soon gave way to intense pulsatile movements of the cytoplasm with transient bulges appearing over the surface of the cell. Within 10 to 15 seconds of the initiation of pulsatile movements, microgametes emerged from the gametocyte surface and rapidly gained in length. The entire process of exflagellation occupied about 1 minute, at the end of which fully formed microgametes

were lashing out vigorously while still attached to the residual parent cytoplasm.

Escape of microgametes - Microgametes, while still attached to the parent cytoplasm exhibited very intense lashing movements alternating with brief periods of immobility. During the spurts of activity they became detached from the residual cytoplasm and escaped, some almost immediately after exflagellation. A majority of gametes escaped during the first 10 minutes but a few took longer and occasionally the last ones to escape did so 40 minutes later. Throughout this period the gametes exhibited active movements.

Microgamete dispersal - Soon after detachment microgametes moved about rapidly and initially the rate of movement was often too rapid to permit their path to be followed from one microscope field to another. Movement was sustained for at least an hour after exflagellation and often longer. Failure of microgametes to escape was not observed.

Microgametogenesis and gamete dispersal in blood obtained from vaccinated mice

Gametocytes of days 2 and 3 of challenge infections in vaccinated mice, in whole blood or when washed and resuspended in their own serum underwent microgametogenesis and gamete dispersal as described below.

Activation and exflagellation - Both these processes occurred normally as described above.

Escape of microgametes - Active lashing movement of microgametes rapidly diminished in intensity and was completely arrested usually within 30 seconds of their formation, but occasionally movement continued for about 3 minutes. Gamete immobilisation usually occurred while they were still attached to the parent cytoplasm, and even those gametes that escaped soon after exflagellation were immobilised within 15 to 20 seconds of their being freed.

Microgametogenesis and gamete dispersal in different permutations of cells and serum

(a) Gametocytes from challenge infections in control mice on days 2 and 3 when washed and resuspended in serum of vaccinated mice obtained at any time during the course of a challenge infection or just before a challenge infection, underwent gametogenesis and gamete dispersal in a manner identical to those of vaccinated mice in whole blood, viz., activation and exflagellation proceeded normally but microgametes were immobilised within seconds of their formation.

(b) Gametocytes from challenge infections in vaccinated mice on days 2 and 3 when washed and resuspended in serum obtained from unvaccinated mice before a challenge infection underwent microgametogenesis and gamete dispersal normally, free microgametes showing sustained activity.

Dose related responses to gamete vaccination

In 3 separate experiments 7 mice were vaccinated intravenously, each with a single dose of the gamete vaccine containing  $2 \times 10^7$  male gametes. In another experiment 4 mice were vaccinated with a single dose of the gamete vaccine containing  $2 \times 10^6$  male gametes. All vaccinated and control mice were challenged 3 weeks after vaccination.

The parasitaemias and gametocytaemias of challenge infections are presented in Figures 5.1 and 5.2 respectively. The infection was fatal in all but 1 of the control mice. Death occurred between days 5 and 7 with parasitaemias of upto 30-40% or between days 12 and 15 with parasitaemias of 50-85%. The mouse that survived the infection cleared its parasitaemia on day 28, the highest recorded count being 58.0%.

All the mice that were vaccinated with a dose containing  $2 \times 10^7$  male gametes were apparently aparasitaemic by day 10 with a mean clearance time of 8.7 days ( $\pm 1.0$  days)\*. The highest mean parasitaemia recorded in this group was 6.36%. In mice that received a lower vaccinating dose containing  $2 \times 10^6$  male gametes the challenge infections were uniformly fatal. Death occurred between the 5th and 19th days, and the highest mean parasitaemia recorded was 61.2%.

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\* Standard deviation



FIGURE 5.1. The mean parasitaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice and mice vaccinated with a single intravenous dose of the gamete vaccine containing ( $\circ$ )  $2 \times 10^6$  male gametes or ( $\bullet$ )  $2 \times 10^7$  male gametes.

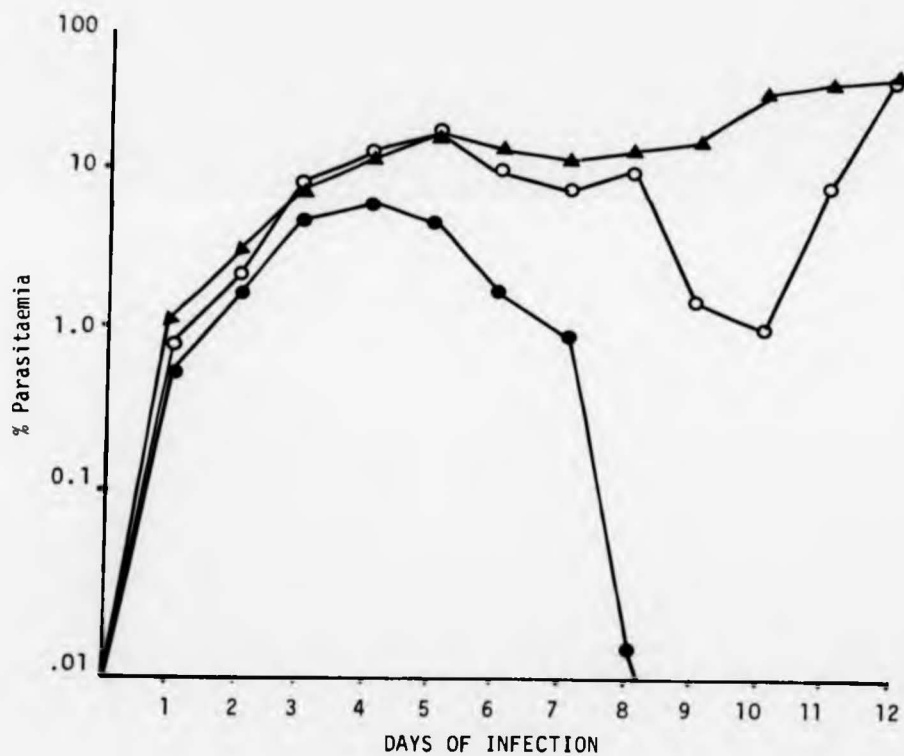
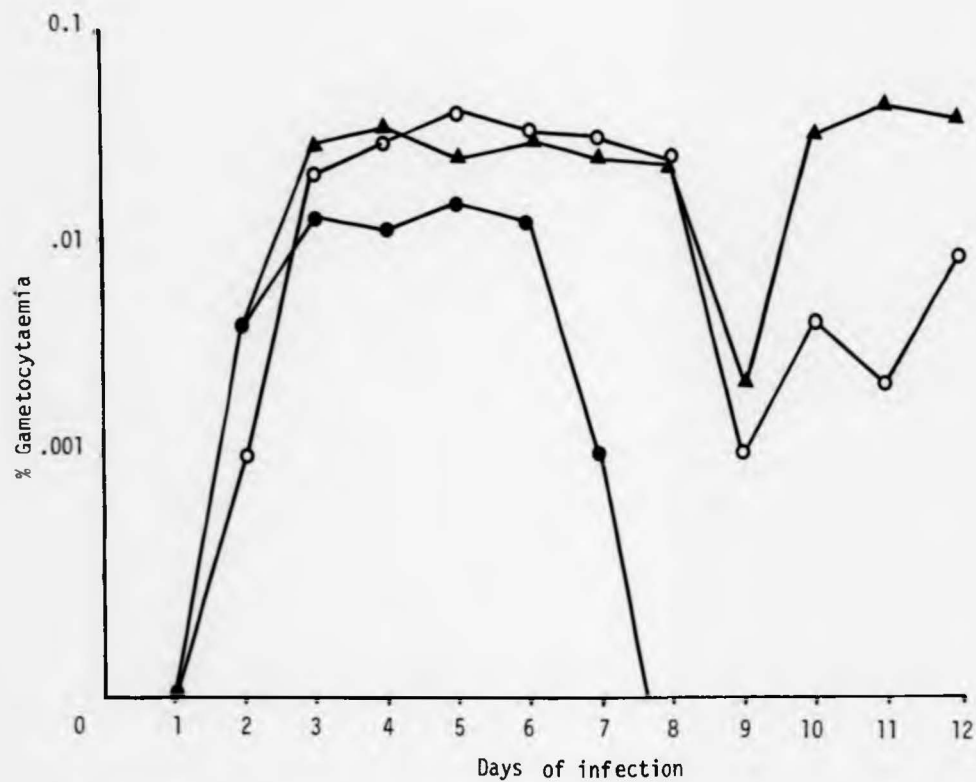


FIGURE 5.2. The mean gametocytaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice and mice vaccinated with a single intravenous dose of the gamete vaccine containing ( $\circ$ )  $2 \times 10^6$  male gametes and ( $\bullet$ )  $2 \times 10^7$  male gametes.



**TABLE 2.** Oocyst production in mosquitoes fed on mice vaccinated with a single dose of the gamete vaccine containing  $2 \times 10^7$  male gametes in experiments 1,2 and 3, and  $2 \times 10^6$  male gametes in experiment 4, and on unvaccinated control mice.

Day	Unvaccinated mice				Vaccinated mice
	Numbers of oocysts per 10 mosquitoes				Number of oocysts per 10 mosquitoes
	Experiments				Experiments 1, 2, 3 and 4
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	
1	0	0	0	0	0
2	857	33	878	859	0
3	2,463	1,645	1,470	270	0
4	45	620	607	02	0
5	0	0	0	05	0
<b>Total counts</b>	3,365	2,298	2,955	1,136	

In all 4 experiments, infections in control mice produced high oocyst counts in mosquitoes whereas in mice that were vaccinated with either dose of the vaccine, challenge infections were totally non-infective to mosquitoes (Table 2).

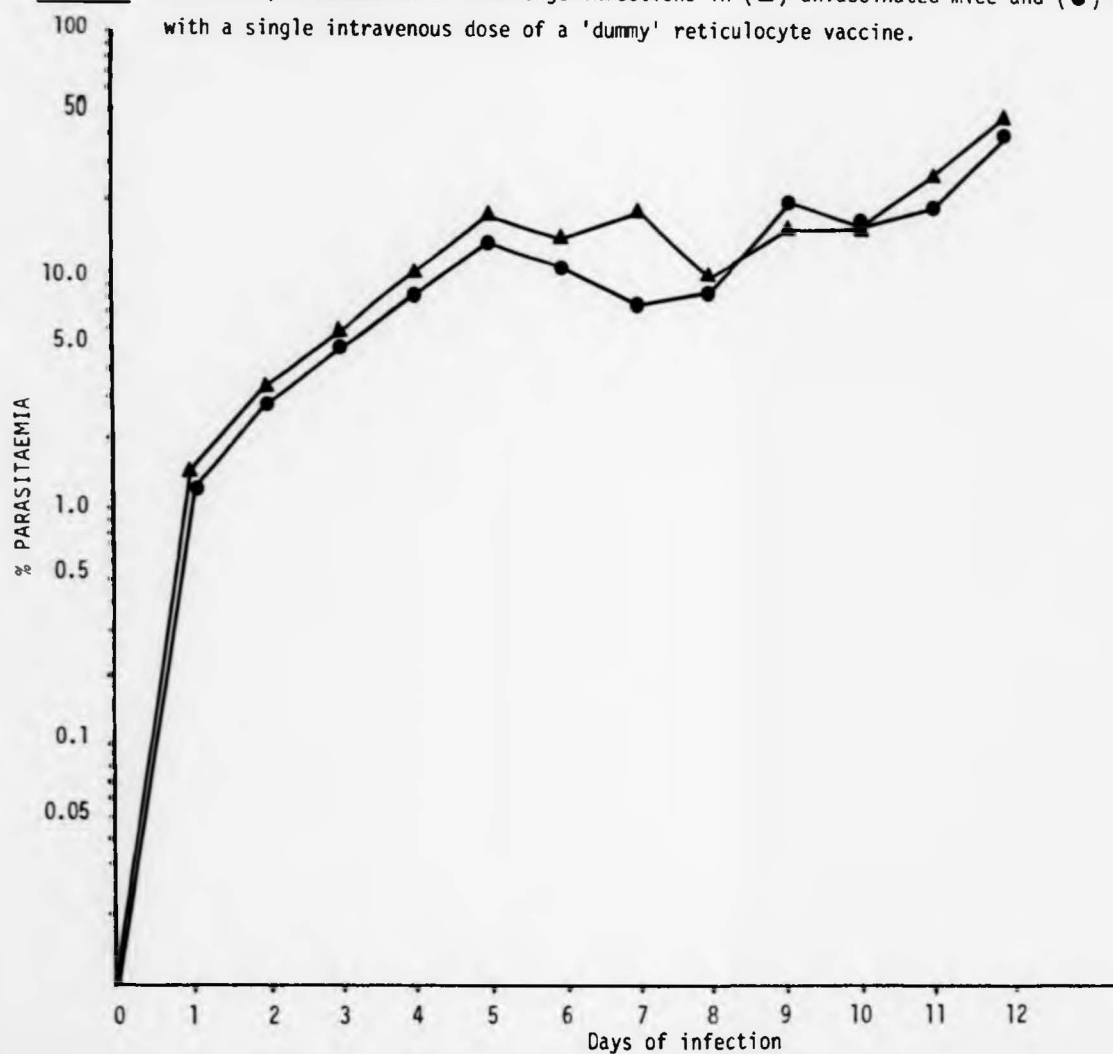
#### Evaluation of a control reticulocyte vaccine

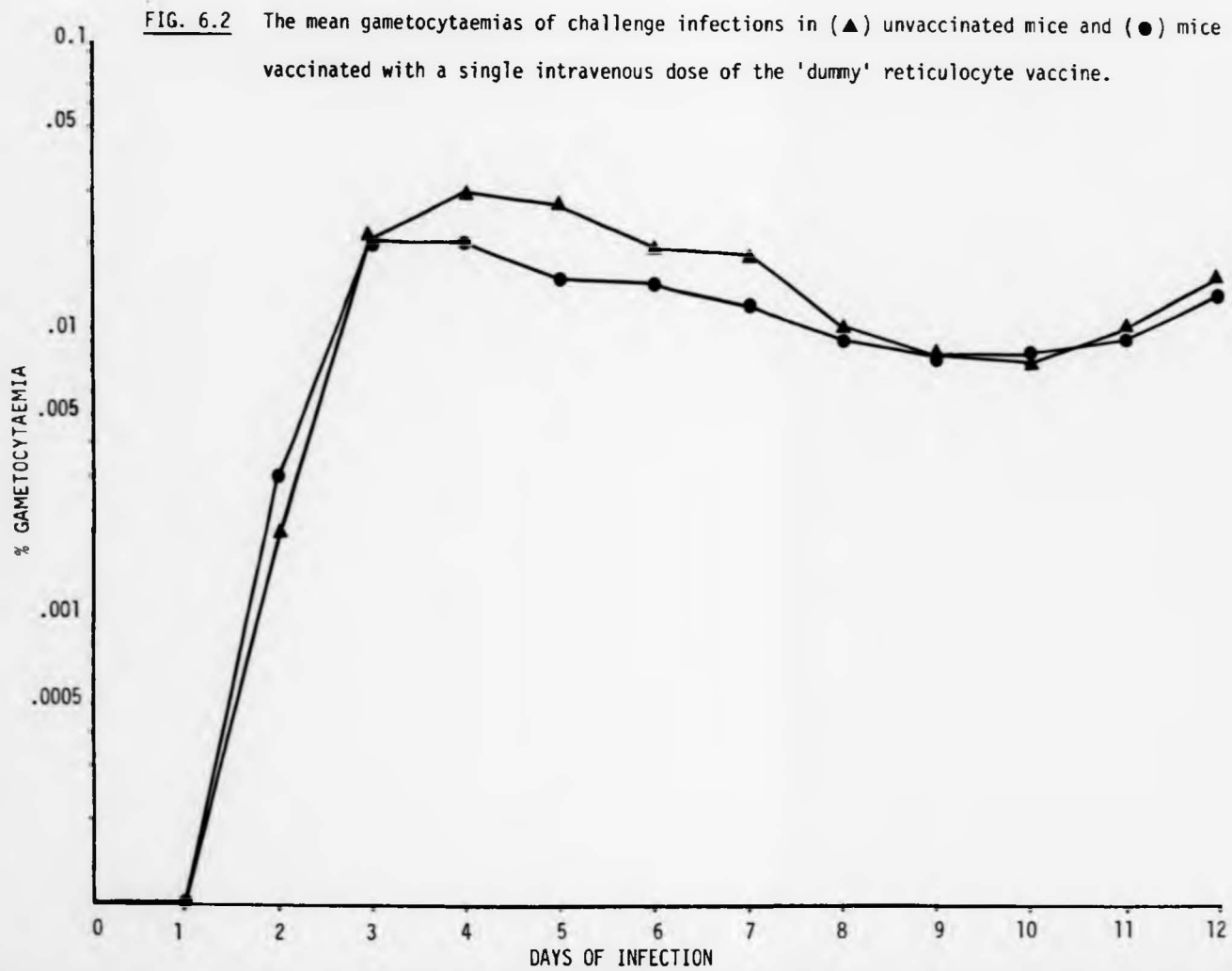
In 2 experiments 7 mice were vaccinated intravenously with a single dose of a 'dummy' reticulocyte vaccine which contained a quantity of reticulocyte membranes that was slightly higher than that contained in the gamete vaccine containing  $2 \times 10^7$  male gametes. A challenge infection was given 3 weeks later.

The parasitaemias and gametocytaemias of mice that received the 'dummy' reticulocyte vaccine and of the unvaccinated controls are represented graphically in Figures 6.1 and 6.2 respectively. All the control unvaccinated mice died of the challenge infection. The highest mean parasitaemia recorded in the control mice was 42.0%. Of the mice that received the 'dummy' reticulocyte vaccine the challenge infection was fatal in 5. The other 2 suffered a prolonged infection which was cleared on days 24 and 25. The highest parasitaemia recorded in these mice was 51.9%.

Oocyst counts in mosquitoes fed on challenge infections in the mice that received the reticulocyte

FIG. 6.1. The mean parasitaemias of challenge infections in (▲) unvaccinated mice and (●) mice vaccinated with a single intravenous dose of a 'dummy' reticulocyte vaccine.





**TABLE 3.** Oocyst production in mosquitoes fed on mice vaccinated with a 'dummy' reticulocyte vaccine and on unvaccinated mice, in 2 experiments.

Day	Vaccinated mice		Unvaccinated mice	
	Number of oocysts per 10 mosquitoes		Number of oocysts per 10 mosquitoes	
	Experiments		Experiments	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
1	0	0	0	0
2	769	811	50	630
3	295	1,846	1,164	518
4	10	0	14	157
5	0	0	06	0
Total counts	<u>1,074</u>	<u>2,657</u>	<u>1,234</u>	<u>1,305</u>

Differences in the oocyst counts between the vaccinated and control groups are not statistically significant.

vaccine were not different from those in mosquitoes fed on control mice (Table 3).

#### Effect of the intramuscular route of vaccination

The gamete vaccine was administered intramuscularly to 5 mice in 2 separate experiments. All mice received 3 vaccinating doses at weekly intervals, each containing  $2 \times 10^7$  male gametes. Vaccinated and control mice received a challenge infection 1 week after the final dose of the vaccine.

Figures 7.1 and 7.2 represent the parasitaemias and gametocytaemias respectively of vaccinated and control mice. The infection was fatal in 1 of the control mice in which death occurred on day 19. In the other 4 mice the infection followed a protracted course before it was cleared. The mean time of clearance of parasitaemia was 24.5 days ( $\pm 4.7$  days)\*. The highest mean parasitaemia recorded in the control mice was 66.4%.

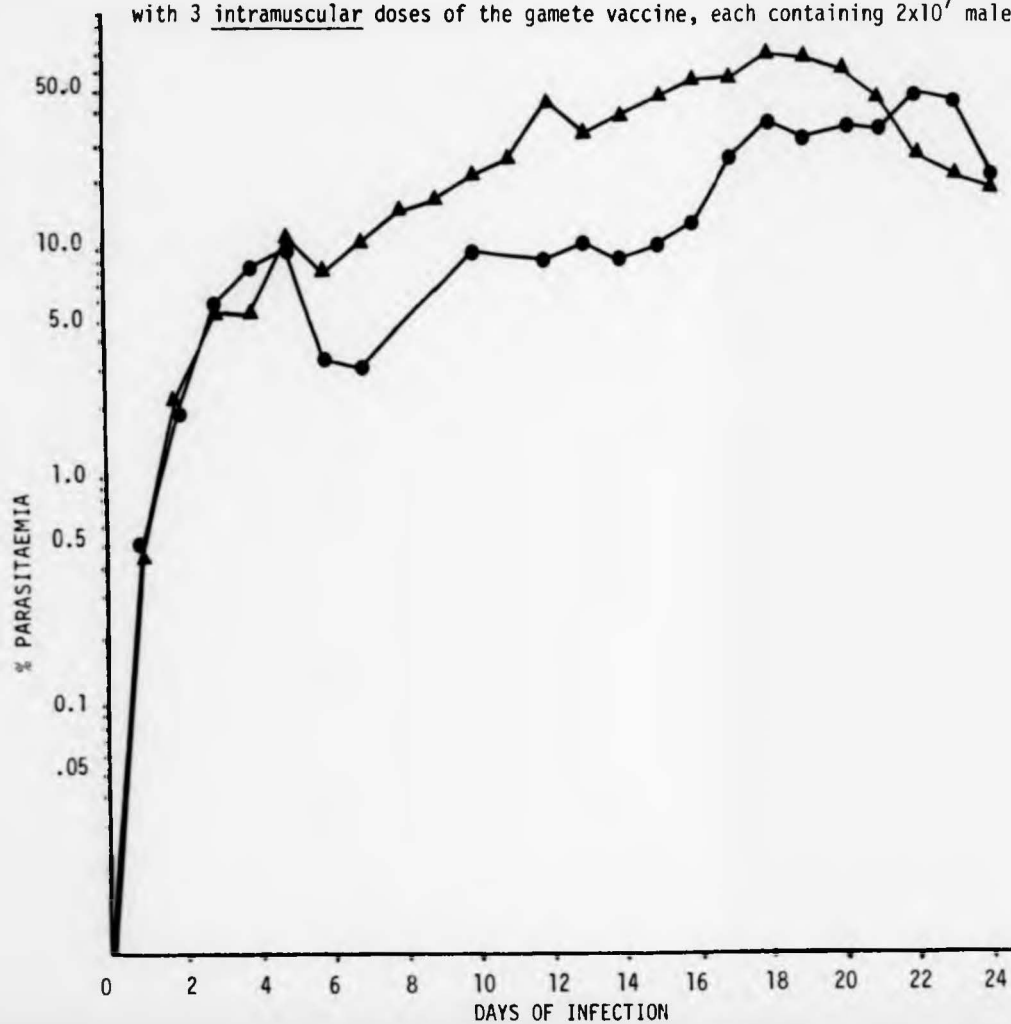
In the vaccinated animals the infection followed a very similar course. 1 vaccinated mouse died of the infection on day 6. The other 4 suffered a prolonged infection before the parasitaemia was cleared. The mean time of clearance was 21.5 days ( $\pm 6.1$  days)\*. The highest mean parasitaemia recorded was 44.8%.

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\* Standard deviation



FIG. 7.1. The mean parasitaemias of challenge infections in (▲) unvaccinated mice and (●) mice vaccinated with 3 intramuscular doses of the gamete vaccine, each containing  $2 \times 10^7$  male gametes.



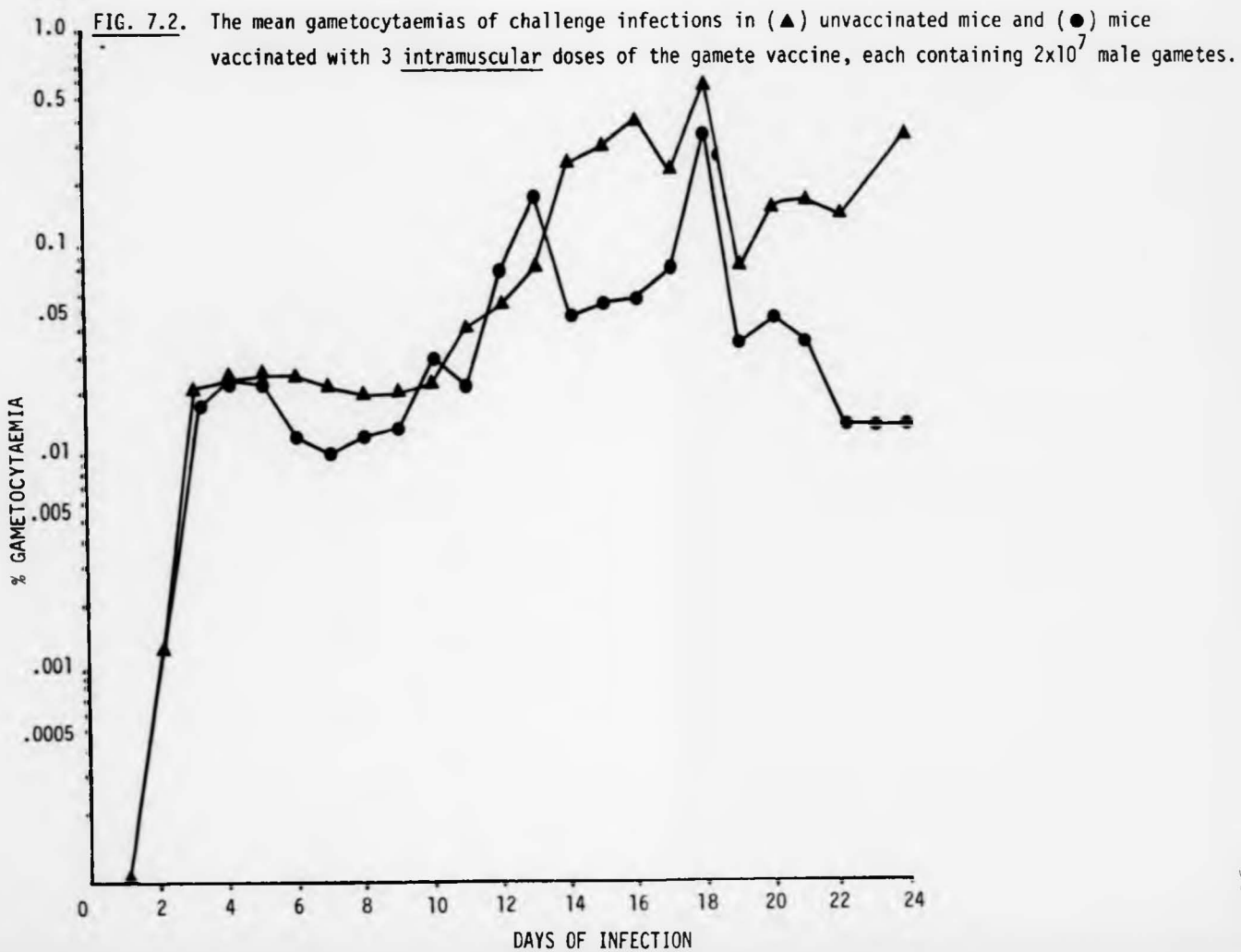


TABLE 4. Oocyst production in mosquitoes fed on unvaccinated mice and mice vaccinated intramuscularly with the gamete vaccine

Day	Unvaccinated mice		Vaccinated mice
	Experiments		Experiments
	<u>1</u>	<u>2</u>	<u>1 and 2</u>
1	0	0	0
2	1,296	135	0
3	1,346	743	0
4	372	182	0
5	10	05	0
Total counts	<u>3,024</u>	<u>1,065</u>	

Mosquito infections obtained by feeding the challenge infections of vaccinated and control mice are presented in Table 4. Oocyst counts in mosquitoes fed on control mice were high whereas mosquitoes fed on vaccinated mice showed no oocysts at all.

#### Duration of immunity

14 mice were vaccinated intravenously in 4 separate experiments, each with 2 doses of the gamete vaccine, each dose containaining  $2 \times 10^7$  male gametes. In each of the 4 experiments 1 mouse was challenged 2 weeks after the final vaccinating dose to confirm the effectiveness of the vaccine by assessing immunity to sexual and asexual stages. Subsequently 4 mice (group A) were challenged 16 weeks and the other 6 mice (group B) 25 weeks, after the last immunising dose.

The parasitaemias of all 4 experiments are summarised and represented in Figure 8.† In group A all the control mice died between 6 and 16 days of the challenge infection. The highest mean parasitaemia recorded was 52.0%. The vaccinated mice cleared their parasitaemia by day 8 with a mean clearance time of 7.25 days ( $\pm 0.96$  days)\*. The highest recorded parasitaemia was only 5.43%.

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\* Standard deviation

† The 'control' graph refers to combined results of all 4 experiments though descriptions refer to the 2 groups separately.

FIGURE 8. The mean parasitaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice and mice vaccinated with 2 intravenous doses of the gamete vaccine and challenged ( $\bullet$ ) 16 weeks and ( $\circ$ ) 25 weeks after vaccination.

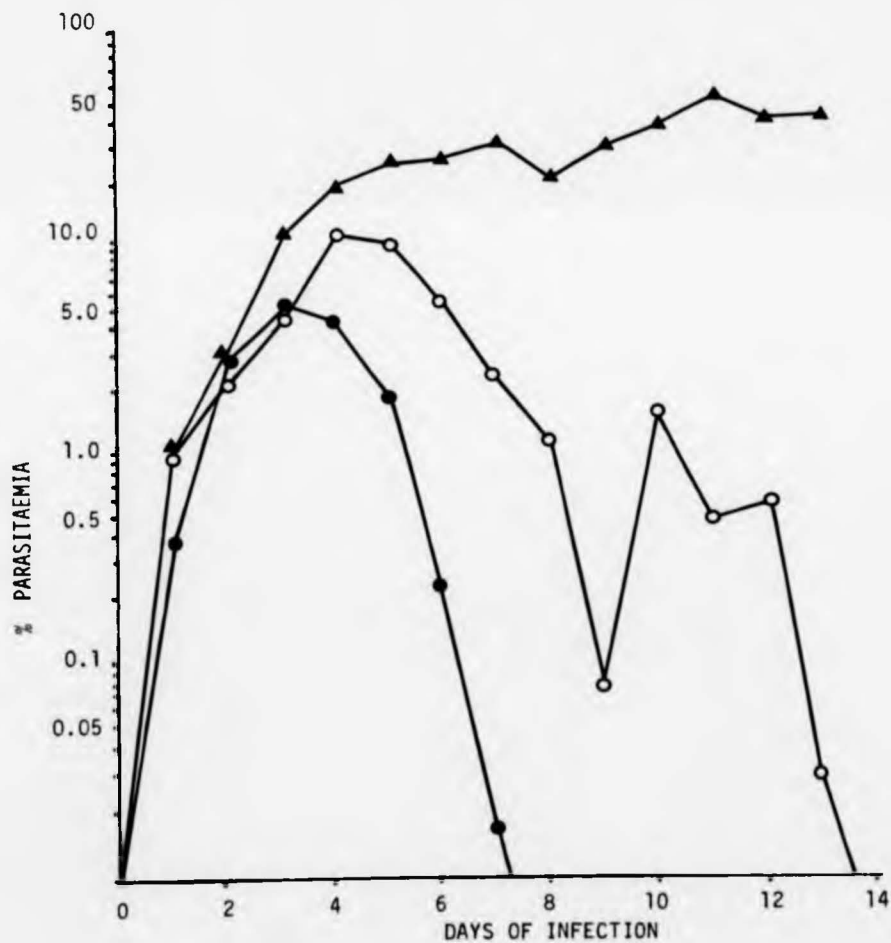


TABLE 5. Oocyst production in mosquitoes fed on mice vaccinated with the gamete vaccine and on unvaccinated mice during challenge infections given 16 weeks (group A), and 25 weeks (group B) after vaccination.

Day	Number of oocysts in 10 mosquitoes							
	<u>Unvaccinated mice</u>				<u>Vaccinated mice</u>			
	<u>Group A</u>		<u>Group B</u>		<u>Group A</u>		<u>Group B</u>	
	Experiments		Experiments		Experiments		Experiments	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
1	0	0	0	0	0	0	0	0
2	1,393	958	76	1,398	0	0	0	01
3	636	563	913	195	0	02	0	0
4	77	103	390	12	0	0	0	0
5	03	0	0	0	0	0	0	0
Total counts	2,109	1,624	1,379	1,605	0	02	0	01

The infection in the control mice in group A was highly transmissible to mosquitoes as seen by the oocyst counts (Table 5). Except for 2 oocysts found in mosquitoes fed on the vaccinated group, the infection was not transmitted.

In group B, all the control mice died of the infection between 6 and 13 days. A mean peak parasitaemia of 70% was reached in the control group. All the vaccinated mice cleared their parasitaemia with a mean clearance time of 11.0 days ( $\pm 2.2$  days)\*. The highest mean parasitaemia was 11.82%.

High oocyst counts were found in mosquitoes fed on control mice. Those fed on vaccinated mice yielded only a single oocyst throughout the infection (Table 5).

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\* Standard deviation

### DISCUSSION

In this study, mice have been successfully immunised against the transmission of P. voelii by using 3 intravenous doses of a vaccine containing formalin-fixed gametes. Subsequent experiments have shown that the transmission of a challenge infection could be completely blocked by a single and smaller dose of the gamete vaccine.

In vaccinated mice, adequate numbers of circulating gametocytes failed to produce any oocysts in mosquitoes whereas in unvaccinated mice and those vaccinated with a control reticulocyte vaccine, gametocytes which were present in comparable numbers produced very high mosquito infections. The precise nature of the reaction which prevents oocyst development in the gut of the mosquito is not known but a possible mechanism may be related to the behaviour of male gametes from vaccinated mice. Observation of microgametogenesis in blood drawn from immunised mice during a challenge infection in vitro revealed a pronounced and effective immobilisation of male gametes almost immediately after their formation, in contrast to the sustained movement of male gametes in blood drawn from unvaccinated mice. This immobilisation of male gametes would impose a severe limitation on fertilisation in vivo and could account for the consequent absence of oocysts in mosquitoes.



It is evident that the ability to immobilise gametes lies in the serum of immunised mice because the phenomenon occurred even when gametocytes from unimmunised mice were washed and resuspended in immune serum. It appears also that gametocytes from immunised mice are potentially viable and unaffected by host immunity as indicated by the fact that they exflagellate normally even in immune serum and that the entire process of microgametogenesis and gamete dispersal proceeds normally in non-immune serum.

Several experiments carried out in chickens immunised against the transmission of P.gallinaceum (Gwadz, 1976) have indicated that gamete immobilising properties are associated with host antibodies that were largely confined to the IgG fraction, and that the reaction is complement independent. In this study the nature of serum factors that are responsible for gamete immobilisation has not been investigated, but in view of the available evidence it is considered very likely that antibody is responsible for blocking transmission.

As the results suggest, it is possible that one of the mechanisms by which host immunity mediates suppression of transmission is by agglutination of male gametes in the mosquito gut. It is also possible that an interaction of host immunity with female gametes could be partly responsible for preventing fertilisation though such a reaction has not been observed in vitro.

Complement dependent lysis has been observed in gametes of P.knowlesi in the presence of immune serum in vitro (Gwadz and Green, 1978).

The intramuscular route of vaccination is effective in inducing transmission blocking immunity. It is not possible to compare the effectiveness of the intramuscular route with that of the intravenous route because a discriminating dose of the vaccine for either route has not been elucidated. Results indicate that transmission blocking immunity lasts at least 6 months. A period beyond this has not been investigated but it seems likely that immunity lasts longer.

In addition to transmission blocking immunity, the gamete vaccine also induced immunity against asexual erythrocytic stages which, though not complete, was also pronounced. It was manifested by a marked lowering of parasitaemia and a 100% survival of vaccinated mice compared to control unvaccinated mice in which the parasitaemia was higher and the infection fatal in a large proportion of mice. There are two possible explanations. First, contamination of the gamete vaccine with asexual stages might be responsible. In experiments carried out on P.knowlesi malaria (Gwadz and Green, 1978), gamete vaccination resulted in immunity to asexual parasites, but in this instance too the antigen mixture consisted of gametes and trophozoites. The alternative

proposition would be that gametes protect against asexual stages because they have protective antigens in common. This particular aspect of gamete vaccination has been investigated further and discussed in Chapter 4.

It appears that the gamete vaccine induces an anti-gamete response more readily than immunity against the asexual stages. Thus, at a lower dose of the gamete vaccine containing  $2 \times 10^6$  male gametes, vaccinated mice were completely susceptible to an asexual infection though its transmission was totally suppressed. Further, the intramuscular route of immunisation confers complete protection against transmission but almost none against the asexual erythrocytic stages. Dissociation of the 2 types of immunity was also apparent when vaccinated mice were challenged 6 months after immunisation, when immunity against asexual stages showed signs of waning whereas the ability to block transmission was almost completely retained.

Fundamental knowledge of the antigenic structure of sexual stages is lacking and it remains to be seen whether gametes and asexual erythrocytic stages share common protective antigens, but it seems more likely that antigenic similarities exist between gametes and gametocytes. It has been noted by Sinden *et al.*, (1978) that expansion of the parasite during gametogenesis necessitates an increase in surface area of at least

30%. Therefore they concluded that the plasmalemma of the activated gametocyte must be a mosaic of old and newly synthesised membranes, and that microgametocytes may also be enveloped in a mixture of membrane types or even exclusively by a new membrane synthesised at the site of their emergence. It is thus possible that new and different surface antigens are expressed by gametocytes when activated but these may or may not be expressed by gametes. In addition to surface antigens, intracellular antigenic components of which little is known also need to be considered. Evidence from studies on P. gallinaceum, in which effective anti-gamete transmission blocking immunity was induced by vaccinating with formalinised or X-irradiated gametocytes, strongly implies immunological similarities between gametes and gametocytes. More supporting evidence comes from fluorescent antibody (FA) studies on P. falciparum malaria. Carter et al., (in press) found that in sera from a population living in a hyperendemic area, FA titres against air dried gametes and gametocytes were invariably closely similar for each serum tested though titres against sexual and asexual parasites frequently differed widely in individual sera. Results to the contrary have however also been obtained from preliminary studies on the same parasite (Sinden et al., 1978).

If, as evidence suggests, antigamete antibodies are able to recognise antigen on gametocytes as well as

gametes, it is surprising that gametocytes are spared the lethal effects of transmission blocking host immunity. It is possible that while they are in the host's circulation gametocytes are completely protected by the erythrocyte 'cloak' but in the lumen of the mosquito gut they emerge from the host erythrocyte and remain exposed to host antibody for a period of at least 7-8 minutes. A possible explanation is that antigenic sites on gametocytes are of a much lower density than on gametes. This is supported by results of immunisation studies where Carter et al.. (1979a) found that preparations containing intracellular gametocytes were much less effective than those containing gametes in inducing transmission blocking immunity.

In establishing a successful transmission blocking vaccine in mice against P. yoelii this study has provided an experimental rodent model for investigating anti-gamete immunity. Results of experimental immunisation in this model have been very encouraging in that an effective block on transmission and a partial suppression of asexual parasitaemia is possible by vaccinating with a relatively crude antigen preparation without the need for an adjuvant. The feasibility of vaccination by the intramuscular route and by a single immunising dose together with the relatively long duration of immunity thus induced are features which would enhance the prospects of gamete vaccination in its applications as a method for the control of human malaria.

CHAPTER 4IMMUNISATION AGAINST ASEQUAL ERYTHROCYTIC STAGESINTRODUCTION

There is a general consensus of opinion that protective anti-malaria immunity is stage specific, though it is largely based on the knowledge that sporozoite induced immunity does not affect the development or viability of the erythrocytic stages (Nussenzweig et al., 1969b). Whether immunity against asexual erythrocytic stages induced by vaccination, protects also against pre-erythrocytic stages is not clearly known. In a study in which rhesus monkeys were immunised with P.knowlesi merozoites in Freund's complete adjuvant (FCA), a sporozoite challenge resulted in all the animals developing blood infections which as expected were effectively controlled (Richards et al., 1977). On account of there being no precise measurement of pre-erythrocytic development in this study, the results only indicate that merozoite vaccination does not completely inhibit pre-erythrocytic development, and gives no indication as to whether there was partial cross protection. However, evidence from both human and experimental malarias seem to suggest that naturally acquired resistance to erythrocytic parasites does not modify the development of exo-erythrocytic stages (Brown, 1969). Transmission

blocking immunity induced by gamete vaccination has so far been excluded from such a scrutiny. In one study (Vänderberg et al., 1972) sera of animals immunised with P.berghoi sporozoites were shown to have no detectable effect on gametocytes or ookinetes, but asexual blood stage vaccines have not been evaluated for transmission blocking immunity and neither has anti-gamete immunity been investigated for cross reactivity against other stages.

It is important to know whether different developmental stages share protective antigens because of the implications for the outcome of vaccination. Thus, to be effective, sporozoite vaccination must result in the elimination of all viable pre-erythrocytic parasites since any which mature would induce blood infections in a host which is fully susceptible to this stage of the parasite. An asexual blood stage vaccine would have the advantage that even if only partially effective it can confer protection on the host by decreasing the severity of the disease. Transmission blocking immunity per se does not confer any direct advantage upon the immunised host unless it protects against asexual blood stages as well.

Experiments presented in the previous chapter showed that the gamete vaccine afforded protection against asexual erythrocytic stages in addition to inducing transmission blocking immunity. Vaccination against

P.knowlesi malaria in rhesus monkeys using a gamete/trophozoite antigen mixture also produced immunity against asexual stages (Gwadz and Green, 1978). In both cases, this could be attributed either to the presence of asexual blood stages contaminating the vaccines or to gametes cross protecting against asexual stages by sharing protective antigens with them.

Indirect information as to whether immunological similarities exist between asexual and sexual stages may be derived from serological studies. In a study already cited in Chapter 2, where sera from humans exposed to hyperendemic malaria were investigated, Carter et al., (in press) found that several sera which had negative or low titre fluorescent antibody (FA) reactions against gametocytes mediated very high titre FA reactions against schizonts. Presumably such sera recognised antigenic components in the asexual parasites which were absent in the sexual stages. The few sera that mediated high titre reactions against gametocytes were invariably associated with similar FA titres to the asexual parasites, and sera which reacted only to sexual stages were not found. Therefore it would not be possible to conclude that any of the sera tested were capable of recognising components unique to sexual stages. In any case such information pertains to specific antibody which may have little or no relevance to protective immunity and therefore necessarily be limited in value.



The experimental work presented in this chapter has been designed to investigate stage specificity of immunity to gametes and asexual erythrocytic stages. In a recent study, Playfair et al. (1977b) introduced a method of experimental vaccination against asexual erythrocytic stages in mice using a killed vaccine. The vaccine consisted of saponin-freed formalin-fixed blood stage parasites and protected mice effectively against homologous stages of P.voelii and P.vinckei and to a lesser extent against P.berghei. They used a different and probably an agametocyaemic strain of the same parasite P.voelii nigeriensis, and methods of antigen preparation and vaccine presentation were similar to those of the gamete vaccine used in this study. Thus, a convenient analogy could be drawn between their vaccine and the asexual erythrocytic stage component of the gamete vaccine. This prompted initial investigations of this study to be based on the methods used by Playfair et al. Vaccination and evaluation methods were subsequently modified to suit the specific needs of this study.

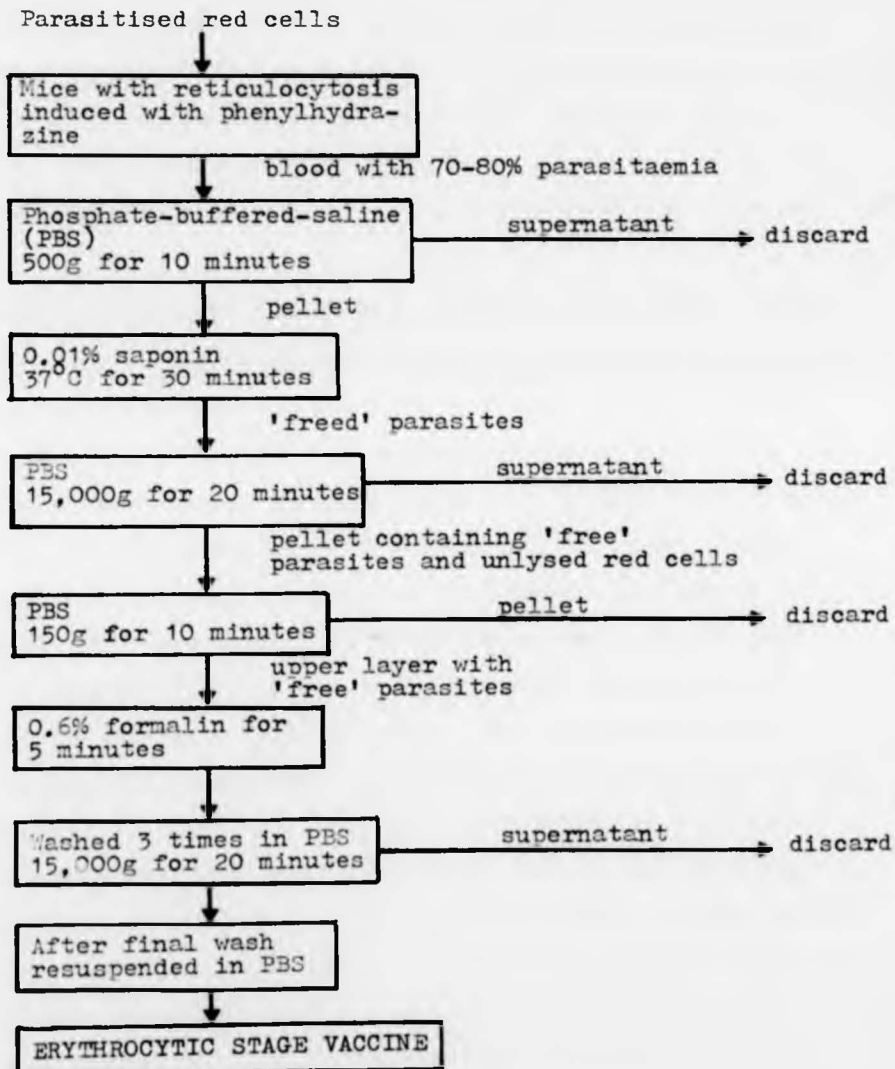
## MATERIALS AND METHODS

### Preparation of an erythrocytic stage vaccine containing asexual and sexual stages

Parasitaemias of 70-80% and gametocyte counts of 0.5 to 1.5% were induced in mice as described in Chapter 3. Mice were bled by cardiac puncture and the red cells were washed in phosphate-buffered-saline (PBS) by centrifuging at 500g for 10 minutes. In order to lyse the red cells they were suspended in 40 volumes of 0.01% saponin and incubated at 37°C for 30 minutes, the freed parasites were then washed once in PBS by centrifuging at 15,000g for 20 minutes. The supernatant was discarded and the pellet containing 'free' parasites and some unlysed red cells were resuspended in PBS and centrifuged at 150g for 10 minutes. The upper brown layer containing 'free' parasites was then removed virtually free of unlysed red cells which had pelleted. The parasites were fixed in 0.6% formalin for 5 minutes at room temperature and then washed 3 times in PBS by centrifuging at 15,000g. Counts were made in a haemocytometer using a phase contrast microscope and adjusted such that 0.2 ml contained the number of parasites required for a single dose.

Figure 9 is a flow diagram of the preparation of the erythrocytic stage vaccine.

**FIGURE 9.** Flow Diagram of Preparation of Erythrocytic Stage Vaccine containing Asexual and Sexual Stages



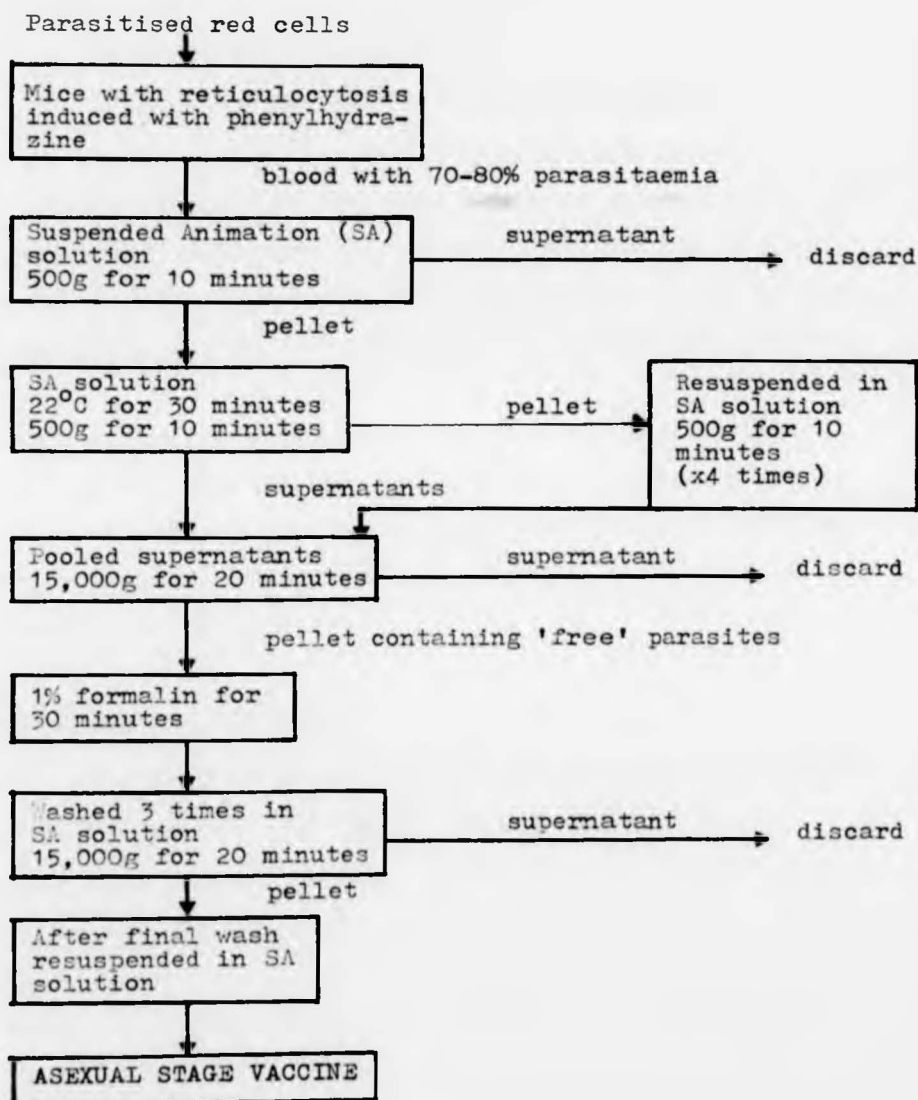
### Adjuvant

In some experiments the erythrocytic stage vaccine was administered with an adjuvant Bordetella pertussis (B. pertussis) that was obtained from Burroughs Wellcome. The preparation consisted of  $4 \times 10^{10}$  organisms per ml in 0.01% thiomersal.  $10^9$  organisms were mixed with the freshly prepared vaccine and injected into each mouse.

### Preparation of a 'pure' asexual erythrocytic stage vaccine

Mice with high parasitaemias and gametocyte counts that were induced as previously described were bled by cardiac puncture into 50 volumes of SA solution. The red cells were washed by centrifuging at 500g for 10 minutes and were resuspended in 3 volumes of SA solution and kept at room temperature for 30 minutes. The freed parasites were separated from the unlysed parasitised and non-parasitised red cells by centrifuging at 500g for 10 minutes. This procedure was repeated 4 times, resuspending the packed material in SA solution so as to recover as many free parasites as possible. The pooled supernatants were centrifuged at 15,000g for 20 minutes and the pellet containing free parasites was resuspended and fixed in a solution of 1.0% formalin for 30 minutes at room temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ). The parasites were then washed 3 times in SA solution at 15,000g. After the final wash, counts were made in a haemocytometer

FIGURE 10. Flow Diagram of Preparation of Asexual Erythrocytic Stage Vaccine



using a phase contrast microscope and adjusted such that 0.2 ml contained the number of parasites required for a single dose.

Figure 10 is a flow diagram showing the preparation of the 'pure' asexual erythrocytic stage vaccine.

The procedure adopted in the preparation was the same as that used in the preparation of the gamete vaccine except that gametogenesis was not induced. Spontaneous lysis of parasitised red cells was the source of asexual parasites, as was thought to be the case in the gamete vaccine. Artificial lysis of red cells was avoided in order to minimise gametocyte release. Giemsa stained thin films revealed that the vaccine contained asexual stage parasites, predominantly merozoites, cell debris and occasional red cells, and appeared to be free of gametocytes and gametes.

#### Assessment of immunity

Immunity to sexual and asexual erythrocytic stages were assessed by methods defined in Chapter 3.

## RESULTS

### Immunisation with an erythrocytic stage vaccine containing asexual and sexual stages

#### (i) Vaccination with an adjuvant

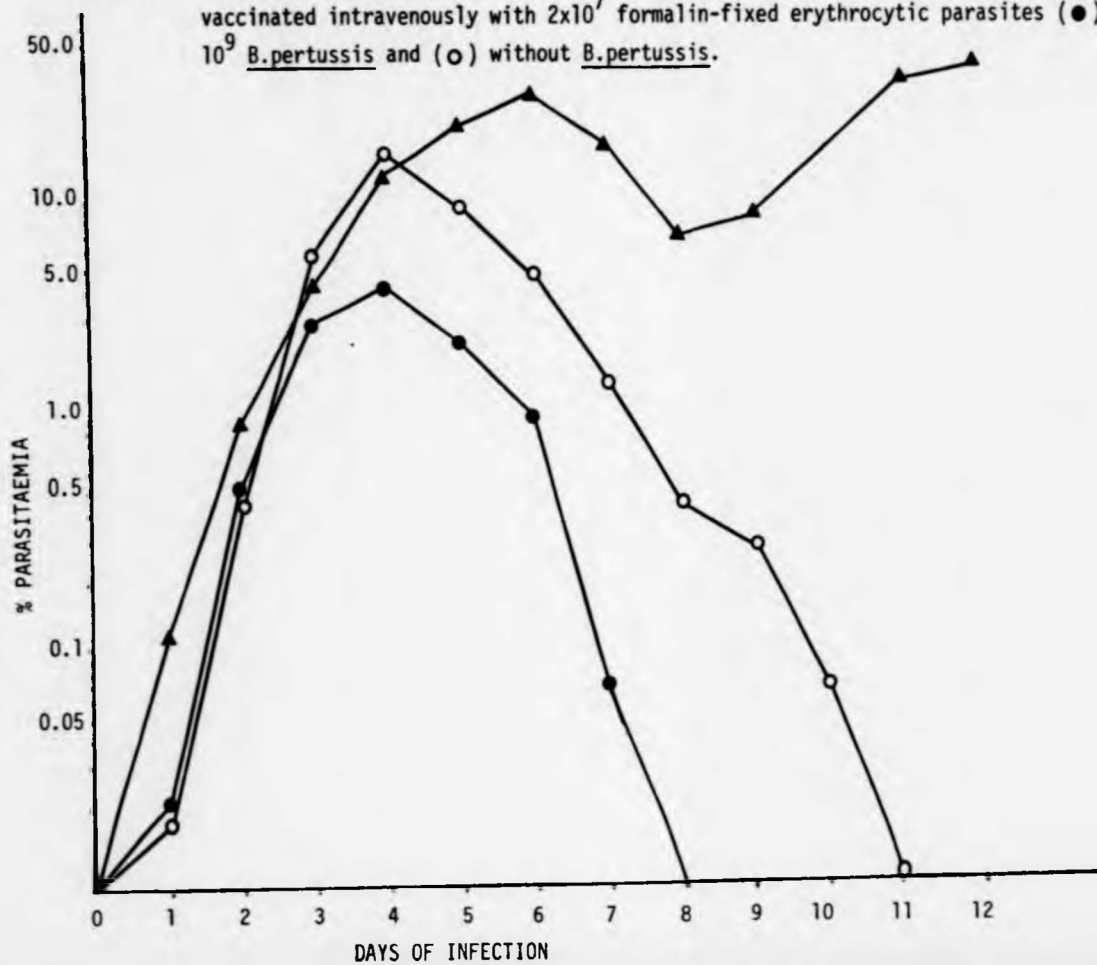
In 2 separate experiments, 7 mice were immunised with a single dose of  $2 \times 10^7$  formalin-fixed parasites and  $10^9$  organisms of B. pertussis intravenously. 5 weeks later the vaccinated mice and an equal number of age matched control animals were challenged with a blood induced infection of  $10^5$  parasitised red cells intravenously. Transmission blocking immunity was not assessed in either of these experiments.

The parasitaemias of the challenge infections in vaccinated and control mice is shown in Figure 11 (the points on the parasitaemia graph of the control mice refer to the combined results of this and the subsequent experiment). The infection was uniformly fatal in all the control mice. The highest mean parasitaemia recorded in the control mice was 65.0%. In contrast, the vaccinated mice cleared their parasitaemia by day 8 with a mean clearance time of 6.8 days ( $\pm 0.26$  days)\*, and the highest mean parasitaemia reached was only 4.11%.

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\* Standard deviation

FIG. 11. Mean parasitaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice and in mice vaccinated intravenously with  $2 \times 10^7$  formalin-fixed erythrocytic parasites ( $\bullet$ ) with  $10^9$  *B. pertussis* and ( $\circ$ ) without *B. pertussis*.





(ii) Vaccination without an adjuvant

9 mice were vaccinated, each with a single dose of  $2 \times 10^7$  formalin-fixed parasites intravenously. A challenge infection was given 3 weeks later by injecting  $10^5$  parasitised red cells intravenously. Transmission blocking immunity was not assessed.

The course of the challenge infections in control and vaccinated mice is shown in Figure 11. The infection was fatal in all the control mice, with a mean parasitaemia reaching as high as 80.0%. The vaccinated mice cleared their parasitaemia completely, the mean clearance time was 9.1 days ( $\pm 0.48$  days).\* The highest mean parasitaemia recorded in the vaccinated mice was 16.7%.

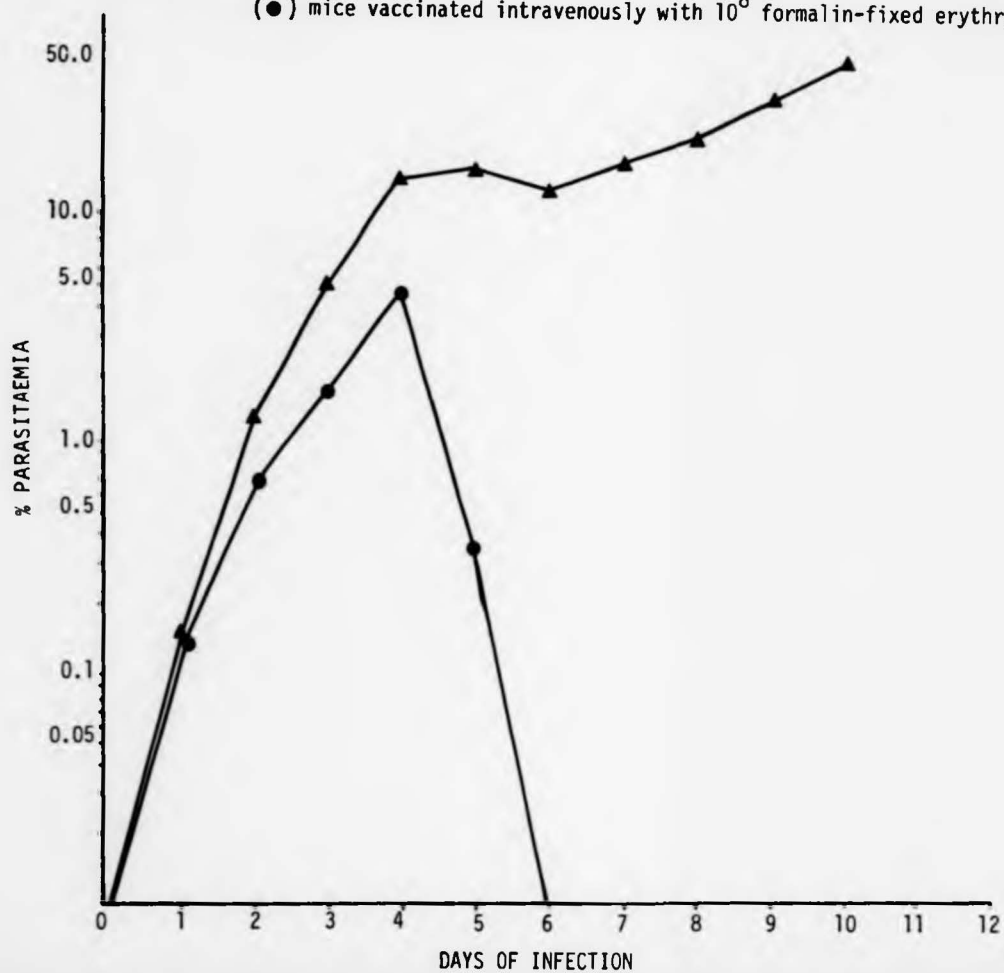
3 mice were vaccinated with a single dose of  $10^8$  formalin-fixed parasites intravenously. A challenge infection of  $10^5$  parasitised red cells was given intravenously to the vaccinated and control mice 3 weeks later and immunity to sexual as well as asexual stages was assessed.

2 of the control mice died of the infection. The other cleared its parasitaemia after a prolonged infection on day 24. The highest mean parasitaemia recorded in the animal that survived was 62.0%. All the vaccinated mice cleared their parasitaemia on day

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\* Standard deviation

FIG. 12.1. Mean parasitaemias of challenge infections in (▲) unvaccinated mice and in (●) mice vaccinated intravenously with  $10^8$  formalin-fixed erythrocytic parasites.



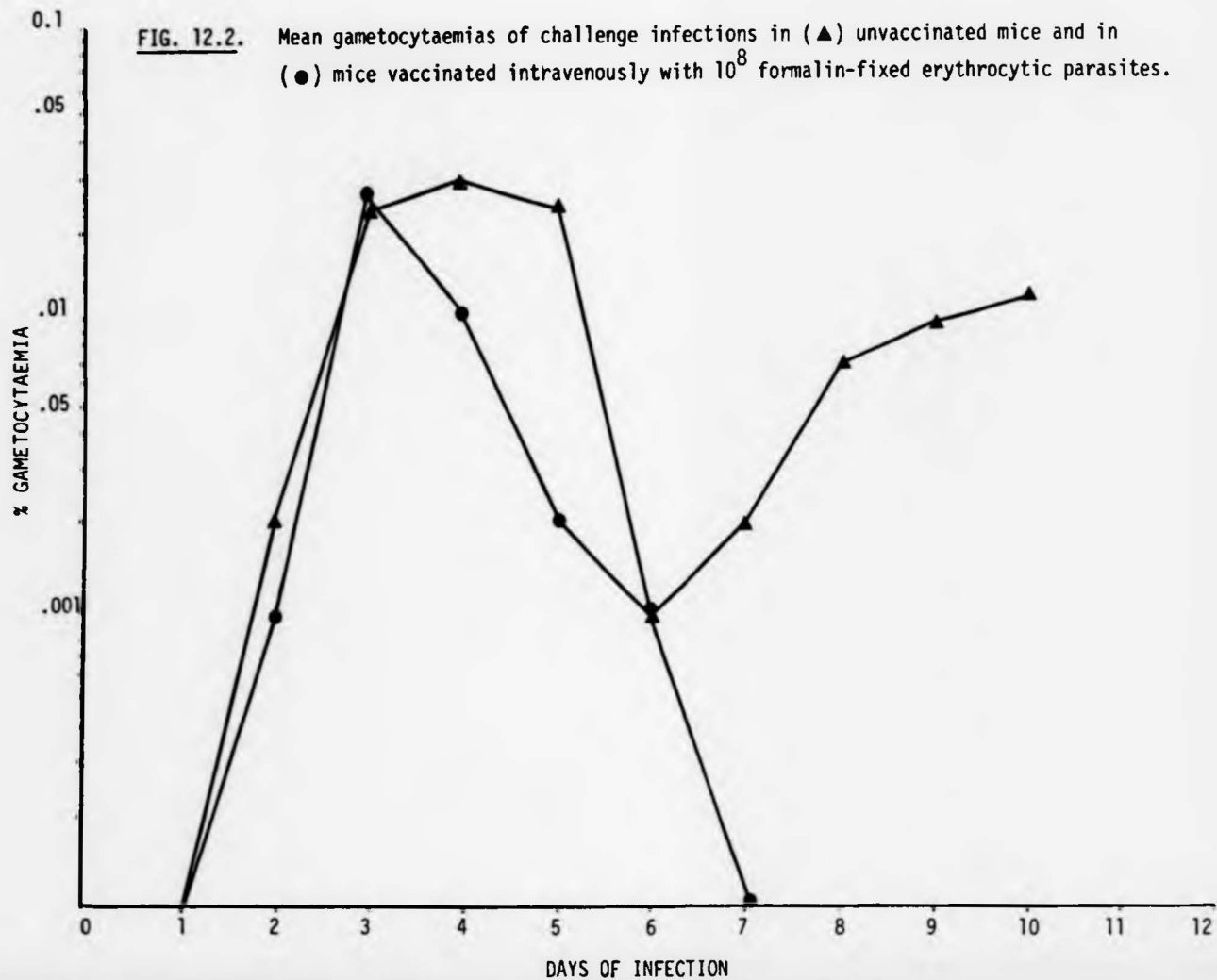


TABLE 6. Oocyst production in mosquitoes fed on mice vaccinated with  $10^8$  erythrocytic parasites (sexual and asexual) and on unvaccinated mice.

Number of oocysts in 10 mosquitoes		
Day	Unvaccinated mice	Vaccinated mice
1	0	0
2	58	0
3	543	340
4	139	0
5	0	0
Total counts	740	340

7. The highest mean parasitaemia recorded was 4.5%. The mean parasitaemias and gametocytaemias of vaccinated and control mice are shown in Figures 12.1 and 12.2 respectively. Cyst counts in mosquitoes fed on vaccinated mice were reduced to 46.08% of that in mosquitoes fed on control mice (Table 6).

Immunisation with an asexual erythrocytic stage vaccine (devoid of sexual stages)

8 mice were vaccinated intravenously with the 'pure' asexual erythrocytic stage vaccine in a single dose; 5 mice with a dose of  $10^6$  and 3 with a dose of  $2 \times 10^6$  formalin-fixed parasites. All the vaccinated mice and the 8 unvaccinated control mice were challenged 3 weeks after vaccination with  $2 \times 10^5$  parasitised red cells intravenously and immunity to sexual and asexual stages was assessed.

In the experiment where the vaccinating dose was  $10^6$  parasites, the challenge infection was fatal in all but 1 of the control mice. Death occurred between 8 and 15 days. The animal that cleared its parasitaemia did so on day 26. A mean peak parasitaemia of 72% was observed in the control group. 2 of the vaccinated mice died on days 6 and 8 of the infection. The others cleared their parasitaemia with a mean clearance time of  $9.6 (\pm 1.1 \text{ days})^*$ . The highest mean parasitaemia in the vaccinated

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\* Standard deviation

FIGURE 13.1

Mean parasitaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice, in mice vaccinated with ( $\triangle$ ) a single dose of the gamete vaccine containing  $2 \times 10^7$  male gametes and single doses of ( $\bullet$ )  $10^6$  and ( $\circ$ )  $2 \times 10^6$  formalin-fixed asexual stage parasites.

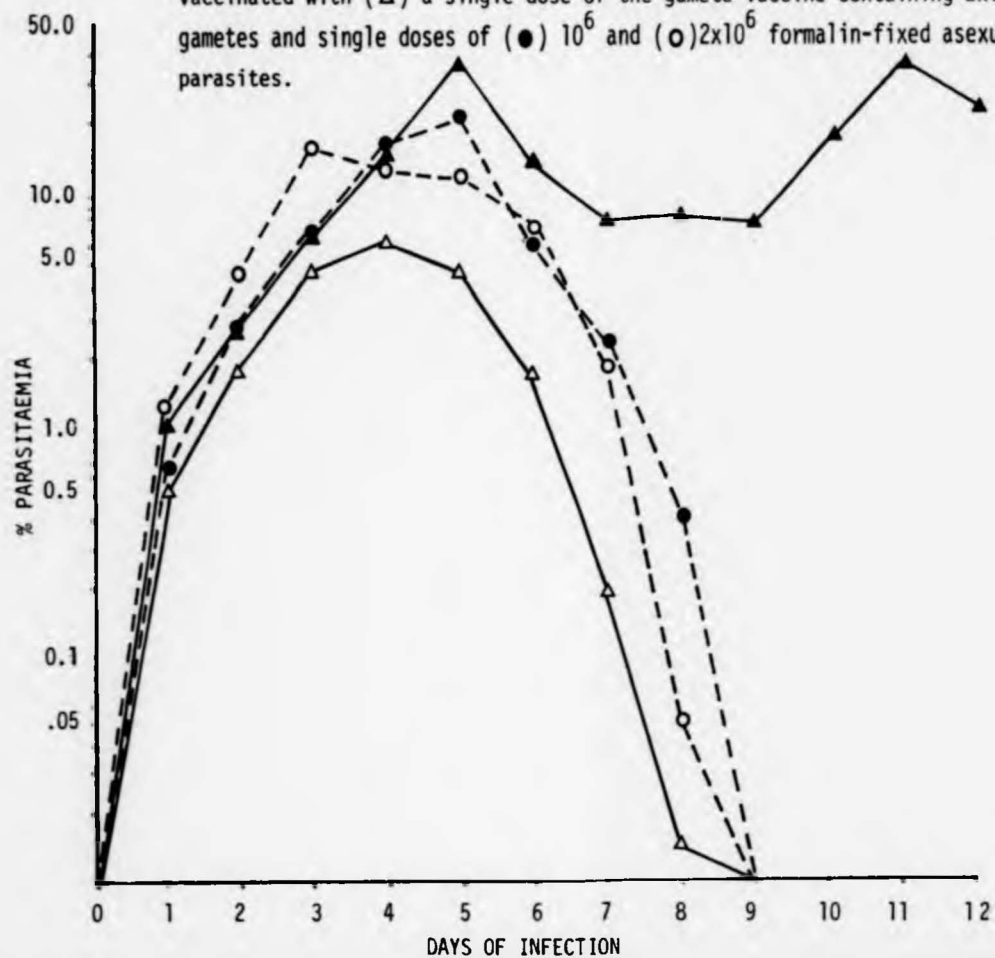
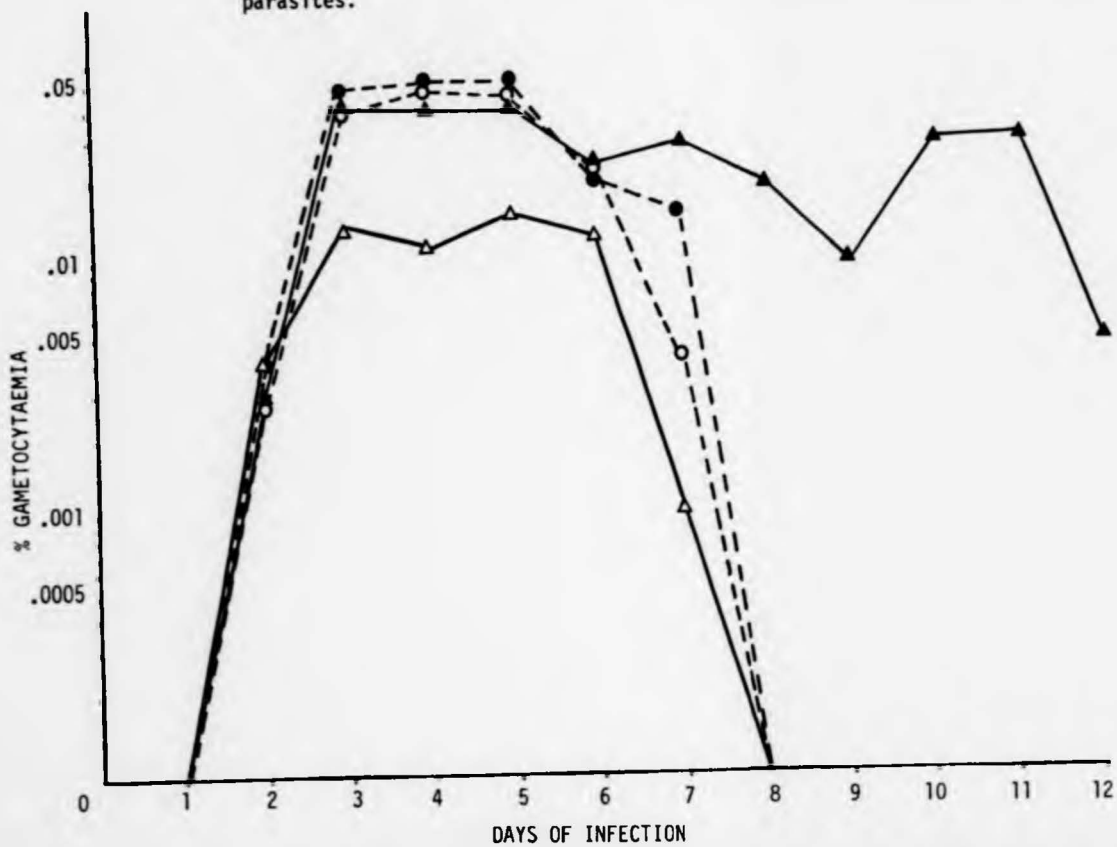


FIG. 13.2. Mean gametocytaemias of challenge infections in ( $\blacktriangle$ ) unvaccinated mice and in mice vaccinated with ( $\triangle$ ) a single dose of gamete vaccine containing  $2 \times 10^7$  male gametes, and single doses of ( $\bullet$ )  $10^6$  and ( $\circ$ )  $2 \times 10^6$  formalin-fixed asexual erythrocytic stage parasites.



**TABLE 7.** Oocyst production in mosquitoes fed on mice vaccinated with the 'pure' asexual erythrocytic stage vaccine containing  $10^6$  (experiment 1) and  $2 \times 10^6$  (experiment 2) parasites, and on unvaccinated control mice.

Number of oocysts in 10 mosquitoes				
Day	<u>Experiment 1</u>		<u>Experiment 2</u>	
	Control mice	Vaccinated mice	Control mice	Vaccinated mice
1	0	0	0	0
2	70	1,505	53	13
3	1,275	306	941	981
4	32	25	05	12
5	50	0	0	0
Total counts	<u>1,427</u>	<u>1,836</u>	<u>999</u>	<u>1,006</u>

Differences in the oocyst counts between the vaccinated and control groups are not statistically significant.



mice was 20.7% (Figures 13.1 and 13.2).

The total oocyst counts obtained by feeding mosquitoes on control mice was 1,427 and those obtained by feeding mosquitoes on vaccinated mice was 1,836 (Table 7).

In the experiment where the vaccinating dose was  $2 \times 10^6$  parasites, the control mice cleared their parasitaemia after day 19. The highest mean parasitaemia recorded was 40.0%. The vaccinated mice cleared their parasitaemia with a mean clearance time of 8.6 days ( $\pm 0.33$  days)\*. The highest mean parasitaemias recorded was 15.1%. In 2 mice there was a recrudescence between days 13 and 19, in which the mean parasitaemia did not exceed 3.2% (Figures 13.1 and 13.2).

The challenge infection of control mice produced in mosquitoes a total of 999 oocysts and that of the vaccinated mice a total of 1,006 oocysts (Table 7).

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\* Standard deviation

## DISCUSSION

These limited experiments indicate that an effective immunity can be raised in mice against P.voelii by vaccinating with formalin-fixed saponin-lysed parasitised blood. The immunity directed against asexual erythrocytic stages was enhanced by using killed B.vertussis as an adjuvant. These findings confirm the results of Playfair et al., (1977b) who obtained protection in mice against P.voelii using a similar vaccine, and there was a striking similarity between levels of immunity induced by comparable doses of the vaccines used in this study and in the experiments performed by Playfair et al., despite the parasites being of different strains.

It is appropriate at this point to consider the results of Playfair and his co-workers on the mechanisms of immunity that operate in mice vaccinated against P.voelii, because such an investigation was not undertaken in this study. They found that immunity involved both antibody and cell-mediated responses; there was good correlation between protection and immunofluorescent anti-parasitic antibody titres in vaccinated mice during a challenge infection. This correlation was maintained even when antiserum was passively transferred to normal recipients, though protection was not as effective (Playfair and De Souza, 1979). Cell mediated immunity in response to vaccination was demonstrated by the classical delayed hypersensitivity skin test, and more importantly,

immunologically primed cells were found to home to the liver more than to the spleen during recovery from a challenge infection (Playfair et al., 1979). The authors proposed that the liver is an important site for parasite destruction, and in drawing a parallel with anti-sporozoite vaccination, stressed the possibility of immunity induced by both types of vaccinations having a common effector pathway.

In the first experiment in which immunity to both stages was assessed, results indicated that the vaccine induced also partial immunity against transmission. The fact that parasitised blood from which the vaccine was derived contained gametocytes makes it very likely that these were responsible for the anti-gamete immunity. In fact, successful immunisation against transmission has been achieved in P. gallinaceum by vaccinating with formolinsed or X-irradiated parasitised blood containing an even lower ratio of gametocytes to asexual stages (Gwadz, 1976).

Immunisation with the vaccine from which all sexual stages were excluded was performed with doses that contained approximately the same number of asexual parasites as were present in a standard dose of the gamete vaccine. Vaccination afforded partial protection against asexual blood stages, and the level of immunity was about the same as that induced by an equivalent dose of the gamete vaccine (Figure 13.1). This difference may be

attributable to the gametes, due possibly to a very low level of cross protection. Immunity against asexual blood stages induced by the gamete containing vaccine was probably due largely to homologous stages contaminating the vaccine, rather than to cross protection from gametes.

Immunisation with the 'pure asexual stage vaccine did not protect mice against transmission of a challenge infection. The dose of asexual parasites used for vaccination was small and even the homologous immunity it induced was partial; it is difficult to predict whether cross protection could be obtained from a larger dose. Nevertheless, these results strongly indicate that protective antigens are not shared to an appreciable extent by sexual and asexual blood stages; anti-gamete immunity is stage specific.

CHAPTER 5A STUDY OF THE INFECTIOUSNESS OF *P. YOELII* TO THE VECTORINTRODUCTION

Infectiousness of malaria to susceptible mosquitoes has been discussed in Chapter 2. It is considered as an entity which is determined by the interaction of many factors, pertaining to both the parasite and its vertebrate host, some of which have been shown to affect it, and others which can be presumed to do so.

During the course of this study, it was revealed that the infectivity of *P. yoelii nigeriensis* in mice to *Anopheles stephensi* mosquitoes conformed to a well defined pattern, which offered a suitable opportunity for the study of infectiousness. In this chapter, studies primarily designed for vaccination have been extended in an endeavour to account for the pattern of infectivity.

## MATERIALS AND METHODS

The gametocyte counts of 50 primary infections of P. yoelii were studied, all of which were initiated with parasitised red cells in a dose of  $2 \times 10^5$  for each mouse, given intravenously. Mosquitoes were fed from days 1 to 6 of the infection on 20 mice and on finding that transmission only occurred from the second to the fifth day, feeding was restricted to this period in subsequent experiments. In 3 infections, mosquitoes were fed on days 9, 12, 14 and 20. In all the experiments mosquitoes were fed between 10.00 and 13.00 hours (GMT) and the experimental procedures involved have been described in Chapter 3.

### Quantification and qualitative assessment of microgametogenesis

Microgametogenesis was quantified by counting the number of exflagellating male gametocytes in wet preparations of blood. Using an automatic pipette, 5 microlitres of tail blood was placed on a microscope slide soon after withdrawal and covered with a 22x22mm coverslip. By gently applying pressure the blood was distributed as evenly as possible throughout the area under the coverslip and the slide was taken for examination immediately. The central area under the coverslip where red cells were tightly packed in a monolayer was observed at a magnification of x1000 using a phase

contrast microscope, and at intervals of a minute the number of exflagellating male gametocytes were counted in 10 microscope fields. For each infection, the highest of these 10 counts was taken as the 'exflagellation count' on that day. A centre of movement created by the microgametocyte was taken as representing a single exflagellating male gametocyte, and was distinguished with ease from the movement of free microgametes which also created disturbances in the red cell layer; these were distinct in being of a fleeting nature and of a lower intensity. Counts were made during the first 10 minutes, by the end of which period all microgametocytes had commenced exflagellation; beyond this period there was not only a sharp decline in the number of such events but also an increase in the number of free male gametes which rendered counting more difficult.

For qualitative analysis of microgametogenesis, a thinner wet blood film was prepared using 2 microlitres of blood spread under a 22x22 mm coverslip, and many fields were examined under phase contrast optics.

Recombination of infected red cells and plasma or serum from different donors

The procedure has been described in Chapter 3.

## RESULTS

The mean gametocytaemias and parasitaemias of mice are graphically represented in Figure 3 (page 70). On day 1, gametocyte counts were below detectable levels ( $<0.001\%$ ) in all infections, and on day 2 ranged from  $0.001\%$  to  $0.01\%$  in  $93\%$  of infections. This rate of increase was maintained till day 3 after which gametocyte counts remained within the  $0.01\%$  to  $0.1\%$  range during the first 2 weeks showing only minor fluctuations. As is evident in Figure 3, the gametocytaemia was almost parallel to the parasitaemia during the course of an infection.

In a few mice that survived the infection beyond 2 weeks, higher gametocyte counts were reached. In  $83\%$  of all infections studied, the highest gametocyte counts reached were below  $0.08\%$ , and in  $12\%$  between  $0.08\%$  and  $0.2\%$ . In only  $5\%$  of infections did it reach values between  $0.2\%$  and  $0.8\%$ , and these were invariably associated with parasitaemias between  $60\%$  and  $80\%$ , though such parasitaemias were recorded in  $27\%$  of infections.

In mice pre-treated with phenylhydrazine (see Chapter 3, page 55) gametocyte counts of  $0.5\%$  to  $1.5\%$  were consistently reached in association with parasitaemias of  $70\%$  to  $80\%$ .



### Infectivity to mosquitoes

The mean oocyst counts per mosquito gut, produced by infections on days 1 to 6 are tabulated (Table 8). The relationship that the infectivity to mosquitoes bears to the gametocyte counts and to the duration of infection is shown graphically in Figures 14 and 15 using 2 parameters of infectivity, viz., the absolute oocyst counts and the percentage of mosquitoes infected. The mean oocyst counts per mosquito gut were zero on day 1, and 74.7 and 179.0 on days 2 and 3 respectively, yet in 6 of the 19 experiments, the mean oocyst counts were higher on day 2 than on day 3. Thereafter, oocyst counts declined to 30.2 on day 4, 11.5 on day 5 and zero on day 6. The percentage of mosquitoes infected on different days of infections also shows a similar pattern (Figure 15). 66% of mosquitoes were infected on day 2, and 75% on day 3. Thereafter, the percentage of mosquitoes infected declined to 34.1% on day 4, 8% on day 5 and zero on day 6.

### Qualitative assessment of microgametogenesis and gamete dispersal in blood

Microgametogenesis and gamete dispersal were observed in 15 infections on each of the first 10 days.

The terms used to describe these processes are employed strictly according to definitions given in Chapter 3 (pages 74 and 77).

TABLE 8. Mean numbers of oocysts ( $\pm$  standard error of mean) produced in mosquitoes by feeding on infected mice from days 1-5 of infections.

	DAYS				
	1	2	3	4	5
1	0	163.8 $\pm$ 36.8	49.6 $\pm$ 14.8	4.0 $\pm$ 1.9	6.2 $\pm$ 2.4
2	0	216.2 $\pm$ 64.8	128.7 $\pm$ 55.5	0	0
3	0	11.4 $\pm$ 6.0	49.6 $\pm$ 20.8	140.6 $\pm$ 47.8	0
4	0	85.7 $\pm$ 43.1	246.3 $\pm$ 47.2	4.5 $\pm$ 3.2	0
5	0	85.9 $\pm$ 36.7	27.0 $\pm$ 11.0	0.2 $\pm$ 0.2	0.5 $\pm$ 0.5
6	0	139.3 $\pm$ 48.5	63.6 $\pm$ 35.6	7.7 $\pm$ 7.4	0.3 $\pm$ 0.3
7	0	87.8 $\pm$ 41.9	147.0 $\pm$ 56.6	60.7 $\pm$ 34.2	0
8	0	3.3 $\pm$ 2.1	164.5 $\pm$ 63.8	62.0 $\pm$ 32.4	0
9	0	5.0 $\pm$ 2.6	116.4 $\pm$ 34.6	1.4 $\pm$ 0.9	0.6 $\pm$ 0.6
10	0	129.6 $\pm$ 51.9	134.6 $\pm$ 67.1	37.2 $\pm$ 15.5	1.0 $\pm$ 0.6
11	0	63.0 $\pm$ 30.5	51.8 $\pm$ 23.9	15.7 $\pm$ 10.0	0
12	0	13.5 $\pm$ 7.2	74.3 $\pm$ 31.1	18.2 $\pm$ 12.1	0.5 $\pm$ 0.4
13	0	7.0 $\pm$ 4.7	127.5 $\pm$ 64.4	3.2 $\pm$ 1.3	5.0 $\pm$ 4.7
14	0	5.3 $\pm$ 2.0	94.1 $\pm$ 37.9	0.5 $\pm$ 0.4	0
15	0	95.8 $\pm$ 57.9	56.3 $\pm$ 18.7	10.3 $\pm$ 6.8	0
16	0	7.3 $\pm$ 3.3	91.3 $\pm$ 55.6	39.0 $\pm$ 21.6	0
17	0	139.8 $\pm$ 61.3	19.5 $\pm$ 7.6	1.2 $\pm$ 0.9	0
18	-	87.8 $\pm$ 41.9	147.0 $\pm$ 56.6	60.7 $\pm$ 34.2	0
19	-	71.6 $\pm$ 19.9	336.8 $\pm$ 75.2	116.1 $\pm$ 21.9	8.0 $\pm$ 6.4

FIGURE 14. Infectivity of 50 primary infections of *P.yoelii* in mice to *A.stephensi* mosquitoes.

▨ - mean numbers of oocysts per mosquito gut.  
 (●) - mean gametocyte counts.

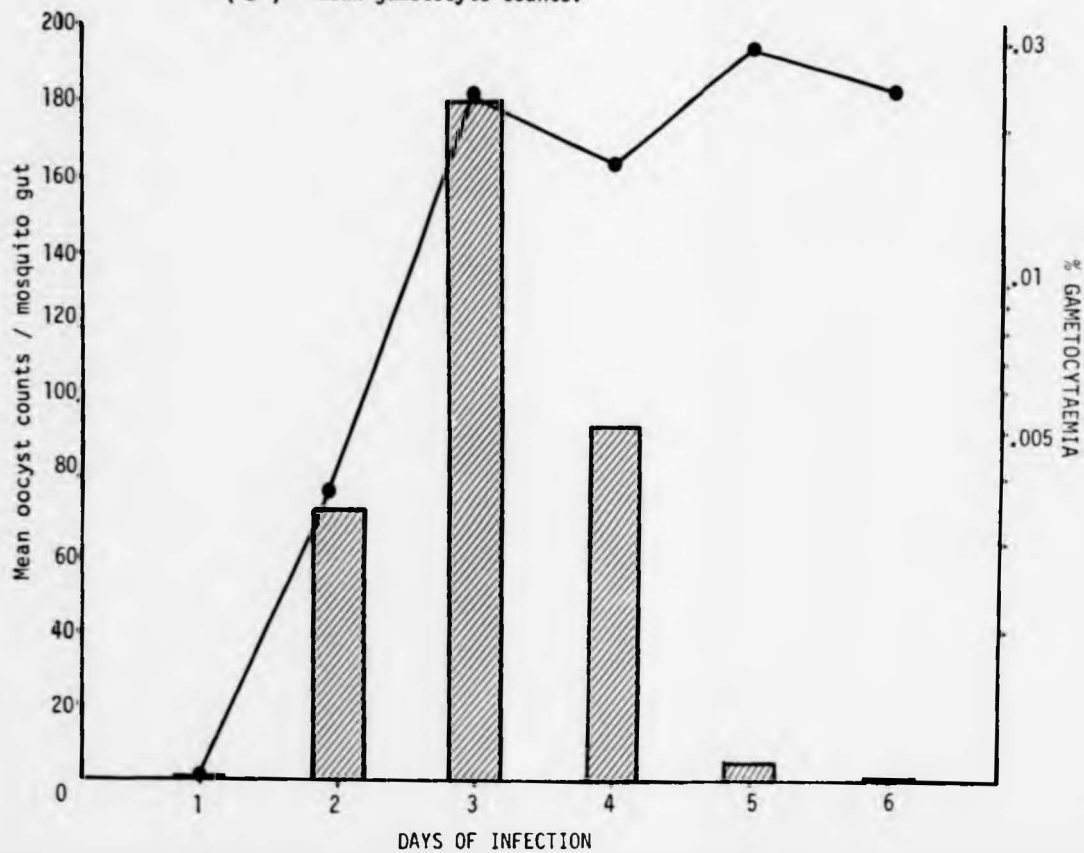
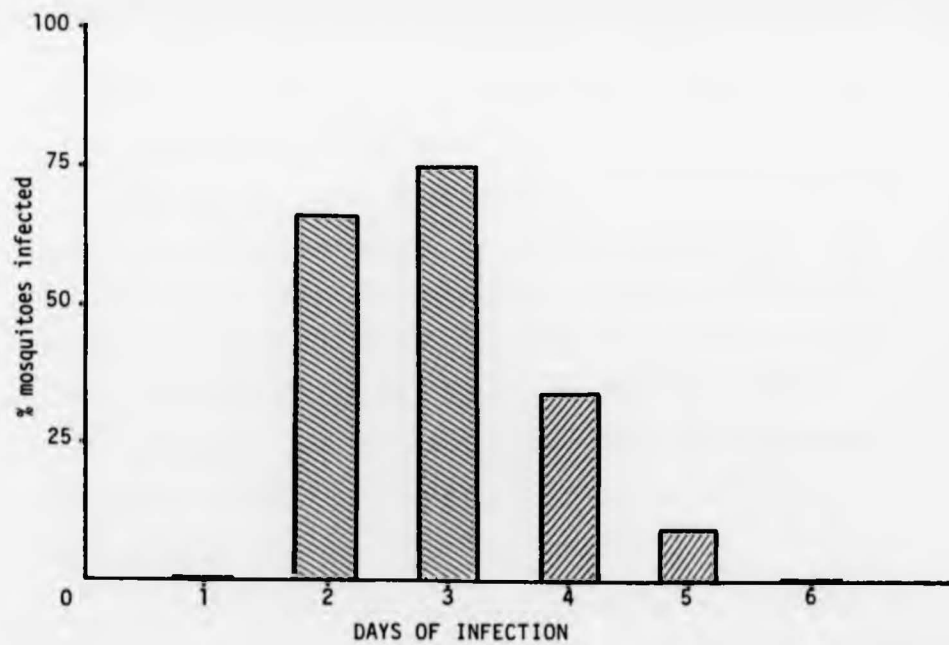


FIGURE 15. Percentage of mosquitoes infected when fed on mice during the first 6 days of infections of P.yoelii.



In addition, a measure of gametocyte quality designated by the term 'viability' is introduced in this study. It refers to a biological state of the gametocyte which is conducive to gamete development, but is used in preference to, and as distinct from the term 'maturity' which also reflects on the ability of a gametocyte to undergo gametogenesis. 'Maturity' has a connotation of age and is a property which reflects solely on the intrinsic developmental process of a gametocyte, whereas 'viability' though it necessarily pre-supposes a functionally mature stage of the gametocyte allows recognition of the fact that extrinsic factors can affect the ability of a gametocyte to develop into gametes, and therefore adds a new dimension to this quality.

The processes of activation and exflagellation can both be used as indicators of gametocyte viability, but the subsequent phases of microgametogenesis, the escape of gametes and their dispersal does not reflect on the state of a gametocyte but rather on that of gametes.

Following are descriptions of microgametogenesis and gamete dispersal on different days of infections.

#### Days 2 and 3

In all infections studied, activation, exflagellation, escape of microgametes and gamete dispersal occurred normally. These have been described in Chapter 5 (page 74).

Day 4

In 10 of the 15 infections, microgametogenesis and gamete dispersal occurred normally.

In the other 5, activation and exflagellation proceeded normally but the behaviour of microgametes was strikingly different thereafter. The vigorous movements of microgametes at the end of exflagellation gradually became diminished and ceased completely while they were still attached to the residual cytoplasm of the gametocyte, this being accomplished in a period of time ranging from 5 to 25 minutes after exflagellation. In most instances, gametes were able to escape despite diminished movement, but free gametes showed very sluggish movements enabling their path to be followed with ease, and became immobile usually in less than a minute after being freed.

Two distinct patterns of gamete immobilisation were identifiable on day 4. One was a general sluggishness and gradual slowing of movements. In the other, microgametes appeared to get fixed to the overlying coverslip at points along their length, thereby impeding movement, which was of a jerky and abrupt manner before completely ceasing.

Day 5

10 of the infections showed no signs at all of microgametogenesis. In the other 5, activation was rarely

identified after 4 minutes, and only in 3 of these infections did it progress to exflagellation. In these, all gametes were immobilised within 10 minutes of exflagellation, either before or after their escape. The phenomenon of surface fixation was observed in these infections.

#### Day 6

In only 2 of the 15 infections were there signs of activation, and even in these, exflagellation was not initiated. The other 13 infections showed no signs at all of microgametogenesis.

#### Days 7 and 8

In 11 infections there were no signs at all of microgametogenesis. In 4, activation was observed but in only 2 did the gametocytes proceed to exflagellate, and in these gametes were immobilised within 10 minutes.

#### Days 9 and 10

On both days and in all infections studied, activation and exflagellation occurred normally, but all gametes were immobilised within 10 minutes of exflagellation. Immobilisation was associated with surface fixation.

#### Quantification of microgametogenesis

The relative numbers of gametocytes that exflagellate in vitro in blood on each of the first 10 days of

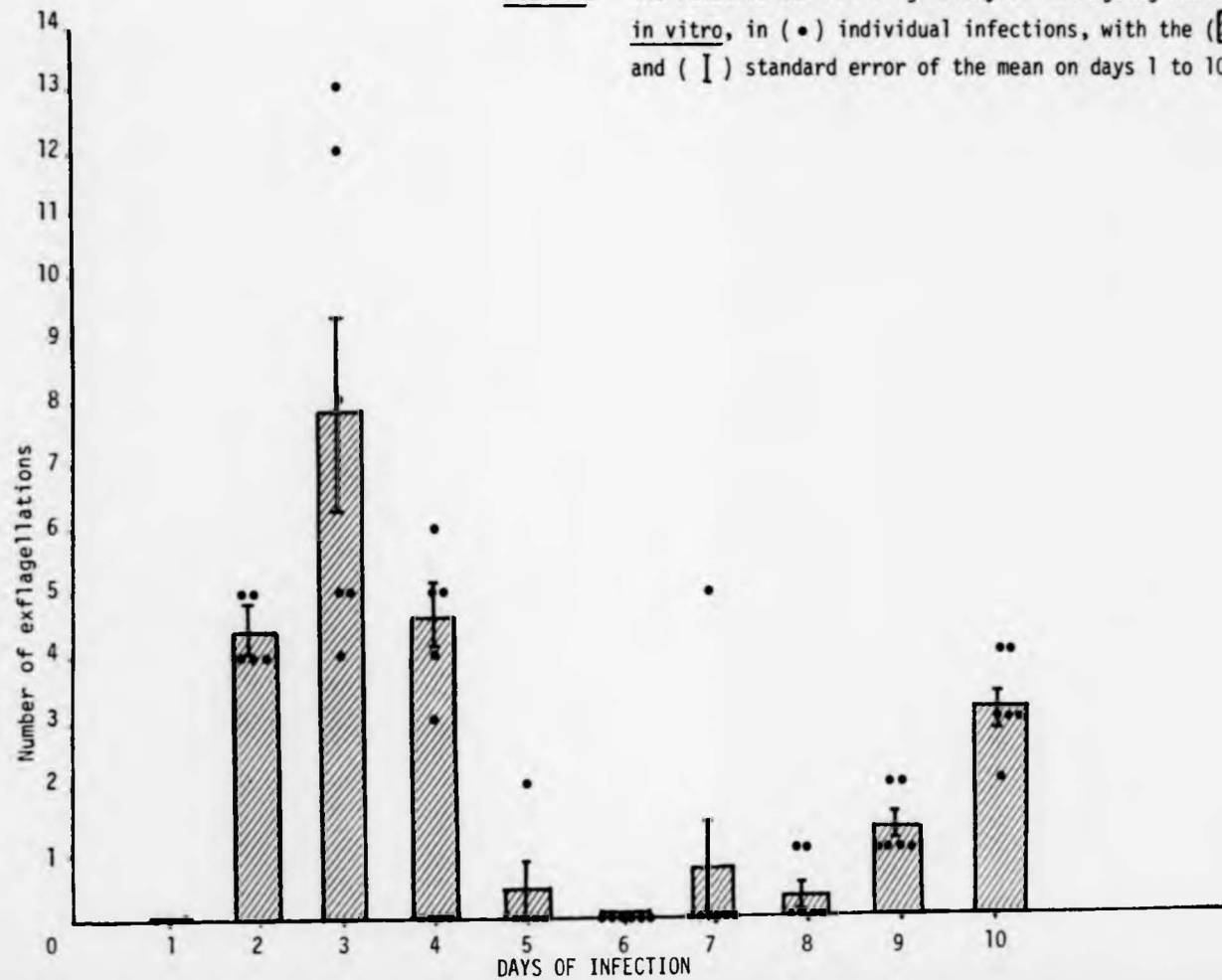
infections are set out in Figure 16. On day 1 exflagellation was not observed. On days 2 and 3 the numbers rose, and thereafter declined to very low levels from 5 to 8 days. On days 9 and 10 the levels began to rise again.

The purpose of quantifying exflagellation was merely to present more effectively one of the findings already obtained by qualitative assessment of microgametogenesis, this being the striking reduction in the number of gametocytes that exflagellated between days 4 and 8 despite there being hardly any changes in the absolute gametocyte counts in the circulation.

For comparative purposes, exflagellating gametocytes were quantified in relation to a fixed volume of blood. However, the variation in red cell counts that occurs as a result of anaemia during the course of an infection has not been accounted for, which raises the question whether the counts on different days (Figure 16) are strictly comparable. However, on day 6, in none of the infections studied was exflagellation seen (despite a large number of microscopic fields being examined); even if the reduction in the red cell count had been allowed for, the exflagellation count would have been zero. Similarly, on days 7 and 8, only in 2 out of 15 infections was exflagellation observed. It is very unlikely therefore that taking account of anaemia



FIG. 16. The relative numbers of gametocytes undergoing exflagellation in vitro, in (•) individual infections, with the (▨) mean and ( I ) standard error of the mean on days 1 to 10.



would have altered the conclusions.

Qualitative assessment of microgametogenesis and gamete dispersal in different permutations of cells and serum

In at least 2 infections, red cells were washed and resuspended in their own serum on each day from the 2nd to the 10th days. Gametogenesis and gamete dispersal was found to proceed in the same manner as in blood on corresponding days.

In 2 infections tested, gametocytes from days 2 and 3, in serum from days 9, 10, 12, 14 and 30, all behaved in the following manner. Activation and exflagellation occurred normally, but during a span of time that varied from about 30 seconds to 30 minutes gametes were immobilised, either while attached to the parent gametocyte or after being freed. The efficiency with which gamete immobilisation occurred varied considerably and bore no apparent correlation with the day of the infection from which serum was obtained.

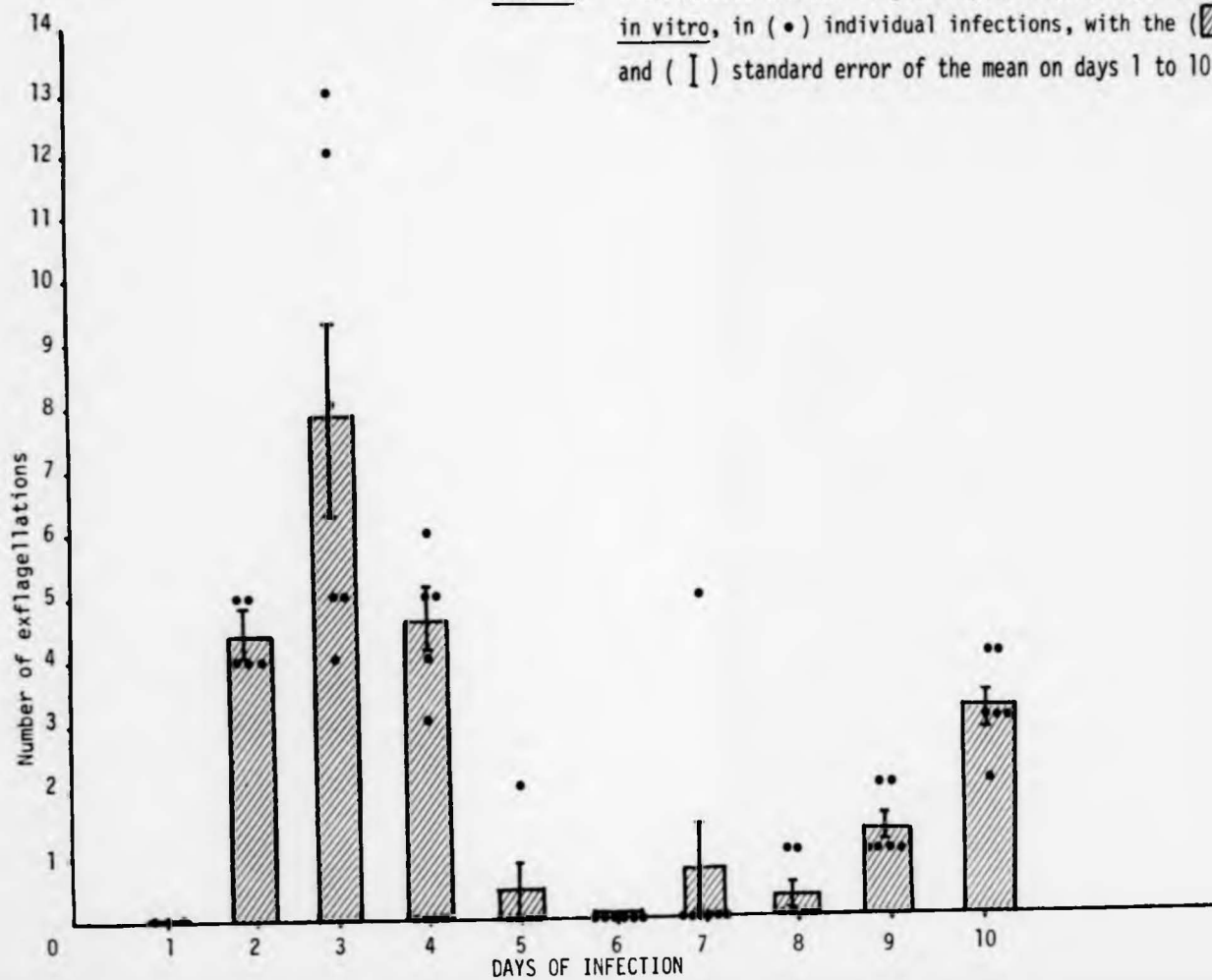
Gametocytes from days 2 and 3, when tested against serum from day 4 from an infection in which whole blood on the same day allowed a normal pattern of gametogenesis and gamete dispersal, behaved as they did in their own serum. In serum from day 4 of an infection in which gametes were immobilised, gametocytes from days 2 and 3 were similarly immobilised.

TABLE 9.

## GAMETOCYTES

SERUM	DAYS	2 and 3	4	5 and 6	7 and 8	9 and 10
		2 and 3	Normal Activation and exflagellation occurred normally. Gametes escaped and dispersed freely.	Normal	Activation occurred only in a few infections. Exflagellation did not occur at all.	Activation or exflagellation did not occur at all.
	a	Normal	Normal			
	4 b	Activation and exflagellation occurred normally. Gametes immobilised.	Activation and exflagellation occurred normally. Gametes immobilised.	Not done	Not done	Not done
	5 and 6	Not done	Not done	On day 5, activation occurred in a few infections, and exflagellation in a very few. On day 6 not even activation occurred in any.	Not done	Not done
	7 and 8	Not done	Not done	Not done	In most infections not even activation occurred. In a few, activation and exflagellation occurred, but gametes were immobilised.	Not done
	9 10 11 12 14 30	Activation and exflagellation occurred normally. Gametes were immobilised	Activation and exflagellation occurred normally Gametes were immobilised	Activation occurred in a few infections, exflagellation did not occur at all.	Activation and exflagellation did not occur at all.	Activation and exflagellation occurred. Gametes were immobilised.

FIG. 16. The relative numbers of gametocytes undergoing exflagellation in vitro, in (•) individual infections, with the (▨) mean and ( I ) standard error of the mean on days 1 to 10.



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Gametocytes from days 2 and 3, when tested against serum from day 4 from an infection in which whole blood on the same day allowed a normal pattern of gametogenesis and gamete dispersal, behaved as they did in their own serum. In serum from day 4 of an infection in which gametes were immobilised, gametocytes from days 2 and 3 were similarly immobilised.

TABLE 9. Summary of microgametogenesis and gamete dispersal in different permutations of gametocytes and serum during the first 10 days of infections. (On day 4, a = 2/3 of infections and b = 1/3 of infections studied).

TABLE 9.

## GAMETOCYTES

DAYS	GAMETOCYTES				
	2 and 3	4	5 and 6	7 and 8	9 and 10
2 and 3	Normal Activation and exflagellation occurred normally. Gametes escaped and dispersed freely.	Normal	Activation occurred only in a few infections. Exflagellation did not occur at all.	Activation or exflagellation did not occur at all.	Normal
a	Normal	Normal			
4 b	Activation and exflagellation occurred normally. Gametes immobilised.	Activation and exflagellation occurred normally. Gametes immobilised.	Not done	Not done	Not done
5 and 6	Not done	Not done	On day 5, activation occurred in a few infect- ions, and exflagellation in a very few. On day 6 not even activa- tion occurred in any.	Not done	Not done
7 and 8	Not done	Not done	Not done	In most infections not even activation occurred. In a few, activation and exflagellation occurred, but gametes were immobilised.	Not done
9 10 11 12 14 30	Activation and exflagellation occurred normally. Gametes were immobilised	Activation and exflagellation occurred normally Gametes were immobilised	Activation occurred in a few infections, exflagellation did not occur at all.	Activation and exflagellation did not occur at all.	Activation and exflagellation occurred. Gametes were immobilised.

SERUM

Gametocytes from day 4, whether immobilised or not by their own sera, when resuspended in serum from days 2, 3 or in uninfected serum, exflagellated normally and gametes dispersed freely. When these gametocytes were suspended in serum from days 9 and 14 of an infection, gametocytes displayed a normal pattern of activation and exflagellation but gametes became immobilised within 10 minutes.

Gametocytes from day 5 and 6 of an infection were resuspended in turn in serum of uninfected mice and of mice infected 2 and 3, and 9, 14 and 30 days previously. Apart from activation observed in a few gametocytes, there were no signs of microgametogenesis in any of the sera.

Gametocytes from day 7, from infections in which they showed no signs of microgametogenesis in homologous sera, remained so when resuspended in serum from days 2, 3, 9, 12 and 14.

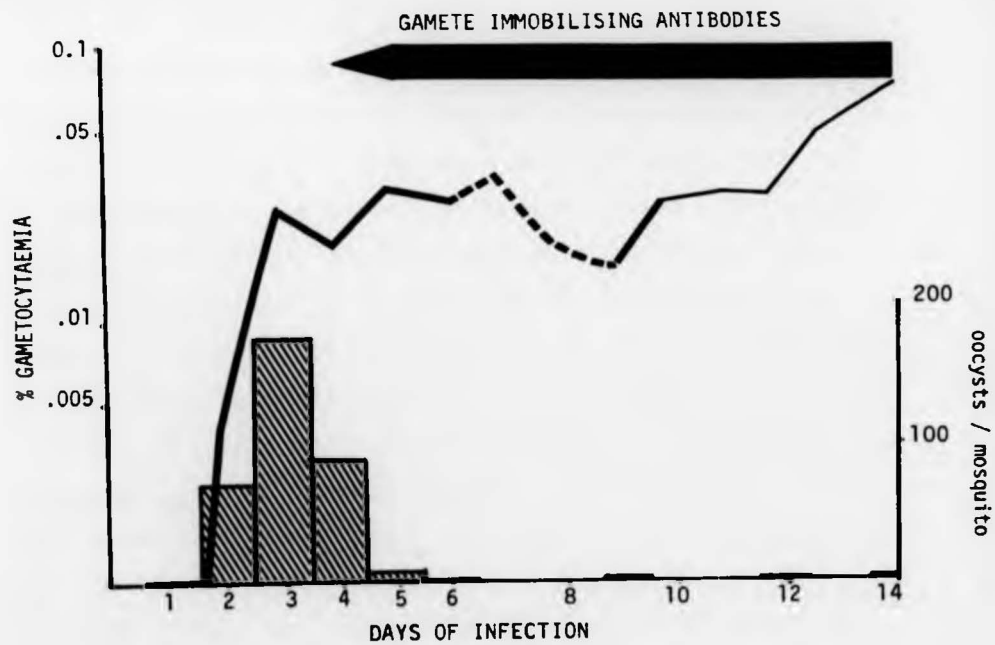
Gametocytes of days 9 and 10 of infections, when resuspended in serum from days 2, 3 or in uninfected serum, showed normal patterns of gametogenesis and dispersal, but in serum from days 9, 12, 14 and 30 gamete immobilisation occurred.

These results are presented in the form of a checker board in Table 9.

Figure 17 is a diagrammatic summary of the results presented in this Chapter, showing the factors determining infectiousness of *P. yoelii* to mosquitoes.



**FIGURE 17.** A diagrammatic summary of factors determining infectiousness of *P.yoelii* in mice to *A.stephensi* mosquitoes, showing the presence of gamete immobilising antibodies, levels of (—) viable and (---) non-viable circulating gametocytes, and the infectivity to mosquitoes (▨ mean number of oocysts per mosquito gut).



## DISCUSSION

### Gametocytogenesis

The numbers of gametocytes first detected on day 2 are far too great to be totally accounted for by young gametocytes that might have been injected with the infecting inoculum, and must be in greater part due to the development of products of schizogony that occurred in the host after inoculation. Therefore, the time taken for a merozoite to develop into a functionally mature gametocyte is very likely to be less than 48 hours. The failure to detect gametocytes or exflagellation, and to infect mosquitoes on day 1 could be accounted for by the presence of insufficient numbers of functionally mature gametocytes on day 1, and does not necessarily exclude a developmental period of 24 hours or less. In P. voelii, the period of development of gametocytes from merozoite to functional maturity has been shown to be about 22 hours (Killick-Kendrick and Warren, 1968). The recent work of Landau et al., (1979) suggests a similar period of development for gametocytes of P. v. voelii and P. y. nigeriensis. Hawking et al., (1972) proposed that the period of development of gametocytes of P. chabaudi was 44 hours or 20 hours, and of P. berghei was 45 or 21 hours. This was calculated firstly on the basis of the time difference between 40% schizogony and 40% exflagellation, assuming that male gametes have a

circadian rhythm of maturity which itself cannot be unreservedly accepted, and secondly, the presumption that gametocytes developed from merozoites formed at only the 2 previous schizogonies. From this study, it can only be suggested that the period of development of gametocytes of P.v.nigeriensis is 48 hours or less.

As is reflected in Figure 3 (page 70), the gametocytaemia runs overtly parallel to the parasitaemia, and this would be inevitable if a constant proportion of merozoites developed into gametocytes at each successive schizogony. A similar parallel between gametocyte counts and the total parasitaemia has been recorded in P.cathemerium (Shah et al., 1934), and a positive correlation between increased parasite density and the appearance of crescents has been recorded in P.falciparum (Miller, 1958). Yet, it is noteworthy that the highest gametocytaemias recorded during the course of natural infections were well below levels reached when comparable parasitaemias were induced artificially using phenylhydrazine. In the latter instance, high parasitaemias were attained in the course of 3 or 4 days which must entail a high rate of increase as compared to the former instance where high parasitaemias were attained during the terminal period of infections, and therefore at a much slower rate. It is conceivable that whatever factors keep the rate of increase of parasitaemia in

check would equally curtail the development of potential gametocytes. Therefore, it is suggested that the absolute parasitaemia as well as the rate of increase of parasitaemia, play a role in determining gametocyte densities in this parasite.

#### Infectiousness to the vector

Infectivity to mosquitoes was restricted to a period between the second and the fifth days of infections, and during this period a positive correlation between gametocyte densities and infectivity could not be shown. Even on days 2 and 3 when there appeared to be an overall positive correlation between gametocyte densities and infectivity, it was not strictly so, as in some experiments oocyst counts were higher on the second than on the third day of infection despite rising gametocyte densities. After day 3, there was a rapid decline of infectivity in the face of a rising gametocyte count, and therefore it is clearly not possible to account for the pattern of infectivity on the basis of gametocyte densities.

From the 5th day of infection onwards, and in some infections as early as the 4th day, gamete immobilising properties were detectable in the plasma. They appear to remain throughout the infection and to outlast it, as such properties were detectable on the 30th day in mice that had cleared their parasitaemia. 2 types of

reactions between gametes and plasma were observed. One was a surface fixation reaction and the other of gamete immobilisation without surface fixation, though a correlation between their presence and the period of infection could not be drawn. Carter *et al.*, (1979b) working on *P. gallinaceum* in chickens, described a surface fixation reaction and another which they termed gamete agglutination, in sera of birds during the course of a primary infection. Both types were found to be associated with the immunoglobulin fraction of immune serum. It is likely then that these anti-gamete factors are antibodies.

On account of the nature of gamete immobilisation observed in vitro, and the strong correlation between the presence of such properties in the plasma and the suppression of infectivity to mosquitoes in vivo, it is very likely that these anti-gamete antibodies are functional, and associated with transmission blocking immunity.

There is also evidence of fluctuations in the viability of gametocytes during the course of the first 10 days of infections, which was the period under study. Despite the presence of gametocytes in the circulation from day 2 onwards, with in fact a gradually rising gametocytaemia, the numbers of viable gametocytes

declined after the 3rd day of infection. On day 6 in all infections studied, and on day 7 and 8 in most infections, none of the gametocytes were viable, but from day 9 onwards the proportion of viable gametocytes began to increase again.

The many theories that can be offered to explain such a pattern of viability during the course of an infection reflect the dearth of knowledge on gametocyte infectivity in rodent malarias, and it will be appropriate to discuss the work of Hawking and his co-workers (1972) on gametocyte maturity in rodent malarias. They proposed that in P.chabaudi and P.berghei, gametocytes were short-lived and remained infective to mosquitoes only for 6 to 10 hours, coinciding with the time of maximum biting activity of mosquitoes at night. The fact that this parasite proved to be highly infective to mosquitoes at around mid-day raises doubts as to whether there could be a peak of infectivity at night. Whether a circadian rhythm of gametocyte maturation exists in P.voelii cannot be ascertained from this study, and it is a cycle of maturation or viability during the course of an infection rather than a circadian rhythm that is implied by these results.

A plausible theory which may explain the pattern of gametocyte viability seen in this parasite is one which supposes that gametocytogenesis occurs in periodic

bouts in the course of an infection, producing long lived gametocytes whose life-span exceeds the period of viability. It is possible that gametocytes are produced from days 1 to 3 of an infection with a life span of 6-7 days. Such a theory would not contradict the pattern of gametocytaemia observed either, because gametocyte densities are maintained at approximately constant levels from the 3rd to the 7th days. A short period of viability of gametocytes of 1 to 2 days may be accounted for by a process of physiological aging that may occur in parasites, associated with a loss of viability, or by factors pertaining to the host erythrocytes, such as those that meet the metabolic requirements of the parasite which become depleted with time and result in the loss of viability. A second bout of gametocytogenesis at about day 9 could account for the increased viability observed at this time; such a process may well continue throughout the infection. This theory is however opposed to that propounded by Hawking *et al.*, (1972) which suggests a continuous production of short-lived gametocytes.

Smalley and Sinden (1977) demonstrated that gametocytes of *P. falciparum* are long-lived and remain infective to mosquitoes for many consecutive days. Despite the many differences that exist between gametocytes of *P. falciparum* and other malarias, biological

processes of related parasites may conform to basic patterns, and gametocytes of this rodent parasite may be similarly long-lived as postulated above.

In a recent study, using the same parasite (P.v.nigeriensis) and P.v.voelii, Landau et al., (1979) recognised a similar pattern of infectivity to mosquitoes and associated it with the appearance of 4 morphologically distinguishable types of gametocytes in the circulation. They proposed that the infectivity and the morphological changes were manifestations of a process of maturation of gametocytes. The pattern of altered viability of gametocytes demonstrated in this study may also possibly be due to such a process, again as proposed above.

Alternatively, if gametocyte production in this parasite occurs daily as Hawking and his co-workers suggested, it is very likely that host factors are responsible for affecting their viability, and host immunity would be a strong contending factor. There is a large body of evidence that antibody does not affect gametocytes in the circulation (see Chapter 2), and as evident from this study, transmission blocking anti-gamete antibodies do not appear to affect the viability of gametocytes. Gametocytes from infections on days 9 and 10, during which time there were circulating transmission blocking anti-gamete antibodies, underwent exflagellation normally in their own serum and proceeded



to completion when washed and resuspended in non-immune serum. Moreover, gametocytes reside within host erythrocytes, which when intact, cannot be penetrated by antibody.

Clark et al., (1975) found evidence of non-specific immunity which causes degeneration of parasites within red cells in species of Babesia and Plasmodia. They postulated a soluble non-antibody mediator of immunity, akin to interferon, liberated by activated macrophages during the course of infections, due to haemoprotozoan antigens (see Allison and Clark, 1977). The damaging effect that such mediators might have on intra-erythrocytic gametocytes could easily result in a loss of viability, and there is a strong possibility of such a mechanism operating in this host-parasite system. Further, in view of the fact that the period of decreased viability coincided with the first crisis period during which time host immunity was becoming established, it is very likely that host immunity by way of non-antibody mediators is responsible for the fluctuations in the viability of gametocytes. The fact that some gametocytes showed an ability to become activated but not to initiate exflagellation may merely reflect the degree to which their viability is affected.

At this juncture it is possible to account for the pattern of infectivity encountered in this model (see

Figure 17). During certain periods of the infection as from days 4 to about 8, the viability of circulating gametocytes is affected and, therefore, during this period, it is responsible for the non-infectiousness of gametocytes to mosquitoes. Transmission blocking anti-gamete immunity is elaborated by the host about day 4 or 5 of an infection, and persists throughout the infection. From day 4 onwards it is also responsible for the lack of infectivity of an infection to mosquitoes. During periods of the infection when viable gametocytes are present as on days 9 and 10 and possibly beyond, functional anti-gamete immunity alone is responsible for rendering them non-infective. Therefore, it is only on days 2 and 3 of an infection, and sometimes on day 4, when viable gametocytes are present and in the absence of transmission blocking immunity that conditions are most conducive to transmission.

In summary, 3 factors that significantly affect infectiousness of malaria to mosquitoes have been demonstrated in this model. Gametocytes are present in the circulation from day 2 onwards in adequate numbers to infect mosquitoes, but 2 other factors, transmission blocking anti-gamete immunity, and gametocyte viability which is possibly also affected by non-specific host immunity, operate to determine the overall pattern of infectivity. Because effective transmission blocking anti-gamete immunity is present throughout an infection

it would presumably be capable alone of ensuring the pattern of infectivity encountered in this model.

CHAPTER 6GENERAL DISCUSSION

The initial objective of this study, which was to establish a rodent malaria model for investigating a transmission blocking vaccine against malaria was successfully accomplished, and further work has resulted in valuable information pertaining to gamete vaccination in malaria. Some of the emphasis has also been laid on unravelling the fundamental biological mechanisms that determine infectiousness of malaria to susceptible mosquitoes, on an understanding of which a strategy of vaccination to block transmission could be profitably based. In this chapter, the study will be viewed from a wider perspective and discussed in terms of its relevance to the possibility of ultimately producing a vaccine against human malaria. A part of the discussion will entail projection of this work against the background of the current status of other experimental anti-malarial vaccines and therefore amount to a comparative evaluation. In an attempt to look ahead at the prospects for an anti-gamete vaccine against human malaria, based on the results of this study as well as on other available information, some of the discussion will necessarily be speculative. But it is hoped that such a

discussion will contribute towards determining the direction of further experimental work in the search for a human malaria vaccine.

This study has demonstrated the feasibility of raising a very effective transmission blocking immunity in mice against P. yoelii by vaccinating with formalin-fixed gametes. Research on anti-malarial transmission blocking vaccines has been undertaken in 2 other experimental models. In the avian parasite P. gallinaceum in chickens, in which the pioneering work in this field was carried out, induction of an effective immunity has also been possible (Gwadz, 1976; Carter and Chen, 1976) and has consequently been used as an experimental model for further investigations. However, on account of the basic differences that exist between avian and mammalian Plasmodia, compounded by obvious immunological dissimilarities in host systems, there are many difficulties encountered in extrapolating these results to human malarias. In the search for a more appropriate experimental system, gamete vaccination was extended to P. knowlesi malaria in rhesus monkeys (Gwadz and Green, 1978), but the requirement for FCA for successful immunisation against this parasite proved to be a serious setback. In view of these circumstances and, as already discussed, because of the many advantages that a rodent model offers for immunological study, successful gamete vaccination in mice against P. yoelii could be seen to

improve considerably the opportunities available for the study of transmission blocking vaccines against malaria.

Results of vaccination in this study have been very encouraging. A relatively crude antigen preparation, used without an adjuvant, induces a very effective transmission blocking immunity. The requirement for adjuvants that are unacceptable for human use has imposed severe constraints of the development of the merozoite vaccine which shows much promise as a potential human malarial vaccine (Mitchel et al., 1974, 1975). Further, as shown in this study, the ability to induce effective anti-gamete immunity by a single vaccinating dose as well as by the intramuscular route of vaccination are features that would enhance the prospects for gamete vaccination against human malarias. The sporozoite vaccine, which remains to date the only experimental vaccine on which even limited human trials have been carried out, confronts major practical difficulties in that repeated and intravenous inoculations are required for an effective immunity to be produced, and even so immunity lasts only for about 3 months (Clyde et al., 1975). Immunity induced by merozoite vaccination lasts upto a year (See Cohen, 1979), and as has been demonstrated in this study, transmission blocking immunity induced by gamete vaccination lasts at least 6 months.

Preliminary investigations into the nature of transmission blocking immunity have shown that extra-cellular gametes are affected by immunity. One possible mechanism of action could be that male gametes are immobilised by antibodies in the mosquito gut, preventing fertilisation from occurring. Many other forms of interaction between host immunity and the parasite could be possible, including effects on the female gametes. Basic immunological aspects of gamete vaccination remains to be investigated.

Results obtained from this study almost certainly imply that transmission blocking immunity is stage specific; functional anti-gamete immunity did not protect against asexual blood stages, nor could transmission blocking immunity be induced by vaccinating with asexual stages alone. It is still possible that some protective antigens common to many stages of the life-cycle exist but not to an extent that would afford cross protection by vaccinating with crude antigen preparations. It seems very likely that sexual stages of P.falciparum also show a similar antigenic distinction; indirect evidence pertaining to the parasite, that was derived from a serological survey which has already been discussed (Carter et al., in press) indicates that this is so. This would imply that vaccination against asexual blood stage parasites, unless sufficiently effective to eliminate a blood infection completely, would not affect

the infectiousness of immunised individuals to mosquitoes, and therefore have little effect on the intensity of malaria transmission. It would also mean that a gamete vaccine used against human malaria would not confer any advantage to the vaccinated individual who will be completely susceptible to the disease. However, in view of the fact that the effectiveness of gamete vaccination as a malaria control measure does not depend on altering the course of the disease in the vertebrate host, this should not be used as a critical parameter in evaluating a transmission blocking vaccine.

During this study, it was demonstrated that unvaccinated mice elaborate anti-gamete transmission blocking immunity during the course of a primary infection with P. voelii. The immunity appeared to be of a similar nature as that induced by gamete vaccination; serum beyond the 5th day of an infection possessed gamete immobilising properties which rendered gametocytes non-infective to mosquitoes. Transmission blocking immunity acquired in response to an infection has also been demonstrated in chickens infected with P. gallinaceum (Carter et al., 1979b); however, such immunity has not been demonstrated at any time either during or following P. knowlesi infections in unimmunised monkeys (Carter, Gwadz and Green, unpublished observation, see Carter et al., 1979b). Evidence pertaining to P. falciparum infections in man is very equivocal and has been discussed



in Chapter 2. Some evidence at least, indicates that repeated infection with P.falciparum evokes an anti-gamete immunity. The implications of such a situation would be that the major reservoir of infection in endemic areas would be found in younger children, and to be effective, a gamete vaccine need be administered only to this sector of the population, a feature which would be of considerable practical advantage to a human malaria vaccine.

If on the other hand, as indicated by a serological survey carried out by Carter et al., (in press) acquired immunity to P.falciparum is devoid of an anti-gamete component, this could also create circumstances which could be advantageous to gamete vaccination. Immunisation with gametes would then introduce a form of immunity with which parasites have no natural experience and against which they have no apparent mechanisms of defence.

It would be interesting to consider what provides the immunogenic stimulus for the elaboration of anti-gamete immunity during an infection by rodent and avian Plasmodia. Gametes do not occur in the vertebrate host and it would not be possible for gamete antigens to be presented to the host. As shown in this study, if asexual and sexual stages do not share protective antigens to a significant extent, it would be only gametocytes that

could provide the necessary antigenic stimulus. There is evidence from this study and from others that gametocytes and gametes share some protective antigens, and it is possible that gametocyte antigens, either expressed on the surface of the host erythrocytes or encountered by the host during the process of parasite destruction, are responsible. If this were so it is even more surprising that gametocytes are spared the lethal effects of anti-gamete immunity, particularly during their brief extracellular existence. A possible reason could be an extreme antigenic lability of gametocytes during this period.

Yet another factor that requires consideration is whether the ease with which the host acquires anti-gamete immunity in response to an infection is a reflection on the likely response to gamete vaccination. In both rodent and avian malaria parasites, the response to vaccination was favourable, and matched by a readily acquired immunity to an infection. In contrast, in the simian malaria model, responses to both vaccination and an infection was poor. These differences exemplify the notorious variability of host-parasite interactions in the various experimental models available for study, which raises the tiresome question as to which animal model is most applicable to P. falciparum in man. Even regarding the P. knowlesi-rhesus monkey system as being an appropriate model for human malaria, (on account of being a primate malaria and a

stringent experimental system) is not totally justified; reference to sporozoite vaccination indicates that results were more consistent among the rodent malarias and the limited human trials that were carried out than they were in the simian malarias. Therefore, considering the encouraging results of gamete vaccination obtained in the rodent and the avian models, the less favourable outcome of vaccination in a simian malaria parasite should not deter research on gamete vaccination for use against human malaria.

Immunoprophylactic methods against malaria need to be carefully evaluated to avoid iso-immunisation of the host, particularly when there is a risk of evoking immunity against an erythrocyte component. In this study, contamination of vaccines with host erythrocyte material could not be avoided on account of the crude methods used in their preparation, but by using valid control experiments, the effect of host components on effective immunity was eliminated. No attempt was made to determine the effects of iso-immunisation on the host, as it was considered beyond the scope of this study. Moreover, vaccines prepared from gametes would, on account of these stages being extracellular, theoretically have a lower risk of contamination with host tissue than some of the erythrocytic stage vaccines.

Also relevant to the subject of immunisation against sexual stages is the observation that circulating

gametocytes of P. yoelii showed a pattern of altered viability during the course of an infection in mice. The reasons for this are not clear, but the likelihood of it being due to non-specific and non-antibody mediators of immunity have been discussed. At present virtually nothing is known about non-specific mechanisms of resistance against sexual stages, and such knowledge might constitute a field of considerable immunological significance.

Prospects for mass production of gametes of P. falciparum in vitro for initial trials and for vaccine production have been enhanced by recent reports of continuous malaria culture (Trager and Jensen, 1976) and the controlled production of gametocytes from such cultures (Carter and Beach, 1977). To date however, factors governing gametocytogenesis in culture are not clearly known, though it has been possible to identify substances that may stimulate gametocyte production in cultures (Carter, R., personal communication). A knowledge of the antigenic structure of sexual stages is basically lacking, but research on the production of monoclonal anti-gamete antibodies from hybrid cells is already under way (Carter, R., personal communication); it may eventually be possible to identify and synthesise dominant gamete antigens which might be used for immunisation.

The current status of experimental vaccines against sporozoites and asexual blood stages have been discussed

previously. They show considerable potential as human anti-malarial vaccines but face formidable practical difficulties before they can be realised. Vaccination with gametes affords a novel approach of immunisation, where immunity is raised against sexual stages of the parasite that occur in the mosquito. Thereby, it aims to interrupt the sexual phase of development in the vector and abolish transmission of the disease.

In this study, a successful gamete vaccine has been demonstrated against a rodent malaria parasite. Whether this success can be repeated in man against P. falciparum remains to be seen.

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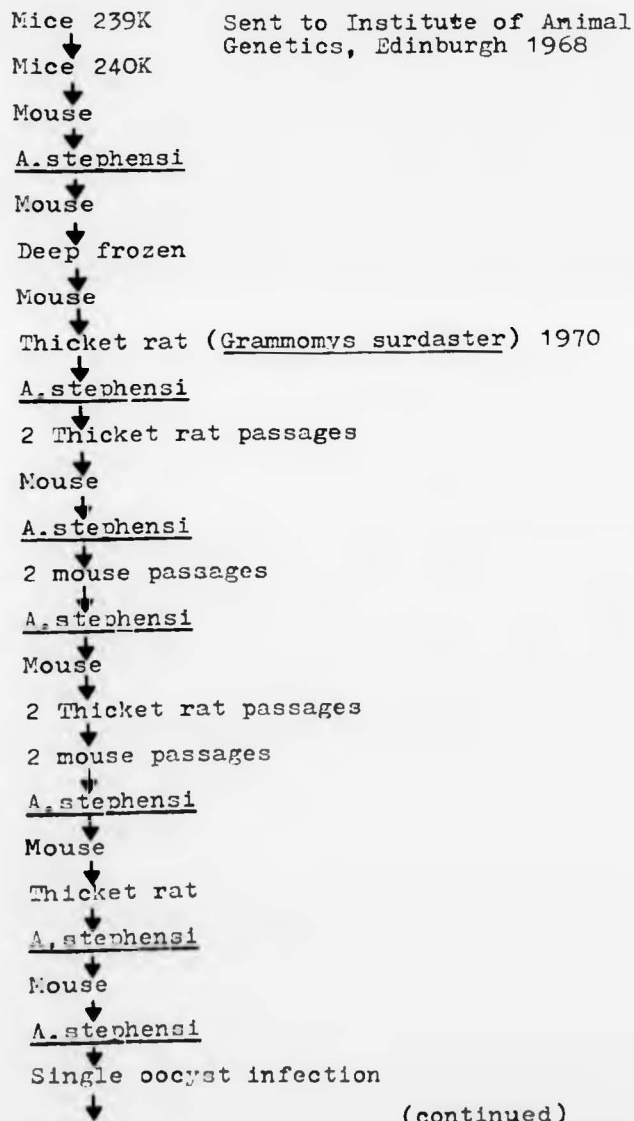
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APPENDIX IHistory of Plasmodium voelii nigeriensis (N67) from 1968 onwards. (Courtesy of Dr. D. Walliker)

(continued)

↓  
Mouse  
↓  
Deep frozen 1970  
↓  
2 mouse passages 1976 (sent to Ross  
Institute)  
↓  
Mice  
↓  
Deep frozen (liquid N<sub>2</sub>)  
↓  
Mouse  
↓  
Deep frozen (liquid N<sub>2</sub>)  
↓  
Mouse  
↓  
Deep frozen (liquid N<sub>2</sub>)  
↓  
2 mouse passages  
↓  
A. stephensi  
↓  
Mouse  
↓  
Cryopreserved (LUMP 1238, obtained from  
the Ross Institute for this  
study)  
↓  
2 mouse passages  
↓  
A. stephensi  
↓  
Mouse  
↓  
Cryopreserved (LUMP 1316) 1977  
↓  
2 mouse passages  
↓  
A. stephensi  
↓  
Mouse  
↓  
Cryopreserved (LUMP 1357) 1978  
↓  
2 mouse passages  
↓  
A. stephensi  
↓  
Mouse  
↓  
Cryopreserved (LUMP 1382) 1979

APPENDIX IIMEDIA AND SOLUTIONS USED

<u>Phosphate-buffered-saline (PBS)</u>	pH 7.3	
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )		0.2 grams
Disodium hydrogen phosphate, hydrated ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ )		1.15 grams
Sodium chloride (NaCl)		8.0 grams
Potassium chloride (KCl)		0.2 grams
Distilled water	upto	1.0 litre

Suspended Animation solution (SA solution)  
(Carter and Nijhout, 1977)

Tris(hydroxy methyl)aminomethane (Tris) (Sigma)		0.21% w/v
Sodium Chloride (NaCl)		0.96% w/v
Glucose		0.2% w/v
	pH adjusted to 7.4 with HCl	

Gamete releasing medium (Carter and Chen, 1976)

5% Sodium chloride (NaCl)		2.5 volumes
10% Glucose		2.5 volumes
1.46% Sodium bicarbonate ( $\text{NaHCO}_3$ )		20 volumes
Inactivated foetal calf serum		100 volumes

APPENDIX 3.1

Experiment: Immunisation with 3 doses of the gamete vaccine. (Figure 4.1 and 4.2).

		Days of infection										
		1	2	3	4	5	6	7	8	9	10	11
Mean parasitaemia ± standard error of the mean	Vaccinated mice	0.68 ± 0.1	2.96 ± 0.5	2.8 ± 0.4	3.1 ± 1.5	1.5 ± 0.8	0.36 ± 0.16	0.1 ± .08	.005 ± .004	.005 ± .004	.001 ± 0	0
	Control mice	0.42 ± .08	1.9 ± 0.2	7.3 ± 2.9	9.9 ± 2.7	11.3 ± 3.9	11.6 ± 7.9	13.6 ± 5.6	11.0 ± 3.3	20.9 ± 6.3	33.5 ± 10.0	19.7 ± 4.3
	Vaccinated mice	0	.008 ± 9x10 <sup>-4</sup>	.008 ± .002	.014 ± .010	.008 ± .005	.007 ± .004	.009 ± .006	6x10 <sup>-4</sup> ± 0	0 -	0 -	0
	Control mice	0	.008 ±	.02 ±	.02 ±	.023 ±	.009 ±	.02 ±	.01 ±	.01 ±	.009 ±	.01 ±
			.002	.002	.008	.008	.005	.008	.002	.002	.001	.003

APPENDIX 3.2

Experiment: Dose related response to gamete vaccination. (Figures 5.1 and 5.2).

		Days of infection											
		1	2	3	4	5	6	7	8	9	10	11	12
Mean parasitaemia ± standard error of the mean	Vaccinating dose $2 \times 10^7$	.52 ± 0.3	1.68 ± 0.1	4.7 ± .02	6.36 ± 1.28	4.85 ± 2.2	1.25 ± 0.65	0.9 ± 0.4	.014 ± .008	.002 ± .001	0	0	0
	Vaccinating dose $2 \times 10^6$	0.75 ± 0.12	2.0 ± 0.5	7.97 ± 1.3	12.8 ± 3.1	17.0 ± 6.9	10.0 ± 2.1	7.8 ± 3.0	10.0 ± 7.9	1.5 ± 0	1.0 ± 0	8.4 ± 0	40.0 ± 0
	Controls	1.12 ± .09	2.28 ± .19	7.6 ± 2.4	11.6 ± 2.1	18.7 ± 3.6	14.9 ± 3.7	12.1 ± 3.5	14.1 ± 6.8	16.7 ± 9.1	30.3 ± 8.8	38.7 ± 10.0	42.6 ± 9.2
Mean gametocytaemia ± standard error of the mean	Vaccinating dose $2 \times 10^7$	0	.004 ± .001	.013 ± .005	.011 ± .002	.015 ± .004	.012 ± .006	.001 ± $3 \times 10^{-4}$	0	0	0	0	0
	Vaccinating dose $2 \times 10^6$		.001 ± $4 \times 10^{-4}$	.02 ± .01	.03 ± .006	.04 ± .008	.032 ± .004	.03 ± .003	.023 ± .007	.001 ± 0	.004 ± 0	.002 ± 0	.008 ± 0
	Controls	0	.004 ± .002	.028 ± .005	.034 ± .006	.024 ± .002	.03 ± .005	.025 ± .004	.023 ± .004	.001 ± $8 \times 10^{-4}$	.032 ± .004	.044 ± .005	.038 ± .002

APPENDIX 3.3

Experiment: Evaluation of a reticulocyte vaccine (as a control) (Figures 6.1 and 6.2).

		Days of infection											
		1	2	3	4	5	6	7	8	9	10	11	12
Mean parasitaemia $\pm$ standard error of the mean	Vaccinated mice	1.1 $\pm$ 0.2	2.5 $\pm$ 0.5	4.4 $\pm$ 0.9	7.5 $\pm$ 1.7	12.3 $\pm$ 3.7	9.7 $\pm$ 2.5	6.5 $\pm$ 3.5	7.2 $\pm$ 3.2	17.0 $\pm$ 5.1	13.9 $\pm$ 2.9	16.9 $\pm$ 5.5	34.1 $\pm$ 8.1
	Control mice	1.3 $\pm$ 0.1	3.0 $\pm$ 0.5	5.3 $\pm$ 0.9	9.1 $\pm$ 2.5	15.7 $\pm$ 4.2	12.3 $\pm$ 5.0	16.4 $\pm$ 7.2	8.5 $\pm$ 3.5	13.9 $\pm$ 8.3	13.5 $\pm$ 7.0	23.4 $\pm$ 9.4	40.3 $\pm$ 13.2
Mean gametocytaemia $\pm$ standard error of the mean	Vaccinated mice	0	.003 $\pm$ $4 \times 10^{-4}$	.02 $\pm$ .005	.02 $\pm$ .006	.015 $\pm$ .002	.014 $\pm$ .002	.012 $\pm$ .002	.009 $\pm$ .003	.008 $\pm$ .005	.008 $\pm$ .002	.009 $\pm$ .003	.013 $\pm$ .001
	Control mice	0	.002 $\pm$ $6 \times 10^{-4}$	.02 $\pm$ .003	.03 $\pm$ .008	.027 $\pm$ .004	.019 $\pm$ .003	.018 $\pm$ .005	.01 $\pm$ .003	.008 $\pm$ .004	.007 $\pm$ .003	.01 $\pm$ .002	.015 $\pm$ $6 \times 10^{-4}$

APPENDIX 3.4

Experiment: Intramuscular route of vaccination (Figure 7.1).

		Days of infection												
		1	2	3	4	5	6	7	8	9	10	11	12	
Mean parasitaemia $\pm$ standard error of the mean	Vaccinated mice	0.54 $\pm$ .09	1.9 $\pm$ 0.2	5.8 $\pm$ 0.9	8.1 $\pm$ 1.2	9.9 $\pm$ 2.7	3.3 $\pm$ 0.8	3.0 $\pm$ 0.9	4.5 $\pm$ 0.9	6.5 $\pm$ 0.6	9.3 $\pm$ 4.4	9.2 $\pm$ 3.1	8.6 $\pm$ 3.1	
	Control mice	0.4 $\pm$ 0.1	2.3 $\pm$ 0.2	5.4 $\pm$ 1.1	5.3 $\pm$ 1.2	11.8 $\pm$ 2.2	8.0 $\pm$ 4.1	11.1 $\pm$ 4.7	14.4 $\pm$ 4.6	16.3 $\pm$ 5.5	20.7 $\pm$ 5.2	24.6 $\pm$ 6.3	41.9 $\pm$ 9.4	
			Days of infection											
			13	14	15	16	17	18	19	20	21	22	23	24
	Vaccinated mice		10.2 $\pm$ 2.8	8.6 $\pm$ 4.6	9.8 $\pm$ 5.0	12.5 $\pm$ 10.0	25.0 $\pm$ 14.8	33.0 $\pm$ 29.5	28.2 $\pm$ 28.2	31.0 $\pm$ 30.9	30.0 $\pm$ 29.0	44.8 $\pm$ 0	40.0 $\pm$ 0	20.0 $\pm$ 0
	Control mice		30.7 $\pm$ 8.4	36.4 $\pm$ 8.2	43.4 $\pm$ 10.0	50.8 $\pm$ 12.3	51.1 $\pm$ 13.2	66.4 $\pm$ 7.0	62.0 $\pm$ 11.5	57.7 $\pm$ 20.9	41.5 $\pm$ 17.4	24.7 $\pm$ 9.9	19.7 $\pm$ 10.1	17.5 $\pm$ 2.5

APPENDIX 3.5

Experiment: Intramuscular route of vaccination (Figure 7.2)

		Days of infection											
		1	2	3	4	5	6	7	8	9	10	11	12
mean gametocytaemia $\pm$ standard error of the mean	Vaccinated mice	0	.001 $\pm$ $2 \times 10^{-4}$	.015 $\pm$ .006	.021 $\pm$ .009	.02 $\pm$ .005	.01 $\pm$ .001	.009 $\pm$ .001	.01 $\pm$ $5 \times 10^{-4}$	.012 $\pm$ .002	.026 $\pm$ .01	.019 $\pm$ .005	.07 $\pm$ .016
	Control mice	0	.001 $\pm$ $2 \times 10^{-4}$	.019 $\pm$ .005	.021 $\pm$ .008	.022 $\pm$ .007	.022 $\pm$ .005	.019 $\pm$ .005	.018 $\pm$ .003	.018 $\pm$ .002	.02 $\pm$ .004	.037 $\pm$ .002	.047 $\pm$ .01
		Days of infection											
		13	14	15	16	17	18	19	20	21	22	23	24
mean gametocytaemia $\pm$ standard error of the mean	Vaccinated mice	0.15 $\pm$ .02	.042 $\pm$ .009	.05 $\pm$ .025	.052 $\pm$ .04	.07 $\pm$ .05	0.3 $\pm$ .02	.03 $\pm$ .02	.04 $\pm$ 0	.03 $\pm$ 0	.012 $\pm$ 0	.012 $\pm$ 0	.012 $\pm$ 0
	Control mice	.07 $\pm$ .02	0.21 $\pm$ .06	0.25 $\pm$ .08	0.34 $\pm$ 0.15	0.23 $\pm$ 0.12	0.5 $\pm$ 0.19	.07 $\pm$ .02	0.13 $\pm$ .08	0.14 $\pm$ 0.1	0.12 $\pm$ .08	0.15 $\pm$ .06	0.43 $\pm$ .09



APPENDIX 3.6

Experiment: Duration of transmission blocking immunity (Figure 8).

		Days of infection												
		-1	2	3	4	5	6	7	8	9	10	11	12	
Mean parasitaemia $\pm$ standard error of the mean	Challenged 16 weeks after vaccination	0.38 $\pm$ 0.1	2.9 $\pm$ 1.0	5.4 $\pm$ 0.8	4.4 $\pm$ 0.5	1.9 $\pm$ 0.4	0.24 $\pm$ 0.1	.017 $\pm$ .01	0	0	0	0	0	
	Challenged 25 weeks after vaccination	0.95 $\pm$ 0.4	2.26 $\pm$ 0.4	4.7 $\pm$ 0.9	11.8 $\pm$ 3.4	10.6 $\pm$ 1.0	5.6 $\pm$ 0.5	2.6 $\pm$ 0.6	1.2 $\pm$ 0.3	.08 $\pm$ .06	1.6 $\pm$ 1.5	0.5 $\pm$ 0.5	0.6 $\pm$ .01	
	Unvaccinated controls	1.05 $\pm$ 0.4	3.1 $\pm$ 0.6	11.5 $\pm$ 1.0	20.3 $\pm$ 2.2	26.1 $\pm$ 3.4	28.0 $\pm$ 5.4	35.3 $\pm$ 4.2	23.1 $\pm$ 6.0	33.3 $\pm$ 3.0	40.2 $\pm$ 2.1	58.0 $\pm$ 6.2	44.0 $\pm$ 7.4	
		Days												
			13	14										
		Challenged 16 weeks after vaccination	0	0										
		Challenged 25 weeks after vaccination	.003 $\pm$ 0	0										
		Unvaccinated controls	46.0 $\pm$ 2.8	49.5 $\pm$ 4.0										

APPENDIX 3.7

Experiment: Immunisation with an erythrocytic stage vaccine (both sexual and asexual)  
in a dose of  $2 \times 10^7$  with and without adjuvant (Figure 11).

Mean parasitaemia  $\pm$  standard error of mean

		Days of infection											
		1	2	3	4	5	6	7	8	9	10	11	12
Vaccinated with adjuvant		.022	0.53	2.0	4.1	2.2	1.0	.07	0	0	0	0	0
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$					
		.006	0.1	0.4	0.4	0.6	0.6	.01					
Vaccinated without adjuvant		.018	0.43	5.9	16.7	9.1	4.7	1.4	0.4	0.2	.07	0	0
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$		
		.005	.02	0.8	2.0	1.0	0.8	0.7	0.2	0.1	.006		
Unvaccinated controls		0.12	0.98	4.3	13.9	21.5	29.1	17.9	6.7	8.1	16.2	32.0	35.6
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
		.06	0.17	0.68	2.3	4.7	6.8	7.0	4.6	3.6	3.7	9.7	8.9

APPENDIX 3.8

Experiment: Immunisation with an erythrocytic stage vaccine (both sexual and asexual)  
in a dose of  $2 \times 10^8$  parasites (Figures 12.1 and 12.2).

	1	2	3	4	5	6	7	8	9	10	11	12
Mean parasitaemia ± standard error of mean	Vaccinated mice	0.13 ± .04	0.67 ± 0.2	1.7 ± 0.5	4.5 ± 1.0	0.35 ± 0.1	.006 ± .004	0	0	0	0	0
	Control mice	0.15 ± .02	1.3 ± 0.4	5.1 ± 0.6	14.7 ± 1.8	15.9 ± 9.1	12.3 ± 0	16.0 ± 0	21.0 ± 0	29.6 ± 0	42.0 ± 0	46.0 ± 0
	Vaccinated mice	0 ± $3 \times 10^{-4}$	.001 ± .01	.027 ± .01	.01 ± .003	.002 ± $3 \times 10^{-4}$	.001 ± 0	0	0	0	0	0
	Control mice	0 ± $5 \times 10^{-4}$	.002 ± .004	.025 ± .01	.03 ± .01	.025 ± .01	.001 ± 0	.002 ± 0	.007 ± 0	.01 ± 0	.012 ± 0	

APPENDIX 3.9

Experiment: Immunisation with a 'pure' asexual erythrocytic stage vaccine (Figures 13.1 and 13.2).

		Days of infection											
		1	2	3	4	5	6	7	8	9	10	11	12
Mean parasitaemia ± standard error of mean	Vaccinating dose 10 <sup>6</sup>	0.88 ± 0.1	2.8 ± 0.2	6.9 ± 0.8	16.5 ± 1.4	20.7 ± 2.8	6.7 ± 1.7	2.3 ± 0.8	0.4 ± 0.3	.001 ± 0	0	0	0
	Vaccinating dose 2X10 <sup>6</sup>	1.25 ± 0.2	4.6 ± 0.5	15.1 ± 4.2	12.6 ± 0.7	11.9 ± 1.35	7.2 ± 1.7	1.8 ± 0.9	.05 ± .09	0	0	0	0
	Unvaccinated controls	1.05 ± .06	2.7 ± 0.4	6.5 ± 0.5	15.8 ± 2.0	30.6 ± 3.5	12.5 ± 1.6	7.7 ± 0.6	7.9 ± 1.0	7.2 ± 1.3	16.9 ± 6.3	30.6 ± 12.4	23.0 ± 9.2
	Vaccinating dose 10 <sup>6</sup>	0	.004 ± 8x10 <sup>-4</sup>	.048 ± .005	.05 ± .005	.05 ± .001	.02 ± .002	.015 ± .004	0	0	0	0	0
	Vaccinating dose 2x10 <sup>6</sup>	0	.002 ± 6x10 <sup>-4</sup>	.038 ± .001	.048 ± .004	.043 ± .003	.022 ± .007	.004 ± .001	0	0	0	0	0
	Unvaccinated controls	0	.003 ± 4x10 <sup>-4</sup>	.04 ± .007	.04 ± .002	.04 ± .004	.025 ± .002	.03 ± .002	.02 ± .002	.01 ± .001	.03 ± .02	.03 ± .02	.005 ± .002

## Immunisation against gametes and asexual erythrocytic stages of a rodent malaria parasite

INVESTIGATIONS into the feasibility of vaccinating against malaria have centred mainly on the use of the sporozoite<sup>1,2</sup> or the asexual blood stages<sup>3,4</sup>. Immunisation using sexual stages of malaria parasites has been demonstrated with *Plasmodium gallinaceum* in chickens<sup>5</sup>. Gamete vaccines have been used to produce an effective immunity against transmission of the avian malaria parasite, with little or no effect on its asexual erythrocytic stages. More recently, this study has been extended to *P. knowlesi* infections in rhesus monkeys<sup>6</sup>; transmission-blocking immunity and resistance to asexual infection were obtained, but were fully effective only when the vaccine was combined with Freund's complete adjuvant. We report here successful immunisation of mice against the sexual stages of *P. yoelii* using formalin-fixed gametes. In addition to very effective transmission-blocking immunity, the vaccinated mice were strongly protected against asexual erythrocytic stages of the parasite.

Eight-week-old female outbred Theiler's Original (TO) mice were used as vertebrate hosts. The parasite, *P. yoelii nigeriensis*, was cryopreserved (stable reference LUMP 1316) soon after cyclical transmission through the vector *Anopheles stephensi*. Parasites from the first passage from the vector were used for initiating an infection.

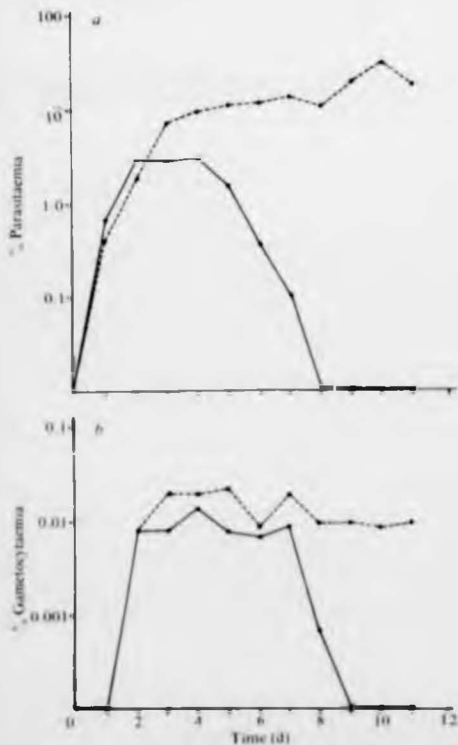


Fig. 1 The course of patent parasitaemia (a) and gametocytaemia (b) in vaccinated (●) and unvaccinated (○) mice.

Table 1 Oocyst production in mosquitoes fed on vaccinated and unvaccinated mice

Day	Unvaccinated mice			Mean no. of oocysts per mosquito	Vaccinated mice No. of oocysts per 10 mosquitoes
	Expt				
	1	2	3		
1	0	0	0	0	0
2	114	1,638	2,162	130.4	0
3	496	498	1,287	76.0	0
4	1,406	40	0	48.2	0
5	0	62	0	2.0	0
Total counts	2,016	2,238	3,449		

In each experiment, 3 vaccinated and 3 unvaccinated mice were given a blood-induced challenge infection. Batches of mosquitoes were fed on each group of mice on days 1-6 of the infection. The mosquitoes were maintained at 24°C and oocyst counts were done 1 week later.

*P. yoelii* preferentially invades reticulocytes. Mice were made anaemic with three subcutaneous injections of phenylhydrazine and infected intravenously (i.v.) with parasitised red cells. As a result of the induced reticulocytosis, the parasitaemia rose to 70-80% with 0.5-1.5% of gametocytes. Mice were bled by cardiac puncture into 50 volumes of a suspended animation (SA) solution (0.21% Tris, 0.96% NaCl and 0.2% glucose, adjusted to pH 7.4) in which gametogenesis was reversibly suppressed<sup>10</sup>. The suspension was centrifuged at 500g for 10 min and the red cells were resuspended in a gamete-releasing medium<sup>8</sup> (2.5 vol of 5% NaCl, 2.5 vol of 10% glucose, 20 vol of 1.46% NaHCO<sub>3</sub> and 100 vol of inactivated fetal calf serum, adjusted to pH 8.0) in a proportion of 1:3 v/v, and the pH readjusted to 8.0. After 30 min at room temperature gametogenesis was complete and the suspension was centrifuged at 500g for 10 min. This procedure was repeated four times, resuspending the packed material in SA solution, so as to recover as many free gametes as possible. The pooled supernatants were centrifuged at 18,000g for 20 min and the pellet containing gametes was suspended in a solution of 1.0% formalin for 30 min at room temperature. The gametes were washed three times in SA solution at 18,000g. After the final wash, counts were made on a haemocytometer using a phase contrast microscope, and adjusted such that 0.2 ml contained  $2 \times 10^7$  male gametes.

The vaccine contained, in addition to male and female gametes, asexual parasites which under the light microscope appeared to be free of their host red cells, and occasional red cell 'ghosts' and white cells. 0.2 ml of the vaccine contained  $2 \times 10^8$  male gametes,  $6-8 \times 10^6$  female gametes and  $10^5-10^6$  asexual parasites.  $2 \times 10^8$  male gametes were obtained from 5.0 ml of infected blood. Each mouse received three doses of the freshly prepared vaccine i.v. at weekly intervals, that is, a total dose of  $6 \times 10^7$  male gametes. One week after the final dose of the vaccine, vaccinated mice and an equal number of age-matched control mice were inoculated i.v. with  $2 \times 10^8$  parasitised red cells. Thin blood films were made daily, stained with Giemsa and the parasitaemia and gametocytaemia ascertained. On days 1-6 after challenge, mosquitoes were fed on vaccinated and control mice, one cage per group. Any unfed female mosquitoes were removed from the cages and 1 week after feeding, 10 mosquitoes from each cage were dissected and the midguts examined microscopically for oocysts.

Results were obtained from three separate experiments involving nine vaccinated and an equal number of control animals. The infection was fatal in all but one of the control mice. Death of the control mice occurred either on days 6 and 7, with parasitaemias of 20-30% (Fig. 1a) or on days 18 and 19, with peak parasitaemias of 50-60%. In contrast, the vaccinated mice were apparently aparasitaemic by day 12, with an average

clearance time of 8.8 d. The highest parasitaemia recorded in the vaccinated mice was 3.1%, reached on day 4 (Fig. 1a). The average gametocyte counts are represented in Fig. 1b. Mosquitoes fed on control mice had high total oocyst counts, these being derived mainly from feeds taken 2, 3 or 4 d after challenge. Mosquitoes fed on vaccinated mice showed no oocysts at all in their midguts throughout the infection (Table 1).

These experiments demonstrate a very effective immunity which blocks transmission. It does not require the use of an adjuvant and we are now investigating whether fewer gametes and/or smaller numbers of immunising doses can be used: preliminary studies indicate that a single dose can be equally effective. Immunity to asexual erythrocytic stages, though not complete, was also pronounced, manifested by a significant lowering of parasitaemia and a 100% survival as compared to the control mice, in which the parasitaemia was higher and the infection fatal in almost all of the mice. Gwadz and Green<sup>9</sup> used a gamete/trophozoite antigen mixture in their experiments with *P. knowlesi* and this also induced immunity against asexual parasites. There are two possible explanations. First, contamination of the gamete vaccines with asexual stages might be responsible. Experiments by Playfair *et al.*<sup>6</sup> and our own experience (unpublished) with saponin-lysed, formalin-fixed asexual stage parasites used as a vaccine without an adjuvant, have resulted in an effective immunity to blood-induced *P. yoelii* infections. The alternative proposition would be that gametes protect against asexual stages because they have protective antigens in common.

It is encouraging that such an effective block on transmission is possible by vaccinating with a relative crude antigen preparation, and without the need for an adjuvant. Further improvements both in methods of vaccine preparation and in its presentation are possible.

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KAMINI N. MENDIS

G. A. T. TARGETT

*Department of Medical Protozoology,  
London School of Hygiene and Tropical Medicine,  
London WC1, UK*

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