Study of a transmission blocking antigen of *Plasmodium berghei*

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by

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ABSTRACT

Ookinetes of the rodent malaria parasite *Plasmodium berghei* were cultured *in vitro* . Methods for the enrichment were developed and these parasites were then used to immunise mice for monoclonal antibody (MAb) production. The anti-ookinete MAbs 12.1, 13.1 and 17.9 produced by Winger *et al.*, (1988) were extensively characterised. The MAbs showed suppression of gametocyte infectivity to mosquitoes by greater than 92% in membrane feed and passive transfer experiments. They also blocked ookinete development *in vitro* by 91.87-100%. Using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot techniques, a protein determinant of M_r 21 kD (Pbs21) was identified as the target of the transmission-blocking MAbs.

Affinity chromatography was developed to purify Pbs21 and the protein was evaluated as a transmission-blocking vaccine candidate. A group of mice was immunised intramuscularly (I. M.) with 20 µg of the acetone-precipitated protein without adjuvant, then boosted once a month later with the same amount of the antigen. As a control, another group of mice was injected with PBS. The efficacy of the vaccine was assessed. Twenty days after the final boost, the mice were infected with *P. berghei* gametocyte-containing blood and offered to mosquitoes. Fourteen days later, oocyst development in mosquito midgut was determined. Full blockade of the parasite development was observed in the mosquitoes that fed on the vaccinated mice. Persistence of the transmission-blocking immunity was also studied. At week 34 post-immunisation, greater than 90% blockade was still achieved.

Sera from the control and immunised mice were collected as appropriate from week 7-39 post-immunisation. Using a heterologous mouse IgG as a standard of known concentrations in ELISA, the level of the 'anti-Pbs21' IgG in the immune sera was determined to be 182.45 and 38 µg/ml at the first and second blockade assessments (week 7 and 34 post-immunisation respectively). In addition, subclass of the specific IgG was found to be IgG1. Using SDS-PAGE and immunoblot analysis, Pbs21 was the immunogenic protein recognised by the immune sera.

The number of epitope(s) of this protective antigen was examined using competitive and two-site ELISA. There were at least 3 B-cell epitopes (epitope I-III) recognised by the transmission-blocking MAbs 12.1, 13.1 and 17.9 respectively in the protein molecule. Epitopes I and II were at a distant sites whilst epitope III topologically overlapped with the 2 epitopes. The conformational structure of the 3 epitopes was extensively studied using immunoblot analysis and ELISA. The observations implied that epitopes I and II were not conformation-dependent. By contrast, epitope III was conformation-dependent.

The role of post-translational modification by glycosylation in the epitopes I-III of Pbs21 was also investigated using periodate oxidation in combination with two-site ELISA. All MAbs still reacted with the periodate-treated protein which suggested that glycosylation might not have a contribution to the immunogenicity of the epitopes.

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ABBREVIATIONS

A, mA	Ampere, milliampere
bp	base pair (s)
cm	centimeter(s)
cpm	counts per minute
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
g	gravitational force
gm	gram(s)
h	hour(s)
HEPES	(*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid])
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
Ir gene	immune response gene
IU	international unit
µCi	microcurie
mg	milligram (s)
min	minute(s)
M_r	relative molecular weight
NGS	normal goat serum
nm	nanometer(s)
NP-40	nonidet P-40
O.D.	optical density
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMSF	phenylmethyl sulfonyl fluoride
rpm	revolution per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	*N, N, N', N'*-tetramethylethylenediamine
TLCK	*N*α-p-Tosyl-L-lysine-Chloromethyl Ketone
TPCK	*N*-Tosyl-L-phenylalanine Chloromethyl Ketone
Tris	(Tris[hydroxymethyl]-aminomethane)
v	volt(s)

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CHAPTER 1

GENERAL INTRODUCTION

Malaria, one of the major infectious diseases, is caused by a parasitic protozoa of the genus *Plasmodium*. The disease is transmitted to the vertebrate host by the bite of the female mosquito.

The genus *Plasmodium* contains about 100 host-specific species of which *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* are naturally infectious to man. Other species infect primates, rodents and birds (Garnham, 1966). Of the 4 species of human malaria parasites, *P. falciparum* and *P. vivax* are the most common. The major cause of mortality due to malaria is *P. falciparum* but infection with *P. vivax*, which gives rise to debilitating relapses of clinical malaria is a major cause of morbidity. Such relapses can recur for many years. The two other species, *P. malariae* and *P. ovale* are generally less prevalent, both causing low-grade chronic diseases.

The disease is a serious health problem affecting millions of people particularly in the tropics. Insecticides and drugs have had only a limited sucess to control the spread of the disease as there is the emergence of insecticide resistance in mosquito populations and of chemotherapeutic drug resistance in parasite strains (especially *P. falciparum*) (Martinez, 1987).

1.1) Life cycle

Life cycle of *Plasmodium* is complicated. The life cycle of the malaria parasite in the mosquito vector and the vertebrate host is illustrated in Figure 1.1.

Malaria infection is initiated by the injection of sporozoites into the bloodstream during a mosquito bloodmeal. Sporozoites rapidly migrate to the liver, where they invade and differentiate inside hepatocytes (as an exoerythrocytic stage) producing thousands of merozoites. Growth of the exoerythrocytic parasites within the liver cells has no observable clinical effects on the host. Rupture of the liver schizont releases the merozoites into the bloodstream, where each is potentially capable of invading an erythrocyte. This initiates the erythrocytic cycle, which gives rise to the symptoms of clinical malaria. Following erythrocyte invasion, the merozoite differentiates through an early ring form, then a trophozoite, with subsequent development into a multinucleate schizont. When the segmenter stage is fully differentiated, the erythrocyte soon ruptures to release new uninucleate merozoites into the bloodstream. The merozoites then re-invade red blood cells and re-initiate the erythrocytic cycle.

Some merozoites differentiate into male and female gametocytes instead of developing along the asexual multiplication pathway, initiating the sexual cycle. When a mosquito feeds on an infected individual, gametocytes are taken up along with the other blood stages. The latter are digested in the midgut of the insect, but the gametocytes undergo further development in mosquito gut and differentiate into male and female gametes. During the 16-24 h following fertilisation, each zygote transforms into a motile ookinete which will then penetrate the epithelial

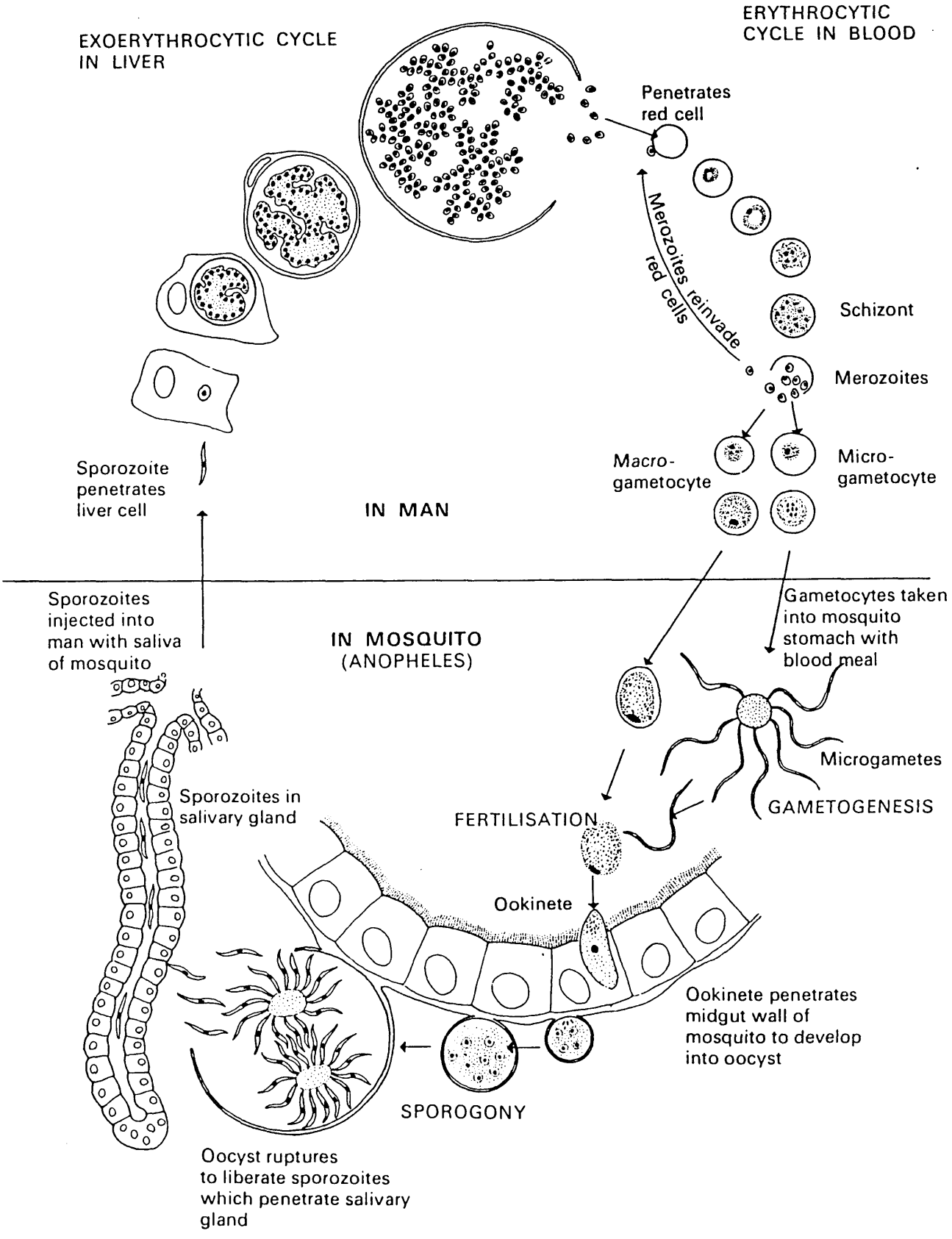


FIGURE 1.1 Life cycle of the malaria parasite (after Vickerman and Cox, 1972).

cells of the midgut and form an oocyst. The oocyst will enlarge and undergo nuclear division (sporogony) to form sporozoites which are released (after a time which depends on species of *Plasmodium*) into the haemocoel upon oocyst rupture and migrate to the mosquito salivary glands. The life cycle of the malaria parasite is then complete.

1.2) The need for a malaria vaccine

The idea of vaccine-based protection of malaria parasites emerged following the failure of insecticide and anti-malarial drug based comparisons. In addition to approaches directed at development of new drugs and means of vector control, there is the prospect of immunisation to protect the host against one or more developmental stages of the malaria parasite or to disrupt the transmission cycle. While the feasibility of immunisation has long been recognised, there are formidable practical limitations centred on means to obtain quantities of purified antigens. Recent advances in biotechnology have removed most of these limitations and it is now possible to produce candidate vaccine antigens in practical quantities. Antigens based upon extracts of whole organisms are neither possible to produce in sufficient quantity, nor desirable from a biological point of view since many plasmodial antigens are irrelevant to protective immunity and may have immunosuppressive effects (Martinez, 1987) and exceptional care would be required to avoid that other (viral) contaminants are absent from the product. Conversely, it is anticipated that quality control of genetically engineered vaccines will be simple, that the probability of inducing immunopathology will be reduced, that the antigenic competition will be minimised and that chemical or biological synthesis will be possible (Mitchell and Anders, 1982).

The malaria parasite has a complex life cycle and not unexpectedly the various stages of the life cycle are antigenically as well as morphologically distinct. Thus, a major activity in current research on malaria is the antigenic analysis of different cycle stages with the aim of establishing the target antigens of host-protective immune responses as a direct consequence of stage specific antigenic expression. Protective immunity in malaria also appears to be stage-specific (as well as species-specific) (Martinez, 1987). Stage-specific antigens are, therefore, characterised in an attempt to establish potential targets for immune attack. Currently, antigens of 5 different life-cycle stages viz., sporozoite, exoerythrocytic (liver), erythrocytic, gamete and (zygote)-ookinete are under investigations to determine their potential as vaccine components (Anders, 1988).

A vaccine against sporozoite stage would prevent sporozoites injected by a mosquito from reaching the liver. This would in turn prevent the onset of blood-stage infection and the transmission of gametocytes to mosquitoes. Vaccine development against the exoerythrocytic stages are at an early stage. Vaccination against the asexual erythrocytic stages of malaria would prevent or reduce the clinical symptoms. Vaccine against the sexual stages would confer no direct benefit on the recipient, but would contribute towards depletion of the reservoir of infected vectors by blocking parasite development in the midgut of mosquitoes ingesting the antibodies.

Research on sporozoite stage was considerably further advanced than many other stages of the malaria life cycle (Lockyer and Holder, 1988). Circumsporozoite surface (CS) proteins (the species-specific proteins covering uniformly the surface membrane of sporozoites) have been characterised in great detail in various species of *Plasmodium* by the use of hybridoma technology and molecular biology (reviewed in Anders, 1988 ; Lockyer and Holder, 1988 ; Nussenzweig and Nussenzweig, 1988). Recently, clinical trials of *P. falciparum* synthetic sporozoite vaccines have been tested using 2 approaches. In one trial, the test material was a recombinant antigen ($R32tet_{32}$) (Ballou *et al.*, 1987) and in the other trial, the antigen tested was a synthetic peptide $(NANP)_3$ linked to tetanus toxoid (Herrington *et al.*, 1987). Overall, the antibody responses were relatively low compared to those found in individuals who have had long-term natural exposure to infected mosquitoes. The vaccinees were subsequently challenged with *P. falciparum* sporozoites by the bite of infected mosquitoes to determine the efficacy of the vaccines. Upon challenge, there was one individual in each trial who was protected and a small number of others who had a prolonged pre-patent period. The protected individuals were among the highest antibody responders (Ballou *et al.*, 1987 ; Herrington *et al.*, 1987).

Antigens of the asexual blood stages have also been extensively studied in a variety of plasmodial species. Several different approaches have been used to identify antigens that are the targets of protective immunity in the asexual blood stages of *P. falciparum.* Among these antigens, a clinical trial has been conducted recently using a synthetic hybrid peptide based on merozoite-specific proteins ; the precursor of the merozoite antigen (PMMSA) & the ring-infected erythrocyte surface antigen (RESA) of *P. falciparum* (Patarroyo *et al.*, 1988). Partial protection of the disease was observed in the volunteers and there was no correlation between the different humoral and cellular immunological parameters measured and the anti-malarial protection observed.

1.3) Sexual stages of the malaria parasite

Studies on the sexual stages of the parasite have been intensified during the last few years because of their obvious role both in transmission and in the search for novel control methods. As described above, transmission of the malaria parasite from the vertebrate host to the mosquito vector was affected by gametocytes which were produced in the blood. These intraerythrocytic forms developed from schizonts. The stimulus for gametocyte production rather than asexual schizogony and merozoite formation remained unknown (Inselburg, 1983). However, it was suggested that conversion to gametocytes was influenced by 2 major factors. Firstly, the innate capacity of a strain or clone to produce gametocytes and secondly, environmental influences which were required for induction of sexual differentiation (Mons, 1986).

The gametocytes were ingested by the mosquito. A drop in temperature of at least 5°C below that of the vertebrate host body temperature together with a rise in pH to 7.8-8.0 in the

mosquito midgut activated the formation of male and female gametes within minutes of ingestion (Sinden, 1983 & 1984). The male gametocytes transformed into eight highly motile male gametes (microgametes) and the female a single macrogamete. Fertilisation of male and female gametes was completed within 8-15 min after ingestion of blood by the mosquito and produces zygotes which in turn transformed into motile ookinetes and subsequently oocysts.

The term 'sexual stages' were applied to all stages involved in the sexual development of the parasites from the gametocytes circulating in the vertebrate host, to the gametes & newly fertilised zygotes and up to the mature ookinetes in the mosquito midgut (Carter *et al.*, 1988a and Carter, 1988b). These stages themselves were not pathogenic to the human host (Sinden, 1983). In recent years, considerable efforts have been made on the immunological aspects of these stages as related to a potential 'altruistic' vaccine development. The vaccine would have no prophylactic value for the individuals but would protect the population of malaria endemic areas by preventing development of the infection within mosquito vectors (WHO, 1985).

1.4) Immunity to the sexual stages of the malaria parasites

Immunity against these stages tends to reduce the infectivity of the parasite to the mosquito. Thus, it has been termed as a transmission-blocking immunity (Carter *et al.*, 1984). The immunity could be induced both artificially and naturally.

1) Artificial-induction of transmission-blocking immunity

Transmission-blocking immunity was demonstrated in avian, simian and murine malaria parasites by immunising animals with 3 kinds of immunogens. They were (a) whole parasitised blood, (b) extracellular gametes & newly fertilised zygotes and (c) transforming zygotes & ookinetes.

Immunity was also induced by monoclonal antibody (MAb) in membrane feed or passive transfer experiments. MAbs against the surface determinants of gametes and zygotes of malaria parasites were developed and demonstrated to block parasite development in mosquitoes e.g. *P. gallinaceum* (Rener *et al.*, 1980 and Kaushal *et al.*, 1983a ; Grotendorst *et al.*, 1984), *P. falciparum* (Rener *et al.*, 1983 ; Vermulen *et al.*, 1985 and Quakyi *et al.*, 1987) *P. yoelii* (Harte *et al.*, 1985b), *P. berghei* (Winger *et al.*, 1988) and *P. vivax* (Peiris *et al.*, 1988).

a) Whole parasitised blood (in which gametocytes were present)

The first evidence for immune suppression of infectivity of malaria parasites to mosquitoes was reported by Huff *et al.*, (1958) who immunised turkeys intravenously with formalin-killed blood infected with *P. fallax*, or chickens with *P. gallinaceum*. It was shown that infectivity of a subsequent blood infection to mosquitoes were reduced although the asexual parasitaemia and gametocytaemia in the hosts were similar to those in control infections. This phenomenon was re-examined by Gwadz (1976), who confirmed that transmission-blocking immunity was induced in chicken immunised intravenously with formalin-killed *P. gallinaceum*-

infected blood which reduced infectivity of a subsequent blood infection to *Aedes aegypti* mosquitoes.

b) Extracellular male and female gametes including newly fertilised zygotes

Carter and Chen (1976) showed that a much more effective transmission-blocking immunity was achieved when chickens were immunised with a relatively minute amounts of partially-purified extracellular male and female gametes of *P. gallinaceum.* Furthermore, immunisation with parasitised blood in which gametogenesis had been induced *in vitro* was reported to be a much more effective immunogen than an equivalent amount in which gametogenesis was not induced (Carter *et al.*, 1979a). Additionally, preparations of purified gamete material from which gametes of one or the other sex were absent were considerably less effective as transmission-blocking immunogens than the mixed gamete preparation . It was also noted that subsequent challenge was a potent boost of waning levels of transmission-blocking antibodies (Carter *et al.*, 1979b).

Immunising with (predominantly) male gametes of *P. knowlesi* also induced transmission-blocking immunity in rhesus monkeys although Freund's complete adjuvant was required in this system (Gwadz and Green, 1978). The immunity prevented parasite development in *Anopheles dirus* mosquitoes. Additionally, persistence of such immunity in rhesus monkeys following immunisation and challenged intermittently was maintained effectively for at least 6 years (Gwadz and Koontz, 1984). This boosting effect from challenge infection presumably resulted from gametocytes and not asexual parasites.

In murine malaria models, transmission-blocking immunity was also induced in mice by vaccination with formalin-fixed gametes of *P. y. nigeriensis* and there was an associated protection against asexual blood stages of the same parasite (Mendis and Targett, 1979 & 1981). However, the cross-protection was shown in the following study to be due to contamination of the inoculum by asexual stage parasites (Mendis and Targett, 1982). In addition, persistence of the immunity was at least 6 months.

Long-term protection of the transmission-blocking immunity was reported to be at least 12 months in the mice vaccinated with male gametes of *P. y. nigeriensis* (Harte *et al.*, 1985a). Interestingly, such transmission-blocking immunity also caused a reduction in the numbers of circulating gametocytes in recipient animals upon subsequent infection.

Transmission-blocking immunity was demonstrated also in hamsters immunised intramuscularly with irradiated-gametes (predominantly male gametes) of *P. berghei* (Kaushik *et al.*, 1982). This immunisation did not affect gametocyte production and morphology.

c) Transforming zygotes or ookinetes

Polyclonal antibodies against zygotes of *P. gallinaceum* and *P. falciparum* were produced and shown to suppress mosquito infectivity (Kaushal *et al.*, 1983a ; Grotendorst *et al.*, 1984 ; Vermeulen *et al.*, 1985a).

All of these experiments described above implied that the sexual stages were immunogenic and capable of inducing a stage-specific immunity. In most of these experiments, highly effective transmission-blocking immunity was induced with little effect on the course of erythrocytic parasite infection following challenge (Carter and Chen, 1976 ; Carter *et al.*, 1979a ; Mendis and Targett, 1979 ; Kaushik *et al.*, 1982).

2) Natural transmission-blocking immunity

It was found that sera of some individuals naturally infected with *P. falciparum* contained antibodies which reacted with gametocytes in IFA but in these early studies transmission-blocking potential was not sought (Smalley and Sinden, 1977).

In Papua New Guinea, antibody responses to the gamete antigens of *P. falciparum* occurred in individuals naturally exposed to malaria (Graves *et al.*, 1988a, b). All adults in the hyperendemic area showed strong responses to asexual-stage antigens through repeated exposure. Only a minority responded to gamete antigens, with no apparent correlation between responsiveness and age. The antibodies suppressed gametocyte infectivity to mosquitoes.

Individuals exposed to natural *P. vivax* malaria infections also developed antibody to the gamete stages which suppressed gametocyte infectivity to mosquito (Mendis *et al.*, 1987a, b ; Ranawaka *et al.*, 1988 ; Peiris *et al.*, 1988). Persistence of the transmission-blocking immunity in *P. vivax* was about 4 months and such immunity could be boosted by following attacks within this 4 month period (Mendis *et al.*, 1987b ; Ranawaka *et al.*, 1988).

1.5) Mechanism of transmission-blocking immunity

As described above, transmission-blocking immunity has been demonstrated against the intracellular gametocytes circulating in the vertebrate host as well as the extracellular gametes, zygotes and ookinetes in the mosquito midgut. Both antibody and cell-mediated mechanisms have been proposed to be involved in such immunity (Carter *et al.*, 1988a).

1) Antibody-mediated immunity

Transmission-blocking immunity mediated by polyclonal or monoclonal antibodies has been demonstrated in many examples. Specific mechanisms of action of these antibodies in preventing transmission can be described as following,

a) Blocking of parasite fertilisation in the mosquito midgut by the antibodies

These antibodies were fertilisation-blocking antibodies which had no effect on the subsequent development of the parasites after fertilisation was completed.

Sera from chickens immunised with gametes of *P. gallinaceum* were shown to suppress transmission of parasites to mosquitoes (Gwadz, 1976 ; Carter *et al.*, 1979b). In the absence of complement, the sera were demonstrated to agglutinate male and female gametes *in vitro* . Two types of antibodies were described in the immune chicken serum :- (i) microgamete

surface-fixation type of antibodies (SF) and (ii) gamete agglutinating type of antibodies (AG) (Carter *et al.*, 1979b). The latter type of antibody (AG) was found frequently even when immunisation was inadequate to suppress transmission. By contrast, the former type of antibody (SF) was directly related to effective transmission-blocking immunity and titres of the antibodies could be boosted by subsequent challenge.

MAbs against male gametes of *P. gallinaceum* were shown to suppress transmission of parasites to *Ae. aegypti* mosquitoes by agglutination of male gametes which was observed under phase-contrast microscope (Rener *et al.*, 1980 ; Kaushal *et al.*, 1983a) and electron microscope (Aikawa *et al.*, 1981). In the absence of active complement, the effect of most suppressive MAbs or combinations of MAbs was shown to be entirely due to their ability to prevent fertilisation (Kaushal *et al.*, 1983a). The same phenomenon was also observed in *P. yoelii* using immune mouse sera (Mendis and Targett, 1982) or MAbs against male gametes (Harte *et al.*, 1985b).

The MAbs against gametes of *P. falciparum* suppressed transmission by blocking fertilisation (Rener *et al.*, 1983). In *P. vivax*, rabbit anti-gamete antibody suppressed transmission of the parasite to the mosquito by blocking fertilisation. Agglutination of male gametes by the antibody was observed in IFA (Munesinghe *et al.*, 1986).

b) Lysis of gametes and newly fertilised zygotes in the mosquito midgut by complement-dependent anti-gamete antibodies

Some MAbs against male gametes against *P. gallinaceum* which were ineffective or moderately effective in suppressing parasite infectivity to mosquitoes appeared to be capable of almost total suppression of transmission in the presence of active complement (Kaushal *et al.*, 1983a). The effects were shown to be associated *in vitro* with the rapid lysis of male & female gametes and zygotes.

The MAbs against gametes of *P. falciparum* reported by Rener *et al.*, (1983) suppressed transmission in the presence of complement not only by blocking fertilisation but also destroying gametes & zygotes (Carter *et al.*, 1988a). The suppressive effect of the MAbs against gametes of *P. falciparum* reported by Quakyi *et al.*, (1987) was also complement-dependent and the mechanism could be directly demonstrated by the rapid lysis of newly formed gametes and zygotes *in vitro*.

c) Inhibition of the post-fertilisation development of the parasite in the mosquito by complement-independent antibodies

Anti-ookinete chicken serum and MAbs against *P. gallinaceum* were produced and shown to prevent subsequent development of fertilised zygotes in mosquito midgut (Grotendorst *et al.*, 1984). The suppressive effect was complement-independent.

In *P. falciparum*, MAbs against macrogametes and zygotes were produced and the influence of the transmission-blocking MAbs on the development of ookinetes was studied (Vermeulen *et al.*, 1985a). Interestingly, the MAb 32F81 did not significantly influence the

number of retort cells and ookinetes formed in the mosquito midgut. Suppression of oocyst development was suggested to be due to prevention of penetration of the mosquito midgut by the ookinetes.

In transmission-blocking immunity induced by natural infections, the suppressive effect of some immune human sera from *P. vivax* patients was demonstrated to be mediated by antibody (Mendis *et al.*, 1987a, b ; Peiris *et al.*, 1988). The presence of antibodies was demonstrated by IFA against live intact gametes of *P. vivax*. In most cases, suppression of transmission was much more effective in the presence of active complement (Mendis *et al.*, 1987a). Interestingly, the same immune human sera could have a dual role in mosquito infectivity, that was a blocking effect at higher concentrations and an enhancing effect at the lower concentrations (Mendis *et al.*, 1987b and Peiris *et al.*, 1988). The mechanism for the enhancing effect was not yet clear. However, it was suggested to be due to improved fertilisation efficiency mediated by very low levels of anti-gamete antibodies (Carter *et al.*, 1988a).

In *P. falciparum*, anti-gamete antibodies were identified in immune human sera by immunoprecipitation of extracts of ^{125}I-labelled gametes of *P. falciparum* (Graves *et al.*, 1988a ; Carter *et al.*, 1989). Some of these sera were observed to promote parasite infectivity to mosquito (Graves *et al.*, 1988a).

2) Cell-mediated Immunity

Transmission-blocking immunity induced by vaccination of *P. y. nigeriensis* microgametes was fully effective for at least 12 months (Harte *et al.*, 1985a). Passive transfer of immune T-cells of the GK 1.5$^+$ Ly 2.2 phenotype reduced transmission of a subsequent infection by 95%. The effect was partly due to a significant reduction in numbers of circulating gametocytes in recipient animals during the infection. This immunity was apparently independent of specific antibody.

Antibody-independent cellular mechanisms of transmission-blocking immunity may also have an important role in human malaria. Peripheral blood from non-immune humans has been shown to contain *P. falciparum* gamete-reactive T cells (Good *et al.*, 1987). Proliferation of T cells and γ-interferon (γ-IFN) production was stimulated *in vitro* by gametes of *P. falciparum*. However, the precise role played by cell-mediated immunity has yet to be determined in human or animal model systems.

1.6) Stage-specific antigens of gametes, zygotes and ookinetes

1) Antigens recognised by the antibodies from artificial-induction of transmission-blocking immunity

Surface antigens of the sexual stages of *P. gallinaceum* were extensively characterised (Howard *et al.*, 1982 ; Kaushal *et al.*, 1983b ; Kaushal and Carter, 1984 ; Carter and Kaushal, 1984 ; Kumar and Carter, 1985a, b).

Surface antigens of male and female gametes were studied (Kaushal and Carter, 1984). The parasites were surface radioiodinated. Triton X-100 extracted proteins were immunoprecipitated by stage-specific rabbit antisera and separated on SDS-PAGE. Four proteins :- PgZ-1 (225 or 250 kD under non-reducing or reducing conditions, respectively), PgZ-3 (the M_r under non-reducing conditions was not known but it was 215 kD under reducing conditions) and PgZ-13a & b (45 & 48 or 56 & 54 kD under non-reducing or reducing conditions, respectively) were present in indistinguishable antigenic forms on both male and female gametes. Three immunogenic proteins, PgZ-14 (42 or 48 kD under non-reducing or reducing conditions), PgZ-17a and b (19 and 17 kD respectively under both conditions) were present on female but not male gametes (as were several weakly labelled, non-immunogenic proteins of less than 45 kD). In contrast, a PgZ-16 (26 kD under both conditions) was present on male but not female gametes.

P. gallinaceum zygote surface antigens were initially studied by Howard *et al.*, (1982). Zygotes were radiolabelld, immunoprecipitated by anti-zygote rabbit serum and separated on SDS-PAGE. Fifteen proteins in the molecular weights ranging from 42-260 kD (under reducing conditions) were detected.

Subsequent study by Kaushal *et al.*, (1983b) also showed that 15 protein bands (namely PgZ 1-15) in the molecular weight range of 40-240 kD (under reducing conditions) were identified on newly fertilised zygotes of *P. gallinaceum*. The major surface antigens were PgZ-1 (240 kD), PgZ-4 (200 kD), PgZ-6 (180 kD), PgZ-11 (80 kD), PgZ-13 (55 kD), PgZ-14 (50 kD) and one band at the bromophenol blue dye marker. PgZ-6 appeared to be a host serum protein selectively adsorbed to the zygote surface antigen. In addition, it was noticed that the major surface protein antigens between newly fertilised zygotes and female gametes were not distinguishable (Kaushal *et al.*, 1983b).

Zygote surface proteins were further defined by Kaushal and Carter (1984). The proteins, on reducing SDS-PAGE were PgZ-1 (250 kD), PgZ-3 (215) kD), PgZ-4 (205 kD), PgZ-6 (165 kD), PgZ-11 (83 kD), PgZ-13a & b (56 & 54 kD respectively), PgZ-14 (48 kD), PgZ-16 (26 kD), PgZ-17a & b (19 & 17 kD respectively). It was noted in this study that the values given for molecular weights of the proteins were in many instances slightly different from Kaushal *et al.*, (1983b). PgZ-13 was resolved into 2 proteins designated PgZ-13a and PgZ-13b. Material previously identified as migrating at the bromophenol blue dye front was also resolved into 2 proteins designated PgZ-17a and PgZ-17b.

Proteins expressed on the surface of *P. gallinaceum* during their development from fertilisation to mature ookinetes were further examined (Carter and Kaushal, 1984). It was found that other stage-specific proteins of gametes & newly fertilised zygotes except PgZ-14, PgZ-17a and b were shed during transformation of zygotes to ookinetes and the proteins could be recovered, immunologically intact and of apparently unchanged molecular weight from the medium in which the cells were transforming. Moreover, three proteins were found to be expressed *de novo* on the surface of the mature ookinetes of which M_r 26 (PgO-1) and 28 kD (PgO-2) on reducing SDS-PAGE (or 31 and 34 kD respectively on non-reducing SDS-PAGE) represented major surface components and M_r 52 kD (PgO-3) (under both non-reducing and reducing conditions) a relatively minor component. PgO-2 was the predominant protein on the mature ookinete (Kumar and Carter, 1985a, b).

Since *in vitro* fertilisation by gametes of *P. falciparum* was inefficient and morphological transformation to the ookinetes was rarely achieved, the full array of proteins expressed on the surface of a mature ookinete of *P. falciparum* was not known (Carter *et al.*, 1988a).

2) Antigens recognised by naturally occurring antibodies

The immune recognition of surface proteins and intracellular proteins of *P. falciparum* gametes by immune human sera from Papua New Guinea was analysed (Graves *et al.*, 1988a ; Carter *et al.*,1989). Gametes were surface iodinated, Triton X-100 extracted and immunoprecipitated with the human sera. Surface proteins of M_r 230, 48 and 45 including minor proteolytic fragments of the 230 kD protein were immunoprecipitated by the sera (Carter *et al.*, 1989). Other proteins of M_r 60 and 40 kD were also immunoprecipitated in small detectable amounts (Carter *et al.*, 1989). To study the reactivity of the human sera to intracellular proteins of *P. falciparum* gametes, the parasites were ^{125}I-labelled after Triton X-100 extraction. Four proteins; 27, 55, 70 and 97 kD of which the 27 kD was the most prominent protein were immunoprecipitated by the sera. A 260 kD protein was also detected in some sera. These proteins appeared to be exclusively internal proteins of the parasites.

1.7) Target antigens of transmission-blocking immunity and their immunogenicity

Whilst little was known about target antigens of gametocytes in the bloodstream of the vertebrate host, target antigens of gametes, zygotes and ookinetes recognised by transmission-blocking MAbs were comprehensively characterised in *P. gallinaceum* (Kaushal *et al.*, 1983a ; Grotendorst *et al.*, 1984 ; Kumar and Carter, 1985b & 1986 ; Kumar, 1985 & 1987). They were also studied in *P. falciparum* (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a & 1986 ; Kumar and Carter, 1985b & 1986 ; Kumar, 1987 ; Quakyi *et al.*, 1987 ; Graves *et al.*, 1988a). More limited information was, however, available for *P. yoelii* (Harte *et al.*, 1985c), *P. vivax* (Mendis *et al.*, 1987b ; Peiris *et al.*, 1988), and *P. berghei* (Sinden *et al.*, 1987, Winger *et al.*, 1988 ; Dearsly, 1989).

1) Target antigens

a) Gamete and newly fertilised zygote antigens

In *P. gallinaceum*, gametes were surface radioiodinated, immunoprecipitated with MAbs and analysed by SDS-PAGE (Kaushal *et al.*, 1983a). A set of 3 proteins with M_r of 220 (equivalent to PgZ-1), 50 and 48 (PgZ-13a and b) under non-reducing conditions (or 240, 56 and 54 kD respectively under reducing conditions) was immunoprecipitated by transmission-blocking MAbs. These proteins were found to occur in apparently indistinguishable antigenic and chemical form on male as well as female gametes. Recently, the molecular weight of these antigens have been stated as 230, 48 and 45 kD under non-reducing conditions (Carter *et al.*, 1988a). These molecular weights will be referred throughout this thesis.

Similarly, 3 proteins of M_r 235, 49 and 46 kD under non-reducing conditions (or 255, 59 and 53 kD respectively under reducing conditions) on the surface of gametes of *P. falciparum* were immunoprecipitated by transmission-blocking MAbs (Rener *et al.*, 1983). Four proteins on the surface of *P. falciparum* gametes of M_r 230, 48, 45 and 10 kD under non-reducing conditions (or 250, 58, 47 and 10 kD respectively under reducing conditions) were immunoprecipitated by transmission-blocking and non-blocking MAbs (Vermeulen *et al.*, 1985a). Carter *et al.*, (1988a) concluded the M_r's of the 3 proteins to be 230, 48 and 45 kD under non-reducing conditions. Thus, it is these molecular weights that will be referred to in this thesis.

The 230, 48/45 kD gamete antigens of both *P. gallinaceum* and *P. falciparum* did not represent precursor-product series (Carter *et al.*, 1984). Instead, the 230 kD protein is physically associated with both 48 and 45 kD molecules and appeared to exist in a stable membrane-bound complex which resulted in co-immunoprecipitation of the polypeptides by various MAbs. There was no immunological cross reactivity due to shared antigenic determinants between the high and the two low molecular weight antigens (Kumar and Carter, 1985b & 1986 ; Kumar, 1985 & 1987). The sole targets of transmission-blocking MAbs were therefore resolved to be the 230 kD protein in *P. gallinaceum* and the 48/45 kD proteins in *P. falciparum*. Recently, the 230 kD protein of *P. falciparum* gametes was also reported to be a target antigen for the transmission-blocking MAbs (Quakyi *et al.*, 1987). In addition, immune human sera from individuals exposed to intense malaria transmission in Papua New Guinea showed a strong correlation between suppression of *P. falciparum* gametocyte infectivity to mosquitoes and the presence of antibodies to the 230 kD gamete surface antigen (Graves *et al.*, 1988a).

The presence of reactivity to 48/45 kD doublet correlated to the transmission-blocking capacity of many MAbs (Vermeulen *et al.*, 1985a). However, non-blocking MAbs against these proteins were also reported. The proteins were physically and antigenically closely related. They could be immunoprecipitated by the MAbs (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a). The proteins were also detected in immunoblot analysis under non-reducing conditions (Vermeulen *et al.*, 1985a).

In *P. vivax*, MAbs against female gametes were produced and shown to suppress transmission (Peiris *et al.*, 1988). On Western blots, the MAb has been demonstrated to react with 2 proteins of M_r 42 and 36 kD from extract of gametes of *P. vivax* separated on non-reducing SDS-PAGE. Thus, these 2 proteins represent antigenic targets for transmission-blocking immunity which may correspond to those 48/45 kD in *P. gallinaceum* and *P. falciparum*.

In *P. y. nigeriensis*, a transmission-blocking MAb immunoprecipitated male gamete antigens of M_r 67, 59, 57, 42 and 35 kD of which the 42 kD appeared to be the major immunogenic protein (Harte *et al.*, 1985c).

b) Transforming zygote or ookinete antigens

Among the 2 major surface proteins (PgO-1 and PgO-2) formed *de novo* during transformation of zygotes to ookinetes of *P. gallinaceum*, only the PgO-1 (26 kD under reducing conditions or 31 kD under non-reducing conditions) was found to be the target antigen of transmission-blocking MAbs against ookinetes (Grotendorst *et al.*, 1984). The PgO-2 (28 kD under reducing conditions or 34 kD under non-reducing conditions) does not appear to feature as a target of transmission-blocking immunity since several independently derived MAbs against this protein had no effect on the infectivity of the parasites to mosquitoes (Carter *et al.*, 1988a).

During the transformation of zygotes to ookinetes of *P. falciparum*, one polypeptide with an M_r of 25 kD (under non-reducing conditions) was identified as the target of transmission-blocking MAbs in immunoblot assays (Vermeulen *et al.*, 1985a).

In *P. berghei*, target antigens of transmission-blocking MAbs were found to be a protein determinant with an M_r of 21 kD (under both non-reducing and reducing conditions) (Winger *et al.*, 1988). Details will be further described in Chapter 2 of this thesis.

For convenience, the target antigens recognised by the specific MAbs or immune human sera will be designated in this thesis using the nomenclature system of Kaslow *et al.*, (1988). Thus, the target antigens on gametes of *P. gallinaceum* and *P. falciparum* will be entitled Pgs230, Pgs48/45 and Pfs230, Pfs48/45 respectively. The target antigen on ookinetes of *P. gallinaceum* described by Grotendorst *et al.*, (1984) will be changed to Pgs31 so as to be consistent with the new terminology. The target antigen of *P. falciparum* ookinetes will be Pfs25 as described by Kaslow *et al.*, (1988) and it will be Pbs21 in *P. berghei*.

2) Immunogenicity of the target antigens

Responsiveness to Pfs230, Pfs48/45 and Pfs25 proteins was controlled by the Ir-gene (Good *et al.*, 1988). Different strains of H-2 congenic mice were injected with *P. falciparum* zygotes. The mouse sera were used to immunoprecipitate either ^{125}I surface labelled zygotes or ^{35}S-cysteine metabolically labelled zygotes. Most strains were nonresponsive to either Pfs230 or Pfs48/45 antigens. It was suggested that the number of T helper epitopes were limited in the 3

antigens. Since antibody was the critical form of the immunity, an infection with a parasite not containing a helper T epitope for a given antigen would not stimulate an antibody response to that antigen. In contrast, antibody response to Pfs25 was not restricted in H-2 congenic mice.

Some of the immune human sera from Papua New Guinea also showed nonresponsiveness to Pfs230 and Pfs48/45 antigens which suggested that the restricted immune response was likely to be genetic differences between individuals in the MHC region controlling response to T helper epitopes (Carter *et al.*, 1989). In contrast, a lack of antibody response in the human sera to Pfs25 was observed, suggesting that this protein was not naturally immunogenic. The protein was synthesised after transformation of gametocytes into gametes which occurred in mosquito midgut, it could not, therefore, act as an immunogen (Carter *et al.*, 1989).

1.8) Biosynthesis of the target antigens of transmission-blocking immunity

Gamete antigens

Biosynthesis of gamete surface antigens of *P. gallinaceum* and *P. falciparum* have been extensively studied during the growth and transformation of gametocytes to gametes and zygotes (Carter *et al.*, 1984 ; Kumar and Carter, 1984 ; Vermeulen *et al.*, 1986). In *P. gallinaceum*, the 3 proteins :- Pgs230, Pgs48/45 were synthesised in the gametocytes but were not synthesised nor otherwise detectable in asexual blood stage parasites (Carter *et al.*, 1984). After gametogenesis, synthesis of the proteins still continued in the female gametes and newly fertilised zygotes for several hours but they failed to appear on the cell surface. Additionally, Pulse-chase experiments indicated that there was a higher-molecular-weight molecule (280-300 kD) that behaved as if a precursor of the Pgs230 (Carter *et al.*, 1988a).

An analogous set of gamete surface proteins of *P. falciparum* (Pfs230, Pfs48/45) was simultaneously synthesised by gametocytes from an early stage in their maturation but were not synthesised by asexual blood stage parasites (Kumar and Carter, 1984 ; Vermeulen *et al.*, 1986). The target proteins synthesised by the gametocytes were expressed on the surface of the extracellular gametes but the gametes themselves no longer synthesised these proteins (Kumar and Carter, 1984). Pulse-chase analysis during biosynthetic labelling of gametocytes of *P. falciparum* did not show any precursor-product relationship between the high and the 2 low molecular weight proteins (Kumar and Carter, 1984 ; Vermeulen *et al.*, 1986).

Pfs230 appeared to be present as a doublet proteins of 240/230 from day 2 of gametocyte development in synchronous cultures (Vermeulen *et al.*, 1986). The 240 kD protein was neither detected in the biosynthetic labelling of non-synchronous cultures (Kumar and Carter, 1984) nor in the ^{125}I-labelling of surface proteins of macrogametes (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a). This protein was suggested to be an intracellular precursor molecule of the Pfs230 (Vermeulen *et al.*, 1986). Similarly, a high molecular weight protein (M_r 260) was also reported to be recognised by the anti-Pfs230 MAbs (Quakyi *et al.*, 1987). Moreover, the immune

human serum from Papua New Guinea also immunoprecipitated Pfs260 protein (but at a barely detectable amount) (Carter *et al.*, 1989). This protein was restricted exclusively to the intracellular compartment and only Pfs230 protein appeared on the gamete surface. However, the relationship between these 2 proteins (Pfs230 and Pfs260) was not clear.

Ookinete antigens

While shedding of gamete surface antigens of *P. gallinaceum* continued, Pgs31 was synthesised *de novo* by the transforming of zygotes to ookinetes (Carter and Kaushal, 1984). Biosynthesis and expression of Pgs31 were studied by Kumar and Carter (1985a, b). The cells were biosynthetically labelled *in vitro* using ^{35}S-methionine and proteins were immunoprecipitated with anti-ookinete rabbit serum or MAbs. Pgs31 which was not synthesised in the gametocytes prior to gametogenesis was detected in early zygote development (2 h post-gametogenesis and fertilisation). The protein was synthesised for a further 8-10 h. Thereafter, synthesis of the protein declined and little or none was synthesised in the mature ookinetes (> 20 h post-gametogenesis).

In *P. berghei*, biosynthesis of Pbs21 was studied (Sinden *et al.*, 1987 ; A. L. Dearsly, pers. comm.). Using EM-immunogold, it was found that there was no synthesis of the protein in gametocytes or the asexual stages (Sinden *et al.*, 1987). Synthesis of the protein was detected on the surface of activated macrogametes 2 h after the onset of the culture i.e. 110-120 min after activation or ca. 100 min after fertilisation. In addition, expression of the protein still persisted for the full life of ookinetes i.e. 24 h (Sinden *et al.*, 1987). These results were consistent with the study using ^{35}S-methionine biosynthetic labelling of synchronous-gametocyte cultures (A. L. Dearsly, pers. comm.). Pbs21 was detected in zygotes after 2 h post-gametogenesis and fertilisation of gametes but not in gametocytes. Synthesis and expression of the protein was also detected on extracellular macrogametocytes and macrogametes which showed no evidence of fertilisation (Sinden *et al.*, 1987). It was therefore suggested that trigger for the expression of the 'Pbs21 gene' might be activation of the macrogametocytes and not fertilisation of macrogametes.

Biosynthesis of Pfs25 was also studied (Vermeulen *et al.*, 1985a & 1986). The protein was not shown to be the product of the higher molecular weight precursor molecule. Unlike Pgs31 and Pbs21, Pfs25 described as being synthesised at a very low but detectable level in gametocytes. The protein was synthesised as early as day 2 of gametocyte development. However, production was sharply increased in the macrogamete/zygote for at least 4-6 h. This observation was not consistent with the recent findings that natural immunity to Pfs25 did not occur since the immune human sera from Papua New Guinea failed to immunoprecipitate the protein from ^{125}I-labelled *P. falciparum* gametes (Carter *et al.*, 1989).

1.9) Chemical, biochemical and immunological structures of the target antigens

Various aspects of chemical, biochemical and immunological properties of the target antigens have been extensively studied in *P. gallinaceum* (Kumar and Carter, 1983, 1985a, b, 1986 ; Carter and Kaushal, 1984 ; Kumar, 1985 & 1987) and *P. falciparum* (Kumar and Carter, 1984, 1985b & 1986 ; Carter *et al.*, 1985 & 1988a ; Fries *et al.*, 1989 ; Graves *et al.*, 1985 & 1988a, b ; Kumar, 1985 & 1987 ; Quakyi *et al.*, 1987 ; Vermeulen *et al.*, 1985a, b & 1986).

1) Chemical structure

Gamete antigens

The distribution of the 230, 48 and 45 kD proteins of *P. gallinaceum* and *P. falciparum* in the detergent-free (aqueous) and detergent-enriched phases was studied using a phase separation technique in Triton X-114 (Kumar, 1985 & 1987). The technique resulted in the separation of the detergent-binding hydrophobic proteins from relatively less hydrophobic ones. Of the 3 transmision-blocking antigens on the surface of gametes of *P. gallinaceum* and *P. falciparum*, Pfs230 and Pgs230 proteins were recovered in the aqueous phase, whereas Pfs48/45 and Pgs48/45 proteins were preferentially found in the detergent phase (Kumar and Carter, 1985b and 1986 ; Kumar, 1985 & 1987). In addition, when phase-separated fractions were recombined, Pgs230 and Pgs48/45 proteins did not reassociate which suggested that phase separation resulted in irreversible changes either in the proteins or in their immediate environment (Kumar, 1985). After phase separation, transmission-blocking MAbs against *P. gallinaceum* gametes reacted only with Pgs230 whereas transmission-blocking MAbs against *P. falciparum* gametes which had been shown to co-immunoprecipitate Pfs230 and Pfs48/45 reacted only with Pfs48/45 proteins (Kumar and Carter, 1986 ; Kumar, 1987).

Hydrophobicity of the 230, 48/45 kD proteins of *P. gallinaceum* and *P. falciparum* was further studied by 2 approaches, (i) the tendency to interact with the lipid bilayers (liposome) and (ii) the tendency to interact with a hydrophobic matrix phenyl-Sepharose on column chromatography (Kumar, 1985 & 1987 ; Kumar and Carter, 1986). The 48 and 45 kD proteins were retained by the hydrophobic column and were incorporated into the lipid bilayers while the 230 kD was not. Thus, it was concluded that the 48/45 kD proteins were strongly hydrophobic and the 230 kD was hydrophilic. This suggested that the 48/45 kD proteins might be buried in the membrane while the 230 kD protein might be more weakly associated with the lipid bilayer (Carter *et al.*, 1988).

Gel-permeation chromatography and cross-linking studies showed that the 3 polypeptides were physically associated in a complex with a stoichiometry of 1:1 between the high and a doublet of low molecular weight peptides. However, individual protein molecules could be presented in an uncomplexed as well as a complexed form in the membrane (Carter *et al.*, 1988a). MAbs against Pfs230 did not precipitate the complex but only their target protein (Quakyi *et al.*, 1987 ; Vermeulen *et al.*, 1985a).

Ookinete antigens

Pgs31 showed strong interaction with the detergent Triton X-114 (Kumar, 1985) and it was a hydrophobic protein (Kumar and Carter, 1983). Similarly, Pfs25 was a hydrophobic protein (Carter *et al.*, 1988a).

Pbs21 also showed strong interaction with Triton X-114 (A. L. Dearsly, pers. comm.).

2) Biochemical structure

Biochemical features of sexual stage antigens of *P. falciparum* and *P. gallinaceum* appeared to be closely analogous (Carter *et al.*, 1984).

a) Post-translational modification of the target antigens

Gamete antigens

Pfs48/45 but not Pfs230 were labelled with either ^{3}H-glucosamine or ^{3}H-mannose (Kumar and Carter, 1984 ; Vermeulen *et al.*, 1986). A higher incorporation of ^{3}H-mannose compared to ^{3}H-glucosamine was observed in Kumar and Carter (1984). Vermeulen *et al.*, (1986) showed that incorporation of only ^{3}H-glucosamine into the proteins was detected but the incorporation was not detected with ^{3}H-mannose labelling.

Ookinete antigen

Post-translational modification of Pgs31 was studied (Kumar and Carter, 1985a, b). The protein could be labelled with ^{3}H-glucosamine, ^{3}H-mannose and ^{3}H-palmitic acid. Moreover, the protein was reported to be heavily labelled by ^{3}H-myristic acid as compared to ^{3}H-palmitic acid (Kumar and Carter, 1985a).

Similar radiolabelling techniques confirmed that Pfs25 was both glycosylated and acylated (Vermeulen *et al.*, 1985a & 1986 ; Fries *et al.*, 1989). Thus, the protein exhibited similar characteristics to Pgs31. Both Pfs25 and Pgs31 were shown to be targets of MAbs that interfered with transmission but not with fertilisation. Both were glycosylated and acylated, had similar apparent molecular weights and are synthesised predominantly immediately after gametogenesis. However, some differences were evident in these 2 proteins. Pgs31 was not synthesised by developing gametocytes and the protein was accessible to surface iodination of zygotes/ookinetes (Kumar and Carter, 1985a, b). Pfs25, however, could not be labelled in this way, probably because of the absence of tyrosine or histidine residues on the surface (Vermeulen *et al.*, 1985a & 1986).

Post-translational modification of Pbs21 was studied (A. L. Dearsly, pers. comm.). The protein was labelled with either ^{3}H-glucosamine or ^{3}H-mannose indicating that it was also glycosylated.

b) Isoelectric point

The isoelectric points (pI) of the target antigens on gametes and zygotes of *P. falciparum* were determined using 2-dimensional (2-D) SDS-PAGE in combination with

immunoblotting (Vermeulen *et al.*, 1986). Approximate pI values for Pfs230 could not be detected on 1-D or 2-D immunoblots since immunoreactivity was destroyed after SDS-PAGE. The values for Pfs48/45 and Pfs25 proteins were equivalent to 6.0 ± 0.1 and 5.6 ± 0.1 respectively.

3) Immunological structure

Gamete antigens

It has already been stated above that naturally-infected individuals could develop an immune response to Pfs230 and Pfs48/45 proteins (Graves *et al.*, 1988a, b). In addition, there was a strong correlation between the suppressive effect of the immune human sera and the presence of anti-Pfs230 antibodies (Graves *et al.*, 1988a). There may be at least 2 B-cell epitopes on Pfs230 protein (Carter *et al.*, 1988a). Conservation of the epitopes on Pfs230 was tested in IFA (Quakyi *et al.*, 1987). The anti-Pfs230 MAbs gave strong positive reactions against surafce proteins of newly fertilised gametes of all 5 isolates tested.

Epitopes on the Pfs48/45 proteins have been characterised using 2-site radioimmunoassays (Carter *et al.*, 1985 ; Vermeulen *et al.*, 1985b). It is concluded that there may be at least 3 separate non-repeated B-cell epitopes (Epitopes I-III) on the proteins (Carter *et al.*, 1988a). The epitopes were sensitive to reduction in SDS-PAGE which indicated that they were determined by tertiary structure, maintained by disulphide bridges between cysteine residues (Vermeulen *et al.*, 1986 ; Carter *et al.*, 1988a).

Antibodies to epitope I of Pfs48/45 blocked transmission when they were present singly in complement-independent fertilisation-blocking reactions. The two other epitopes (Epitopes II and III) were recognised by MAbs that must be present together in order to suppress infectivity in complement-dependent, fertilisation irrelevant fashion (Carter *et al.*, 1988a). Diversity of B-cell epitopes was observed in Pfs48/45 (Carter *et al.*, 1988a). Epitope II was variant in a minority of *P. falciparum* isolates (Graves *et al.*, 1985). Recently, Epitope III was found to be a naturally immunogenic epitope recognised by the majority of persons who had antibodies to Pfs48/45 proteins in Papua New Guinea (Graves *et al.*, 1988b). Diversity of B-cell epitopes has also been found among *P. vivax* isolates for an apparently analogous pair of gamete surface proteins of 42 and 36 kD (Carter *et al.*, 1988a).

Pfs230 and Pfs48/45 also contained T-cell epitopes (Carter *et al.*, 1988a). Although the epitopes which were CD4 (T helper) epitopes have not been determined for Pfs230 and Pfs48/45 antigens, it was implicated that they were limited in numbers since an immune response to the antigens in the mice injected with zygotes of *P. falciparum* was MHC-restricted (Good *et al.*, 1988).

Ookinete antigens

There are at least 2 B-cell epitopes in Pfs25 (Vermeulen *et al.*, 1985b ; Carter *et al.*, 1988a) recognised by either transmission-blocking and non-blocking MAbs (Vermeulen *et al.*, 1985b). None of the epitopes were repeatedly expressed. All relevant epitopes were sensitive

to reduction which implied that the epitopes were conformation-dependent (Vermeulen *et al.*, 1985a & 1986 ; Fries *et al.*, 1989). Pfs25, upon reduction, lost its immunological reactivity in immunoblot assays and the reactivity was not restored by renaturation (Fries *et al.*, 1989). Reaction of the protein with the MAbs after deglycosylation in Western blot analysis or after modification by periodate treatment in 2-site ELISA indicated that the carbohydrate moiety added during post-translational modification neither formed an integral part of nor contributed to the stability of the epitopes in Pfs25 (Fries *et al.*, 1989). By contrast, acylation had a crucial role in the immunological reactivity of the epitopes recognised by the transmission-blocking MAbs (Fries *et al.*, 1989).

Recently, the gene encoding Pfs25 has been cloned (Kaslow *et al.*, 1988). Deduced amino acid sequence (217 amino acids) consisted of a putative signal peptide at the N-terminus, a short hydrophobic anchor at the C-terminus (15 amino acids) which showed a tendency to have a phosphatidyl-inositol-like membrane anchor, 4 potential glycosylation sites for N-linked sugars and 4 epidermal growth factor (EGF)-like domains .

A notable feature of the deduced amino acid sequence was that it contained 11% cysteine residues. This correlated with the high uptake of labelled cysteine in biosynthetic labelling (Carter *et al.*, 1988a) and was consistent with previous reports about the biochemistry of the protein that the epitopes recognised by the transmission-blocking MAbs were conformation-dependent (Vermeulen *et al.*, 1986 ; Fries *et al.*, 1989).

The biological function of Pfs25 was not known. As the protein was acylated and together with the identification of the EGF-like domains in the deduced amino acid sequence, the protein might facilitate lipid bilayer interaction, or have a function in membrane fusion events-the processes that might be involved in binding to receptors on mosquito epithelial cells to facilitate invasion (Kaslow *et al.*, 1988 ; Fries *et al.*, 1989).

The gene encoding Pgs31 gene has also been cloned using an oligonucleotide probe directed against one of the EGF-like domains of Pfs25 (Kaslow *et al.*, 1989a). The Pgs31 gene coded for a polypeptide of 215 amino acids, 2 amino residues less than Pfs25. The organisation of Pgs31 was strikingly similar to Pfs25 at a structural level. The deduced amino acid sequence contained a putative signal sequence at the amino-terminus, a hydrophobic region at the carboxyl terminus, 4 tandemly repeated EGF-like domains and 10.5% cysteine residues. By comparing Pgs31 with Pfs25, 6 conserved regions consisting of 6 or more amino acid residues have been identified. Most of the conserved regions were outside EGF-like core consensus sequences.

1.10) Rodent malaria model

In the past, much of the experimental work on parasite biology was carried out on the avian malaria parasites. At present, however, they are less widely used following the development of both rodent and primate malaria models (WHO, 1987). The rodent malarias have provided invaluable models in fundamental to sophisticated analyses of the biology of the malaria parasites. Perhaps the main contributions of the rodent malaria parasites to various aspects of research have been the inexpensiveness and the ability to produce high infection levels for almost all the parasite stages in both the host and vector. With many of the stages of the life cycle now cultured *in vitro* (i.e. the ookinete, exoerythrocytic and erythrocytic stages), the rodent malarias may be expected to provide convenient models for the development of techniques to be applied subsequently to the less readily available human parasites.

Furthermore, the existence of many inbred stains, the characterisation of surface markers for identification of lymphocyte subsets, and the capacity to manipulate the murine hosts experimentally, have provided some of the most significant information on the repertoire of host immune responses. These enable the mouse to be an ideal host for *in vivo* studies.

Murine model systems have already made significant contributions to the efforts to further our understanding of the human malarias. Many aspects of studies on immunity to *P. berghei* sporozoites provide the basis for our understanding of the antigenic structure of the circumsporozoite (CS) proteins and the complexity of the host immune responses to the antigen (reviewed in Nussenzweig and Nussenzweig, 1988). The exoerythrocytic (EE) stage in *P. berghei* is also being extensively characterised (A. Suhrbier, pers. comm.). Antigens homologous to *P. falciparum* vaccine candidates have already been demonstrated in rodent malarias, i.e. the precursor of the major merozoites surface antigen (PMMSA) of *P. yoelii* (230 kD) and the ring-infected erythrocyte surface antigen (RESA) of *P. chabaudi* (reviewed in Anders, 1988 ; Lockyer and Holder, 1988). In *P. yoelii*, microgamete surface antigens of which the M_r 42 kD is the major immunogenic protein has also been demonstrated as a transmission-blocking vaccine candidate (Harte *et al.*, 1985c).

Techniques have been attempted to establish evolutionary relatedness of species of plasmodia. For instance, the base composition of DNA from various plasmodial species (McCutchan *et al.*, 1984). Rodent parasites displayed a dG.dC content of approximately 18%. In contrast, *P. vivax* was reported to have a dG.dC content of about 30%. Interestingly, *P. falciparum* was found to have a base composition more like that of the rodent and avian parasites than the other primate parasites. Thus, on this single criterion, murine malarias may bear a closer evolutionary relatedness to *P. falciparum*.

However, several disadvantages could be emerged from studies on the rodent malarias. In some instances, conclusions drawn from immunological or pharmacological experiments with rodent malarias in mice or rats may not be directly applicable to the human disease because of differences in host immune responses, anatomy or metabolism.

1.11) The aims of the thesis

As described, infectivity of malarial gametocytes to mosquitoes can be interrupted by transmission-blocking immunity. The targets of such immunity are on the extracellular gametocytes, male and female gametes, zygotes and ookinetes. Each cell type offers distinct antigenic targets. This study concentrates upon the transmission-blocking antigens on the ookinetes. Identification of the second parasite stage in the mosquito midgut is important since the MAbs against gametes and ookinetes were shown to have an additive effect on suppression of malaria transmission (Vermeulen *et al.*, 1985a).

The rodent malaria *P. berghei* is used as a model to extend the pilot studies of Sinden *et al.*, (1987), Winger *et al.*, (1988) and Dearsly (1989). Informations on the target antigen of ookinetes were established but incompletely characterised and need further investigations. In addition, the parasite offers several advantages. The ookinetes can be successfully cultured *in vitro* (as has already been described by Sinden *et al.*, 1987). They can be harvested which enables the possibility of immunisation studies with purified antigens in a rodent host.

Thus, the objectives of the thesis are to further characterise and purify ookinete-specific protein(s) of *P. berghei* which are the targets of transmission-blocking MAbs. The potential of the antigen(s) as a vaccine candidate is further assessed. Protective immunity in the host is determined. Additionally, antigenic epitopes on the target antigen(s) are characterised to further understand the immunochemical properties which might enable a better way to control the disease and apply to the human malaria parasites.

CHAPTER 2

MONOCLONAL ANTIBODY PRODUCTION AND CHARACTERISATION

2.1) Introduction

A major breakthrough in immunology has been the production of monoclonal hybridomas (Kohler and Milstein, 1975 and 1976). The method is proved to be one of the most powerful tools to study many protein antigens.

Monoclonal antibodies (MAbs) against all stages of vertebrate infection malaria parasites have been produced e.g. sporozoites (reviewed in Nussenzweig and Nussenzweig, 1988) and asexual stages (reviewed in Miller *et al.*, 1984). They have been used to identify the target antigens of protective immunity in several species of *Plasmodium.*

MAbs against determinants of gametes and zygotes of several malaria parasites have also been produced i.e. *P. gallinaceum* (Rener *et al.*, 1980 ; Kaushal *et al.*, 1983a), *P. falciparum* (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a ; Quakyi *et al.*, 1987), *P. yoelii* (Harte *et al.*, 1985b) and *P. vivax* (Peiris *et al.*, 1988). The MAbs were used to characterise and isolate the target antigens of transmission-blocking immunity.

In *P. berghei*, MAbs against ookinetes have been produced (Winger *et al.*, 1988) and shown to suppress transmission of the disease (results partly-presented in this chapter). Pbs21 was identified as the target antigen of the transmission-blocking MAbs (results presented in this chapter and Winger *et al.*, 1988). This chapter will describe the production of MAbs against the affinity-purified Pbs21 and characterisation of the anti-Pbs21 MAbs in various aspects.

2.2) Materials and Methods

2.2.1) The malaria parasite

Plasmodium berghei (ANKA isolate) was a kind gift from Dr. D. Walliker (Edinburgh). The parasite was cryopreserved and maintained by syringe passage in mice with regular mosquito transmission. The cloned line (2.33L, a gift from Dr. D. Walliker ; Edinburgh) which produced only the asexual stages of the parasite was also maintained for the study of the asexual stages.

1) Thawing and maintaining of the parasite

One aliquot of *P. berghei* gametocyte-infected blood (500 µl) which was stored in liquid nitrogen was rapidly thawed in a 37°C water bath for 3 min. The blood was injected intraperitoneally (I. P.) into 2 Theiler's Original (T.O) mice (Harlan Olac Ltd.) (0.2 ml of the blood/mouse). Three days after the infection, the exflagellation of the parasite was checked by mixing 2 drops of blood from tail-bleeding with 100 µl of complete ookinete medium which was RPMI 1640 (GIBCO) containing 0.025 M HEPES (Sigma), 0.0037 M Hypoxanthine (Sigma), 0.024 M $NaHCO_3$ (Sigma), PSN antibiotic mixture (GIBCO ; 0.05 mg/ml Penicillin, 0.05 mg/ml Streptomycin, 1 mg/ml Neomycin at a final concentration) and 20 % (v/v) heat-inactivated foetal calf serum (FCS ; GIBCO) pH 8.3. To increase the number of reticulocytes which are preferentially infected by the parasite, the mice were treated with 0.2 ml/mouse of 6 mg/ml of phenylhydrazine in 0.85% NaCl 3 days prior to infection. After etherisation, the mice were bled by cardiac puncture into a heparinised syringe {0.1 ml of 1 mg/ml of Heparin (Sigma ; H-9133) in 0.85% NaCl per ml of the blood}.

The parasite was maintained by syringe passage until the passage number 7 before another aliquot of the induced parasitised blood (P1) was thawed to initiate the infection.

2) Mosquito feeds

Cyclical transmission was performed regularly so as to maintain the infectivity of the parasite to the mosquito. *Anopheles stephensi* bred in our laboratory were used as the vector throughout the study. Female mosquitoes were potted (50-90 mosquitoes/pot) and starved for 24 h before a blood feed. Non-phenylhydrazine-treated mice were infected with *P. berghei* (10^7 parasitised cells/mouse). Three days after the infection, the mice were anaesthetised with Rompun-Vetalar (1:2, Bayer and Parke-Davis Veterinary) and the mosquitoes fed for 1/2 h. Unfed mosquitoes were removed and the rest was kept at 19°C and 80% relative humidity for 14 days for oocyst development.

3) Cryopreservation of the parasite

Infected mosquitoes were allowed to feed onto 2 naive mice for 1/2 h. The mice (passage number P_0) were kept until 10-20% parasitaemia was obtained before being passaged to another group of 10-20 mice as a stock of the parasite (passage P_1). At about 10-20% of the

parasitemia, the mice were bled as described above. Five-hundred µl of the blood was pipetted into cryotubes (GIBCO-NUNC) containing 50 µl of DMSO (Sigma). Following mixing, the tubes were frozen at -80°C in a foam box where the rate of cooling is approximately 1°C/min and kept at -80°C overnight before they were transferred and stored in liquid nitrogen.

2.2.2) *In vitro* culture of the ookinetes of *P. berghei*

The culture was set up routinely according to the methods described by Sinden *et al.*, (1987). Twenty T.O mice were treated with phenylhydrazine (as described in section 2.2.1), 3 days prior to infection with *P. berghei* ANKA isolates (as a source of gametocyte-infected blood) or 2.33L (as a source of erythrocytic stages-infected blood). On day 4 post-infection, the mice were bled. The blood as kept on ice until the process was finished (20-30 min), then passed through a cellulose CF-11 (Whatman) column to remove the contaminating white blood cells. The blood was diluted 10 times with complete ookinete medium and cultured at 19°C for 24 h in air for ookinete development.

2.2.3) Ookinete enrichment

The culture (see section 2.2.2) was centrifuged at 600g (1, 500 rpm ; Sorvall 6000) and 4°C for 10 min to pellet the cells. Twenty volumes of cold 0.17 M NH_4Cl were added into the cell pellet to lyse the red blood cells. The reaction was kept on ice for 10 min before the lysis was stopped by the addition of an equal volume of complete ookinete medium. Intact parasites were pelleted at 300g (800 rpm ; Sorvall 6000) for 10 min, following washing twice with 5 ml of ookinete medium without serum then kept frozen at -80°C until use. If the asexual stages and gametocytes were needed, the parasite-infected blood was subjected to the ammonium chloride-treatment after passing through CF-11 column as described for ookinete enrichment.

2.2.4) Hybridoma production

The monoclonal antibodies (MAbs) 12.1.3, 13.1.15 and 17.9.7 (which will be designated as 12.1, 13.1 and 17.9) were produced against whole cell ookinetes of *P. berghei* by Dr. L. A. Winger as described earlier (Winger *et al.*, 1988). All the MAbs recognised the protein of an M_r of 21 kD (Pbs21) under both reducing and non-reducing conditions (Winger *et al.*, 1988). More MAbs against the affinity-purified Pbs21 (section 3.2.2) were derived so as to further understand the antigen.

1) Immunisation protocol for fusion

Fusion 1 ; A Balb/C mouse was immunised intraperitoneally (I. P.) with 20 µg of the affinity-purified antigen in complete Freund's adjuvant (CFA ; GIBCO). After a month, it was boosted with the same amount of the antigen in incomplete Freund's adjuvant (I. P.) and finally boosted intravenously (I. V.) 2 weeks later with freeze-thawed ookinetes of *P. berghei* (10^7 cells). The mouse was then splenectomised on day 4 after the final boost.

2) Preparation of macrophage feeder layer

Lymphoid cells often grow poorly or die when grown at low density and the reasons are not well-understood (Goding, 1983). The problems can be overcome by culturing together with a cell 'feeder' layer. Here, peritoneal cells (lymphocytes and macrophages) are used as the feeder.

Balb/C mice were sacrificed by cervical dislocation. The abdominal skin was opened under sterile conditions. Eight ml of cold complete HT medium (RPMI-1640) (GIBCO) containing 0.05 M glutamine, 0.001 M sodium pyruvate, 50 IU/ml gentamycin, 10% (v/v) myoclonal foetal calf serum (MFCS), 0.01 M hypoxanthine, 0.003 M thymidine and 0.024 M sodium bicarbonate (all tissue culture reagents were obtained from GIBCO) were injected into the peritoneal cavity using a 21G needle, taking care to avoid puncturing the gut. Following massage of the peritoneal cavity, about 5 ml of the medium were drawn back into the syringe and the cells were transferred to a centrifuge tube which was kept on ice to minimise macrophage adherence. Usually, about 2-5 x 10^6 macrophages can be obtained from one mouse and were used for each 96 x 200 µl well plate or 3 mice were used per each 24 x 2 ml well plate. After centrifugation at 600g for 10 min, the cells were resuspended in either complete HAT medium (the same as HT medium except $4x10^{-7}$ M aminopterin was added) for cell fusion or HT medium for thawing and feeding cells then aliquoted into tissue culture plates, i.e. 0.1 ml per each well of 96-well plate or 1 ml per each well of 24-well plate. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The macrophage plates were usually prepared 1 day prior to use (All tissue culture plasticwares were obtained from GIBCO-NUNC).

3) Preparation of spleen cells for fusion

An immunised Balb/C mouse was sacrificed as described above. The abdominal skin was opened before the spleen was carefully dissected out under sterile conditions and placed in 5 ml of cold sterile HT medium. Using 5 ml syringe and a 23G needle, the spleen was gently teased and perfused with the medium so as to release the cells within. This process was repeated with a further 5 ml of HT-medium. The medium containing cells was then collected into a 15 ml tube and centrifuged at 600g for 10 min. The supernatant was discarded and the cell pellet was mixed with 5 ml of cold sterile 0.17 M NH_4Cl in order to lyse the contaminating red blood cells. The cells were left on ice for 10 min before the lysis was stopped by the addition of 5 ml HT medium containing 20% FCS. The remaining cells were pelleted at 600g for 5 min and washed once with 10 ml of serum-free HT medium and re-centrifuged. The number of viable cells was counted under the microscope on a Neubauer haemocytometer by mixing 10 µl of the cell suspension with 10 µl of 0.4% (w/v) of Trypan blue (Edward Gurr Ltd.) in saline solution. Usually, about 10^8 cells were obtained from each spleen.

4) Preparation of myeloma cells

P2-X63-Ag8-653 (FLOW) which is a non-IgG secreting cell but which allows the production of antibody-secreting hybridomas when fused with spleen cells (Kearney *et al.*, 1979) was used as a fusion partner. The cells were maintained in the logarithmic phase before use. On the day of fusion, the cells (about 10^7 cells) were harvested by gently tapping the tissue culture flasks and pipetting the medium with a sterile pipette so as to loosen the adhered cells. The cells were then centrifuged at 600g for 10 min and washed once in 10 ml of serum-free HT medium. The number of viable cells was counted in the Neubauer haemocytometer. The cells were finally resuspended in an appropriate volume of serum-free HT medium (10^6-10^7 cells/ml).

5) Cell fusion

Different protocols for cell fusion have been described, with similar results (Goding, 1983). They vary mainly in relation to the duration of exposure or the concentration of polyethylene glycol (PEG) . Factors which seem to be of minor importance include temperature (20-37°C), cell numbers, the MW of PEG and the presence or absence of serum or erythrocytes during fusion (Goding, 1983). The precise ratio of myeloma to spleen cells is not crucial and good results have been achieved with the ratios of spleen : myeloma cells from 1:1 to 10:1 (Goding, 1980). The method outlined here is a modification of Kennett (1979).

The spleen cells (About 10^8 cells) were mixed with the myeloma cells P2-X63-Ag8-653 (10^7 cells). The mixed cells were washed once with 10 ml of serum-free HT medium then centrifuged at 600g for 5 min. The supernatant was aspirated and the cell pellet was resuspended by gently tapping the fusion tube (Falcon no. T-4446). Two-hundred µl of the serum-free HT medium containing 50% (v/v) PEG (MW 1300-1600 ; Sigma P-7777) and 10% (v/v) DMSO (Sigma) at a final concentration was slowly added to the cell pellet then centrifuged at 300g for 6 min. The whole process was completed within 8 min following the addition of PEG. The cells were immediately diluted with 5 ml of serum-free HT medium, followed by another 5 ml of HT medium containing 20% (v/v) MFCS. The cells were pelleted at 600g for 5 min prior to resuspension in 100 ml of HAT medium containing 10% (v/v) MFCS then plated by pipetting 1 ml of the cell solution into each well of 24-well plates (4 plates) containing macrophages as a feeder layer.

6) Hybrid selection

After a week in HAT medium, it is safe to assume that all the parental myeloma cells will be dead, and any growing cells will be hybrids (Goding, 1983). At this point, HAT selection was terminated. The culture supernatant was aspirated and 1 ml of fresh HT-medium containing 10% (v/v) MFCS (complete medium) was added to each well. The cells were fed by medium replacement with complete medium every other day. On day 14 after fusion, the hybrid

cells could be observed macroscopically. The screening assays were then performed using ELISA and IFA as described below.

a) Screening by an Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay offers a number of advantages over Radioimmunoassay (RIA) or Immunoradiometric assay (IRMA). It is a simple and reliable method. No radioisotopes are required and the reagents are inexpensive and have a long shelf-life (Goding, 1983).

The MAbs against ookinetes of *P. berghei* have been produced using immunofluorescence assay (IFA) to screen for the positive clones producing the specific antibody (Winger *et al.*, 1988). Here, ELISA was developed as a tool to screen for the positive clones producing the antibody against Pbs21. Specificity of ELISA was first assessed using the ascitic fluids 13.1 and 7.8 (an irrelevant MAb against merozoite of *Babesia divergens* ; a gift from Dr. C. M. Winger). The method was modified from Voller *et al.*, (1974). Wells of microtitre plates (FLOW ; Cat. No. 76-381-04) were pre-coated with 50 µl of 1 µg/ml of the affinity-purified Pbs21 in PBS (see section 3.2.2). The plates were left overnight at room temperature to enhance the maximum binding of the antigen to the plate. Following 3 washings with 200 µl/well of 0.5% normal goat serum (NGS ; Sera Lab) in PBS (PBS-NGS), residual binding sites of the wells were saturated with 200 µl of 5% NGS-PBS at 37°C for an hour.

The wells were washed as above and subsequently incubated with a doubling dilution series of the ascitic fluids 13.1 and 7.8 (1:5000-1:51, 200). Titration was always performed in 2.5% NGS-PBS. The plates were left at 37°C for an hour before the wells were washed for 3 times with 200 µl of 0.05% Tween, PBS solution (PBS-Tween). As the second antibody, the wells were incubated with 100 µl of 1:1000 dilution of the affinity-purified goat anti-mouse IgG-Alkaline Phosphatase conjugate (Sigma A-5153) in PBS-Tween and left at 37°C for an hour. Following twice washings with PBS-Tween and another twice with carbonate-bicarbonate buffer (0.05 M carbonate/bicarbonate buffer pH 9.6, $1x10^{-4}$ M $MgCl_2$), the wells were incubated with 100 µl of 1 mg/ml of p-Nitrophenyl phosphate (Sigma P104-0) in carbonate-bicarbonate buffer as a substrate for the enzyme. The plates were left at 37°C for 1/2-1 h before the optical density (O.D.) of the product (p-nitrophenol) was read at 414 nm on an EIA reader (BIO-RAD 2550).

After the specificty of ELISA was established, it was then applied to screen for the positive clones producing the antibody against Pbs21. Wells pre-coated with the antigen were incubated with 100 µl of myeloma culture supernatants (which were removed aseptically) at 37°C for an hour. Following 3 washing with PBS-Tween, the wells were incubated with goat anti-mouse IgG-Alkaline Phosphatase conjugate and subsequently with the substrate. The $O.D_{414}$ was measured as described.

b) Screening by an Immunofluorescence Assay (IFA)

Screening of hybridomas by immunofluorescence has also been widely used and the sensitivity of the assay appears to be similar to radioimmunoassay (RIA) and ELISA (Goding, 1983). Immunofluorescence has the definite advantage that one may detect and describe antibodies against subpopulations of cells or subcellular structures.

The positivity of the hybrid cells to Pbs21 was tested using ELISA and confirmed by IFA to observe the fluorescence around the ookinetes. *In vitro* culture of ookinetes of *P. berghei* was set up as above (Section 2.2.2). After 24 h at 19°C, 200 µl of the culture (at least 10^5 cells/well) were pipetted into each well of 96-well plates (Conical-shaped bottom ; FLOW) which were kept on ice throughout the process. The cells were centrifuged at 600g for 5 min and the supernatant was aspirated. One-hundred and fifty µl of the culture supernatants was pipetted into each well. The plates were kept at 4°C for an hour. After centrifugation, the cells were washed 3 times with ookinete complete medium (200 µl/well) then added with 50 µl/well of fluorescein isothiocyanate (FITC)-conjugated to an affinity-purified goat-antimouse IgG (Sigma F-9006) at a dilution of 1:50 in complete ookinete medium. The plates were kept at 4°C for another hour before the cells were pelletted and washed as described then resuspended in a 1:1 dilution of ookinete complete medium and Citifluor mountant. Fluorescence was observed under the UV microscope (Leitz) at x250 magnification.

7) Cloning by limiting dilution

As soon as positive wells are identified, the hybrid cells are cloned. Cloning is important to reduce the risk of overgrowth by non-antibody producing cells, and to ensure that the antibodies are truly monoclonal. Cloning by limiting dilution is always done in the presence of macrophage feeder layer since growth of the hybridoma is critically dependent on the cell density.

Feeder layer plates were prepared as above (Section 2.2.4.2) in complete HT medium. Positive hybrid wells were resuspended, counted and checked for the cell viability. Dilutions of the cells were made and plated at 1 and 2 cells per 100 µl well in 96-well plates containing macrophage feeder layer (1 plate/category or 2 plates/positive isolate). A week later, the cells were fed with fresh complete HT medium. Ten days after the cloning, growth of the single clones was checked using an inverted phase microscope (Leitz).

The positivity of the clones were confirmed using ELISA and IFA as described above. After expansion of the clones, the remaining positives were further subcloned twice so as to ensure the clonality.

8) Expansion of hybrid cells

Once the active hybrids have been identified and subcloned, they are expanded for freezing and antibody production. The cells were subsequently cultured in larger volumes. Thus, cultures were expanded from 200 µl to 1 ml, consequently into 10 ml in 25-ml and 50 ml in

250 ml culture flasks, taking care to maintain the logarithmic growth of the cells. The use of macrophage as a feeder layer at this step was not as critical as for cloning of cells by limiting dilution.

9) Storage of cells in liquid nitrogen

Hybridoma cells may be frozen in liquid nitrogen and stored for several years with good recovery (Goding, 1983). About 10^6 cells in 0.9 ml were aliquoted into 1.2 ml cryotubes (GIBCO-NUNC) containing 0.1 ml of DMSO. They were mixed and immediately placed into a foam-box where the rate of cooling is approximately 1°C/min and kept at -80°C overnight. The cells were then transferred and stored in the liquid nitrogen (-196°C) the next day.

10) Thawing of cells

The vial containing cells was thawed rapidly in a 37°C water bath for 3 min. The cells were washed twice in 10 ml complete HT-medium then resuspended in 1 ml of the medium before plating into each well of 24-well plate containing macrophage as a feeder layer.

11) Growth of hybridomas in animals

It is generally easier to grow the cells as tumours in animals if very large amounts of antibody are needed. Typical antibody levels in the culture supernatant range from 5-50 µg/ml compare to 5-15 mg/ml in the ascitic fluid (Goding, 1983).

A number of syngeneic mice (Balb/C) were injected I. P. with Pristane (2,6,10,14-tetramethyl pentadecane, Sigma ; 0.5 ml per mouse) to increase the rate of ascites formation. Ten days later, the mice were injected with the hybrid cells (1-5 x 10^6 cells per mouse). Within 2 weeks after the injection of the cells, the tumour was usually developed and the mice were sacrificed. The peritoneal fluid was removed using a 10 ml syringe with a 21G needle and left at 4°C overnight then centrifuged at 13,000g (MSE) for 10 min. The supernatant was removed, aliquoted and kept at -80°C.

2.2.5) Characterisation of the monoclonal antibodies

1) Assessment of antibody subclasses

Using ELISA, classes and subclasses of the monoclonal antibodies were determined. Wells pre-coated with Pbs21 were incubated with either neat culture supernatant or appropriate dilutions of ascitic fluids. As the second antibody-enzyme conjugate, a panel of different anti-immunoglobulin subclasses conjugated to Alkaline-Phosphatase i.e. goat-antimouse IgM (Sigma A-7784), IgG1, IgG2a, IgG2b and IgG3 (Serotec) was added. The rest of the process was performed as described in section 2.2.4.6 a.

2) Effect of the monoclonal antibodies on parasite development

The effect of the MAbs raised against ookinetes of *P. berghei* to parasite development were studied both *in vivo* and *in vitro* so as to complete the investigations described in Winger *et al.*, (1988).

a) In vivo study

Mosquito infectivity in the presence of various MAbs was studied using artificial membrane feed or live feed after passive transfer to mice of the ascites.

i) Membrane feeds

Before blood feeds, batches of 50-90 female *Anopheles stephensi* were potted. *P. berghei* gametocyte-infected mice which showed vigorous exflagellation were anaesthetised (as described in section 2.2.1.2) then exsanguinated by cardiac puncture into a heparinised syringe. Five-hundred µl of the blood was mixed in the tubes containing 250 µl of heat-inactivated individual ascites (1.2, 13.1 and 17.9) and were kept in a 37°C water-bath. Similar observations using 12.1 have already been described by Winger *et al.*, (1988). As negative controls, ookinete complete medium, UPC 10 which was a commercially-available clarified ascites (Sigma M-7769) or the ascites C 13.4 (an irrelevant ascites raised against the protein coat of *B. divergens* merozoites, a gift from Dr. C. M. Winger) were included. UPC 10 was used as an IgG2a subclass control for the MAb 13.1. MAb 7.8 (an irrelevant ascites raised against *B. divergens* merozoites) was used as an IgG1 subclass control for the MAb 17.9 and was already described in Winger *et al.*, (1988).

The mixtures were introduced into water-jacketted membrane feeders covered with a very thin Parafilm membrane and the temperature was kept constant at 37°C. Mosquitoes were allowed to feed at 19°C for 1/2 h under 3% O_2, 4% CO_2 and 93% N_2 gases. Following the feeding, unfed mosquitoes (about 10-20%) were removed and the rest were left at 19°C and 80% relative humidity for 14 days for oocyst development. %Transmission and %blockade were calculated as following,

$$\%\text{Transmission} = \frac{\text{Mean number of oocysts/gut in the presence of individual ascites}}{\text{Mean number of oocysts/gut in the presence of medium control}} \times 100$$

$$\%\text{Blockade} = 100 - \%\text{Transmission.}$$

ii) Passive immunisation study and live feeds

P. berghei gametocyte-infected mice were anaesthetised and offered to mosquitoes for 1/2 h then subsequently given 100 µl of the ascites (12.1 and 17.9) I. V. The observations using the MAbs 7.8 and 13.1 were already described in Winger *et al.*, (1988). As negative control, UPC 10 (which had the relevant subclass to the MAb 12.1) was also included. An hour later, another batch of mosquitoes was allowed to feed on these MAb-treated mice. Fed

mosquitoes were handled as above for oocyst observation. %Transmission and %blockade were calculated as following,

$$\%\text{Transmission} = \frac{\text{Mean number of oocysts/gut from post-administration of ascites}}{\text{Mean number of oocysts/gut from pre-administration of ascites}} \times 100$$

$$\%\text{Blockade} = 100 - \%\ \text{Transmission}$$

iii) Determination of oocysts

Surviving mosquitoes (typically about 90% of those that fed) were killed on ice then dissected under the microscope (Wild Heerbrugg). The gut was pulled out from each mosquito into a drop of ookinete complete medium on a glass microscope slide and a cover-slip (13 mm diameter) was placed on top. Number of oocysts was scored under the microscope (Leitz) at x200 magnification to measure the infectivity of the mosquitoes. The degree of transmission was calculated as percent reduction of oocysts as described above.

b) In vitro study

Ookinete development in the presence of various MAbs was studied *in vitro*. As described in section 2.2.2, *P. berghei*-infected blood from phenylhydrazine-treated mice was passed through a CF-11 column at 4°C.

One ml of the blood which contained residual mouse serum was added into each well of 24-well plate. The plate was kept on ice to prevent the occurrence of exflagellation before the antibodies (12.1, 13.1 and 17.9) were added so as to keep the time constant between each MAb. To inactivate the heat-labile component (complement), the ascitic fluids were heated at 56°C for 1/2 h then added to the culture at a final concentration of 1:10. As negative controls, either the medium or irrelevant MAbs 7.8 and C 13.4 were included. Following culturing at 19°C for 24 h, the parasites were smeared on glass microscope slides which were fixed in absolute methanol for 2 min and stained with Giemsa for 10 min [1 volume of Giemsa's stain (Gurr's improved R66 ; BDH) + 9 volume of phosphate buffer (3.52×10^{-3} M Na_2HPO_4, 1.54×10^{-3} M KH_2PO_4 pH 7)]. Ookinete development was observed under the light microscope (Leitz) at x1000 magnification. The parasites were counted in the tail of blood films (at least 10^4 non-infected red blood cells were counted). Individual parasite stages were determined and reported as percent total cells. %Inhibition of *in vitro* ookinete formation was calculated as following,

$$\%\text{Inhibition of ookinete development} = \left[1 - \frac{\%\text{Ookinetes in the presence of an individual MAb}}{\%\text{Ookinetes in the presence of medium control}}\right] \times 100$$

3) SDS-PAGE and immunoblot analysis of the monoclonal antibodies

SDS-PAGE is perhaps the most widely used method of protein analysis. When combining the SDS-PAGE with immunoblot methods, antigens specifically identified by the antibody can be studied.

a) SDS-PAGE

Normally, ookinete proteins were separated on SDS-PAGE. In some experiments, asexual stages and gametocyte proteins were also studied. Ammonium chloride-enriched asexual stages, gametocytes and ookinetes of *P. berghei* parasite pellet equivalent to one mouse was solubilised in 300 µl of either non-reducing (0.004% w/v bromophenol blue, 0.01 M EDTA, 10% v/v glycerol, 0.005 M iodoacetamide, 0.002 M PMSF, 5% SDS and 0.01 M Tris pH 6.8), or reducing (the same as non-reducing buffer except that 5% v/v of 2-mercaptoethanol was included) Laemmli buffers (Laemmli, 1970). Following boiling for 5 min and centrifugation at 13,000g for 5 min, 30 µl of the solution was loaded onto a 15% separating gel (Acrylamide : Bis-acrylamide ratio = 30:0.8) with a 3% stacking gel (the recipe of SDS-PAGE is described in Appendix 1). The gel was run overnight in SDS-PAGE buffer (0.025 M Tris pH 8.3, 0.192 M Glycine, 0.1% SDS) using BIO-RAD vertical slab gel apparatus with the cooling system at a constant current (4 mA/gel) (Pharmacia power pack). As molecular weight (MW) markers, the protein standard (Sigma ; SDS-7) which was a mixture of bovine albumin (66 kD), egg ovalbumin (45 kD), rabbit muscle Glyceraldehyde-3-phosphodehydrogenase (36 kD), bovine erythrocytes Carbonic anhydrase (29 kD), bovine pancrease Trypsinogen (24 kD), soybean Trypsin inhibitor (20 kD) and bovine α-Lactalbumin (14 kD) was also loaded in the outer lanes of the gel.

After electrophoresis, the gel was subjected either to staining with Coomassie blue using the method modified by Weber and Osborn (1969) to visualise the protein pattern or Western blot transfer. If the gel was stained, it was then placed in Coomassie staining solution for an hour then destained with agitation until the background was clear (Appendix 1).

b) Immunoblot study

The method was modified from Towbin *et al.*, (1979) and Burnette (1981). After electrophoresis, the gel was removed from the apparatus and laid down onto pre-soaked Scotch-Brite scouring pads in between the gel holding cassette. Pre-wetted nitrocellulose membrane (BIO-RAD ; pore size 0.45 µm) in distilled water was placed onto the gel, avoiding the formation of air bubbles between the gel and the nitrocellulose. The other porous pad was placed on top and the whole assembly was clamped together. The proteins were transferred for 5 h in Western blot transfer buffer (0.19 M glycine, 0.025 M Tris pH 8.3, 20% methanol) at 60 V and 21 mA using the transblot cell (BIO-RAD) with a tap water cooling system.

Following blot transfer, the MW marker strips were cut off, stained in amido black solution (0.5% w/v amido black in 90% v/v methanol and 2% v/v glacial acetic acid) for 3 min

and destained in the destaining solution (90% methanol and 2% glacial acetic acid) until the background was clear. The strips were finally washed in distilled water before drying between glass plates.

The remaining strips were removed from the gel, and left in 5% NGS in milk solution (5% skimmed milk in mouse tonicity PBS :- 0.016 M Na_2HPO_4, 0.004 M NaH_2PO_4 and 0.15 M NaCl pH 7.4) at 37°C for an hour to saturate the remaining protein binding sites of the nitrocellulose membrane.

The nitrocellulose membrane was cut off into strips before incubation with either neat culture supernatant or 1:50-1:100 dilution of ascitic fluid in 5% NGS in milk solution (2 ml for each lane of 14 cm^2) in individual sealed-plastic bags at room temperature for 2 h.

Following the incubation with the first antibody, the nitrocellulose strips were washed individually for 4 times (15 min each) with milk solution. ^{125}I-labelled sheep anti-mouse IgG (Amersham ; IM.131) diluted in milk solution (1-5x10^6 cpm/ml/lane) was added as a second antibody. The reaction was left at room temperature for 1 h before the strips were washed as described. This was followed by 2 (15 min) washes in PBS, 0.05% Triton-X 100, 0.05% Tween-20 and twice more (15 min each) with 0.4 M NaCl in PBS .

The nitrocellulose strips were air-dried and carefully placed in cassettes with intensifying screens (Dupont-Cronex Lightning Plus) then left overnight at -80°C with the X-ray film (Fuji). The film was developed in developer (ILFORD PQ Universal) at 19°C for 5min and fixed in the fixer (ILFORD Hypam) until it was clear. The film was dried following rinsing in tap water for 20 min.

2.3) Results

2.3.1) *In vitro* culture and enrichment of the ookinetes of *P. berghei*

Figure 2.1 shows a Giemsa-stained thin film of ookinetes in whole blood culture. Different parasite stages were counted before and after ammonium chloride enrichment (1, 000 non-infected red blood cells were counted). The results including relative enrichment of ookinetes following ammonium chloride treatment are shown in Table 2.1. Gametocytes and ookinetes were enriched by 27.49 and 50 folds respectively while %non-infected red blood cells were significantly reduced. After the enrichment, the ring and trophozoite stages were reduced to 1.06 and 3.08% respectively. This might be due to loss of the parasites by low speed centrifugation during the process (800g). These results implied that the ammonium chloride method offered a simple approach for the enrichment of ookinetes which were adequate for further studies i.e. surface antigen purification (Chapter 3), pre-fusion boosting, SDS-PAGE and immunoblot analysis.

2.3.2) Hybridoma production

Specificity of ELISA was assessed and the results are shown in Figure 2.2. The immunological activity of Pbs21 was demonstrated using the existing specific MAb 13.1 but not the irrelevant MAb 7.8. After the specificity of ELISA was established, the method was then applied to screen for positive clones from fusion. The results from fusion 1 are as following,

Fusion 1 ; Of 30 parental clones, 8 (namely, 1.1-1.8) were selected as positive clones by ELISA against Pbs21 (i.e. $O.D_{414}$ of positive clones was between 0.19-1.31 while $O.D_{414}$ of the negative clones was 0.002-0.01). Following cloning twice, 5 clones (1.1, 1.2, 1.4, 1.6 and 1.8) remained positive. Ascites from these hybridomas was produced. Positivity of the ascites was re-assessed but unfortunately only 1.2 remained positive on both ELISA against Pbs21 and IFA using live preparation of *P. berghei* ookinetes.

Although the MAbs 12.1, 13.1 and 17.9 have already been presented in Winger *et al.*, (1988), antibody titres (determined by IFA) and immunoglobulin subclasses (determined by ELISA) of these MAbs, as well as these of 1.2 and 2 irrelevant MAbs, 7.8 and C 13.4 are summarised in Table 2.2. The MAbs 7.8 and C 13.4 are specific to intact merozoites and merozoite protein coat (24-29 kD) of *B. divergens* respectively (C. M. Winger, pers. comm.). These 2 MAbs do not show specific surface binding with ookinetes in IFA, or positive reaction with Pbs21 in ELISA.

2.3.3) Effect of the monoclonal antibodies on parasite development

The following experiments were carried out so as to complete the investigations described in Winger *et al.*, (1988). Discrepancies between the numbers in individual experiments described here and in Winger *et al.*, (1988) were observed. However, the overall perspective was the same.

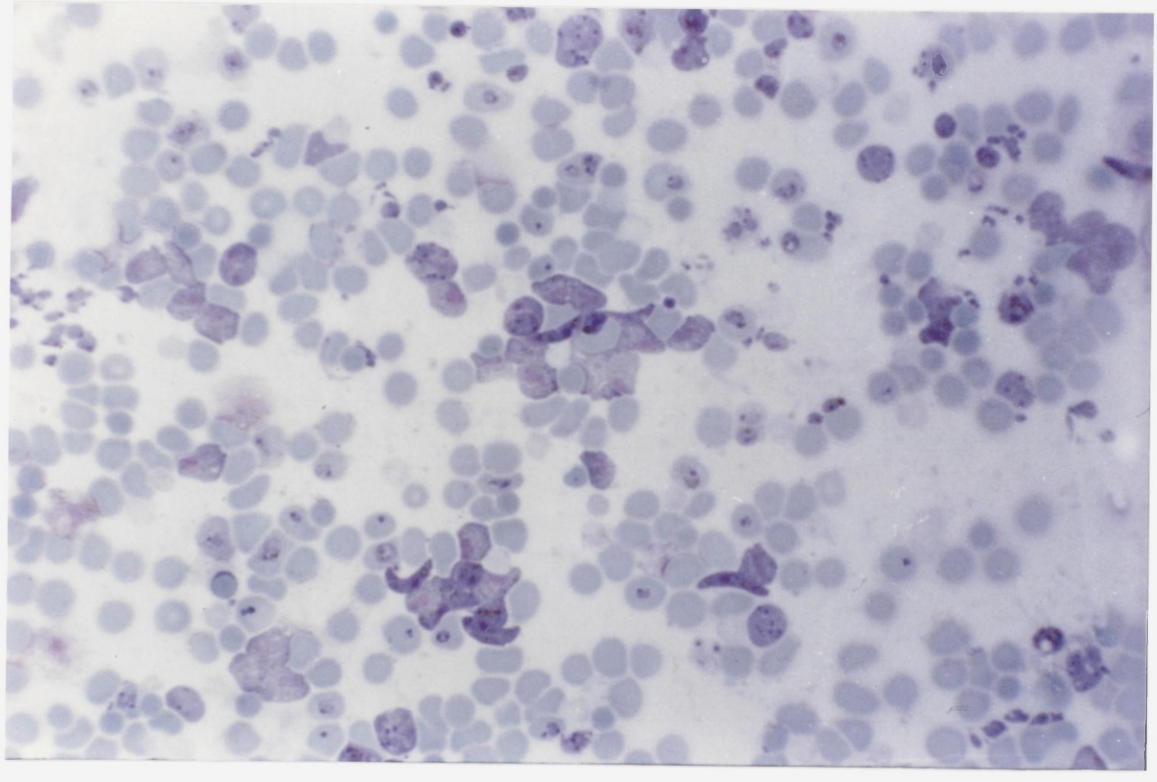


FIGURE 2.1 Giemsa-stained thin film of *P. berghei* zygotes-ookinetes in whole blood culture. Magnification x400.

TABLE 2.1 Enrichment of *P. berghei* ookinetes by ammonium chloride treatment.

Ookinetes were cultured *in vitro* and enriched using ammonium chloride. Bloodsmears were taken before and after the enrichment. Different parasite stages were counted from 10 fields on the slides with equally distributed cells (at least 1, 000 non-infected red blood cells were counted). Values (expressed as percent total cells) are the arithmetic mean ± S. D. of three separate experiments.

Conditions	% Parasite stages					
	NRBC*	Ring	Trophozoite	Schizont	Gametocyte	Ookinete
Before Treatment	58.24 ± 28.02	3.53 ± 1.59	33.61 ± 23.29	1.8 ± 2.54	2.12 ± 1.47	0.69 ± 0.31
After Treatment	1.38 ± 1.84	1.06 ± 1.39	3.80 ± 3.43	1.83 ± 2.51	58.28 ± 23.02	34.50 ± 19.03
Enrichment Factor**	0.02	0.30	0.11	1.02	27.49	50.00

* = Non-infected red blood cell.

** = Enrichment factor is calculated from $\frac{\%\text{Individual parasite stage after the ammonium chloride treatment}}{\%\text{Individual parasite stage before the ammonium chloride treatment}}$

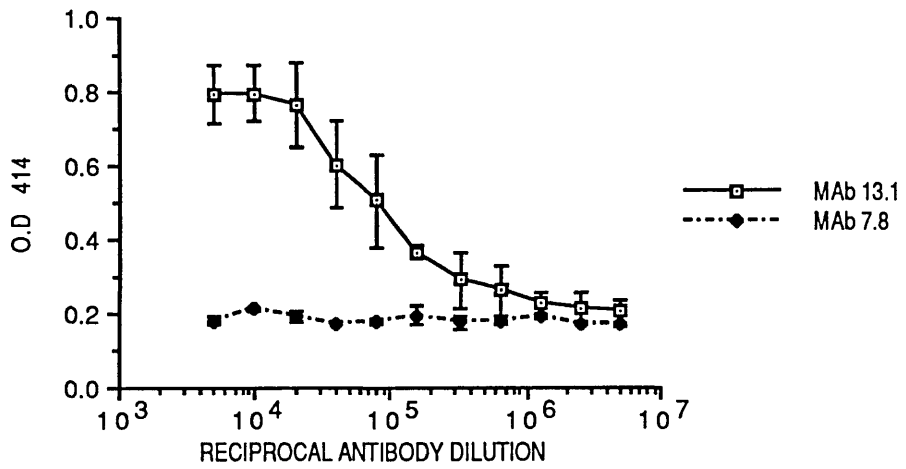


FIGURE 2.2 Indirect one-site ELISA demonstrating specificity of the assay against Pbs21 using the ascitic fluid 13.1. Wells pre-coated with 1 µg/ml of the antigen were incubated with a doubling dilution series of the ascitic fluids 13.1 and 7.8 (1:5000-1:51,200), subsequently with goat anti-mouse IgG-alkaline phosphatase conjugate and finally with the substrate as described in section 2.2.4.6 a. All values given are the arithmetic mean of duplicate samples ± S. D.

TABLE 2.2 Titres and subclasses of the monoclonal antibodies.

Monoclonal antibodies	Antibody titre by IFA	Subclasses by ELISA
1.2	$\leq 1:10^3$	IgG3
12.1	$>1:10^4$	IgG2a
13.1	$>1:10^4$	IgG2a
17.9	$\leq 1:5x10^3$	IgG1
7.8*	$\leq$ 1:100	IgG1
C 13.4**	$\leq$ 1:100	IgG1

* = The MAb against merozoites of *B. divergens*.

** = The MAb against protein coat of *B. divergens* merozoites.

1) ***In vivo*** **study**

a) Membrane feeds

Oocyst development was observed in the presence of the MAbs 13.1 and 17.9 . As negative controls, complete ookinete medium and UPC 10 were included. The results are shown in Table 2.3.

The degree of transmission in the medium control was defined as 100%. The MAbs 13.1 and 17.9 were shown to inhibit oocyst development by greater than 96 and 99% respectively. 12.1 also inhibited the formation of oocysts by ~ 94% (Winger *et al.*, 1988). UPC 10 which was used as an IgG2a subclass control for the MAbs 12.1 and 13.1 did not significantly suppress mosquito infectivity when compared to the medium control (using proportion analysis, $p < 0.5$) (Kirkwood, 1988). The MAb 7.8 was used as an IgG1 subclass control for the MAb 17.9 and the degree of transmission by 7.8 was defined as 100% (Winger *et al.*, 1988). However, the absolute effect of the MAb 7.8 on oocyst development was not known since the medium control was not included in the experiment described by Winger *et al.*, (1988).

Oocyst development in the presence of the MAb 1.2 compared to the negative control MAb C 13.4 was also studied (Table 2.3). The degree of transmission by the MAb 13.4 was defined as 100%. Although the immunoglobulin subclass of C 13.4 was not relevant to 1.2, no inhibitory effect of the MAb 1.2 on oocyst development was observed. Instead, an enhancement of transmission was detected. From this observation and together with the inappropriate antibody titre in IFA ($\leq 1:10^3$; Table 2.2), the MAb 1.2 was decided not to be further characterised in passive immunisation study.

b) Passive Immunisation

The inhibitory effect of the MAbs on oocyst development was further investigated in passive immunisation experiments. Numbers of oocyst development before and after the mice were given the ascitic fluids 12.1 and 17.9 are presented in Table 2.4. As negative control, UPC 10 was included.

The MAb 12.1 absolutely inhibited oocyst development while 17.9 inhibited by greater than 95%. The MAb 13.1 also suppressed oocyst development by 93% (Winger *et al.*, 1988). The inhibitory effect by the control MAb UPC 10 on mosquito infectivity was not significantly different from the control feed (ascites pre-injection) ($p < 0.5$). In parallel, 7.8 did not significantly suppress mosquito infectivity ($p < 0.4$) (Winger *et al.*, 1988).

The results from both membrane feed and passive immunisation experiments in the present studies and Winger *et al.*, (1988) implied that oocyst development was inhibited by all MAbs 12.1, 13.1 and 17.9. The inhibitory effect was not restricted to subclasses of the MAbs as suppression of mosquito infectivity in the presence of relevant subclass controls (UPC 10 and 7.8) was not significant.

TABLE 2.3 Effect of the ascitic fluids on oocyst development in membrane feed.

P. berghei gametocyte-infected blood was mixed with the ascitic fluids in 2:1 ratio (500 µl of the blood : 250 µl of the ascitic fluids) and introduced into water-jacketted membrane feeders. Mosquitoes were fed through a thin film membrane for 1/2 h. Following being kept at 19°C for 2 weeks, the mosquitoes were dissected for oocyst determination. Two experiments are reported. Experiment 1 was the observations of the ascitic fluids 13.1 and 17.9 compared to negative control (medium) and subclass control ascites UPC 10. Experiment 2 was the observation of the ascitic fluid 1.2 compared to negative control C 13.4.

Ascites	*Mean oocysts/gut ± S.D (range)	Infected mosquitoes/ total	%Transmission	%Blockade
Exp. 1				
None	3.88 ± 6.75 (0-32)	33/56	100	0
UPC 10	2.69 ± 4.49 (0-17)	17/36	69.33	30.67
13.1	0.13 ± 0.62 (0-4)	3/46	3.35	96.65
17.9	0.02 ± 0.15 (0-1)	1/44	0.52	99.48
Exp. 2				
C 13.4**	23.7 ± 36.62 (0-143)	24/33	100	0
1.2	48.52 ± 70.44 (0-249)	19/29	204.73	-104.73

* = The arithmetic mean number of oocysts/gut from total mosquitoes.

** = The MAb against protein coat of *B. divergens* merozoites.

TABLE 2.4 Effect of the ascitic fluids on oocyst development in passive immunisation study.

P. berghei gametocyte-infected mice were anaesthesised and offered to mosquitoes for 1/2 h then subsequently given 100 µl of the ascitic fluids 12.1 and 17.9 I. V. UPC 10 was included as the control of the assay. Another batches of mosquitoes were fed on these ascites-treated mice for 1/2 h. The mosquitoes were dissected for oocyst determination as described earlier.

Ascites	Mean oocysts* /gut ± S.D		Infected mosquitoes/total		% Transmission	% Blockade
	pre-administration of ascites (range)	post-administration of ascites (range)	Ascites pre-administration	Ascites post-administration		
UPC 10	101.47 ± 143.34 (0-740)	67.18 ± 93.4 (0-384)	50/64	27/32	66.21	33.79
12.1	11.07 ± 10.25 (0-36)	0	15/49	0/71	0	100
17.9	40.47 ± 79.36 (0-370)	1.73 ± 6.01 (0-29)	35/53	8/45	4.27	95.73

* = The arithmetic mean number of oocysts/gut from total mosquitoes.

2) *In vitro* study

In vitro ookinete development in the presence of the MAbs 12.1, 13.1 and 17.9 was observed. As negative controls, complete ookinete medium and the MAbs 7.8 & C 13.4 were included. The results reported as percent total cells (at least 10^4 non-infected red blood cells were counted) are summarised in Table 2.5. %Inhibition of ookinete development *in vitro* by the MAbs is schematically presented in Figure 2.3.

The MAbs 12.1 and 17.9 completely inhibited *in vitro* ookinete formation while 13.1 suppressed the parasite development by greater than 91%. In contrast, the control MAbs UPC 10, 7.8 and C 13.4 did not significantly inhibit *in vitro* ookinete development ($p < 0.5$).

2.3.4) Characterisation of the monoclonal antibody specificity using SDS-PAGE and immunoblot techniques

1) Target antigen(s) of the transmission-blocking monoclonal antibodies

The antigenic determinants recognised by the MAbs 12.1, 13.1 and 17.9 were studied on both non-reducing and reducing SDS-PAGE. Following Western blot analysis and autoradiography, it is observed that the MAbs 12.1 and 13.1 recognised a protein determinant of M_r 21 kD (or Pbs21) under both non-reducing (Figure 2.4, lanes 9 and 11) and reducing conditions (Figure 2.4, lanes 10 and 12, respectively).

As negative controls, neither 7.8 nor UPC 10 interacted with Pbs21 (Figure 2.4, lanes 3 & 4 and 7 & 8, respectively). However, 7.8 recognised the proteins of M_r 24.5 and high molecular weight proteins of ~76-162 kD (Figure 2.4, lanes 3 & 4). In addition, normal mouse sera (NMS) did not react with Pbs21 (Figure 2.4, lanes 1, 2).

Unfortunately, 17.9 failed to recognise Pbs21 under both non-reducing and reducing conditions in this experiment (Figure 2.4, lanes 5 & 6). It is not known whether loss of the detection was due to (i) reduction of Pbs21 protein synthesis at this stage of development. Normally, ookinetes were harvested within 20-24 h after culturing at 19°C. (ii) the speculation that the antibody recognised an epitope which is prone to degradation by freeze-thawed process. Normally, the preparation of ookinetes after the ammonium chloride enrichment was kept at -80°C until use. For SDS-PAGE, the parasite pellet was thawed and resuspended in Laemmli buffer as described in Materials and Methods. Unlike 17.9, the MAb 13.1 still recognised Pbs21 after the parasite pellet was freeze-thawed for 4 times (data not shown).

The following experiment was therefore carried out (Figure 2.5). Fresh cultures of *P. berghei* at different time course (0, 1, 2, 3, 6, 11 and 24 h post-gametogenesis and fertilisation) were set up so as to follow the expression of Pbs21 using the MAb 17.9. Ookinetes were enriched as described above and resuspended directly in either non-reducing or reducing Laemmli buffers. Following SDS-PAGE and immunoblot analysis, the MAb was found to react with Pbs21 under non-reducing conditions (Figure 2.5, panel B) but not the protein under reducing

TABLE 2.5 Effect of the monoclonal antibodies on ookinete development *in vitro.*

One ml of *P. berghei* gametocyte-infected blood was added into the wells containing the heat-inactivated ascitic fluids (12.1, 13.1 and 17.9) at a final concentration of 1:10. As negative controls, either the medium or irrelevant ascites 7.8 and C 13.4 were included. Following culturing at 19°C for 24 h, the parasites were smeared on glass microscope slides and Giemsa stained. Ookinete development was observed under the light microscope (Leitz) at x1000 magnification. The parasites were counted in the tail of blood films (at least 10^4 non-infected red blood cells were counted). Individual stage of the parasite was reported as percent total cells.

Ascites	%NRBC*	%Ring	%Trophozoite	%Schizont	%Gametocyte	%Ookinete	% Inhibition of ookinete formation
None (Medium)	81.96	0.77	6.28	2.03	0.73	4.18	0
UPC10	82.83	0.31	7.32	2.58	0.50	3.86	7.66
7.8**	82.15	0.14	9.63	3.51	0.84	3.72	11.0
C 13.4***	78.19	0	10.20	6.07	0.39	5.14	-22.97
12.1	84.54	0.40	12.40	2.25	0.40	0	**100**
13.1	89.34	0.68	8.12	1.10	0.76	0.34	91.87
17.9	87.18	1.22	9.17	1.79	0.65	0	**100**

* = Non-infected red blood cell.

** = The MAb against merozoites of *B. divergens.*

*** = The MAb against protein coat of *B. divergens* merozoites.

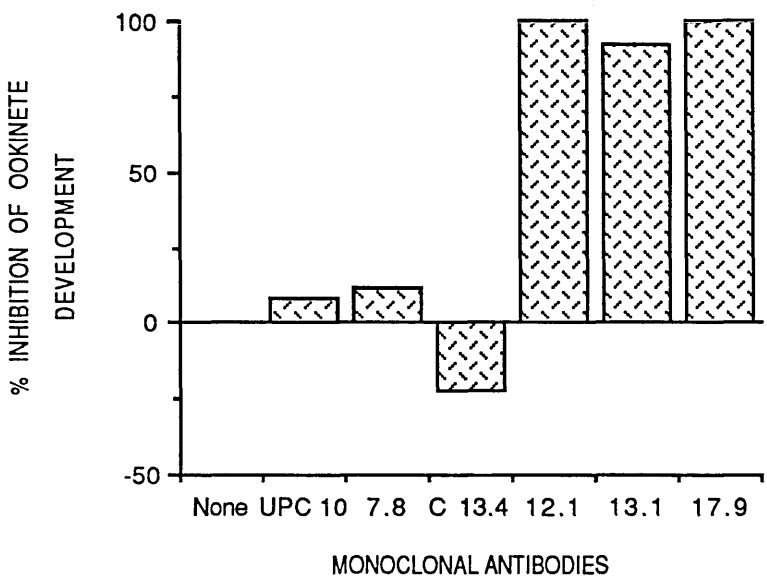


FIGURE 2.3 %Inhibition of ookinete development *in vitro* by the ascitic fluids.

One ml of *P. berghei* gametocyte-infected blood was added into the wells containing the heat-inactivated ascitic fluids 12.1, 13.1 and 17.9 at a final concentration of 1:10. As negative controls, either the medium or irrelevant ascites 7.8 and C 13.4 were included. Following culturing at 19°C for 24 h, bloodsmears were taken and Giemsa stained. Ookinete development was observed under the light microscope (Leitz) at x1000 magnification. The parasites were counted in the tail of blood films (at least 10^4 non-infected red blood cells were counted). Percent inhibition of ookinete development *in vitro* by the ascitic fluids was calculated as reported in Table 2.5.

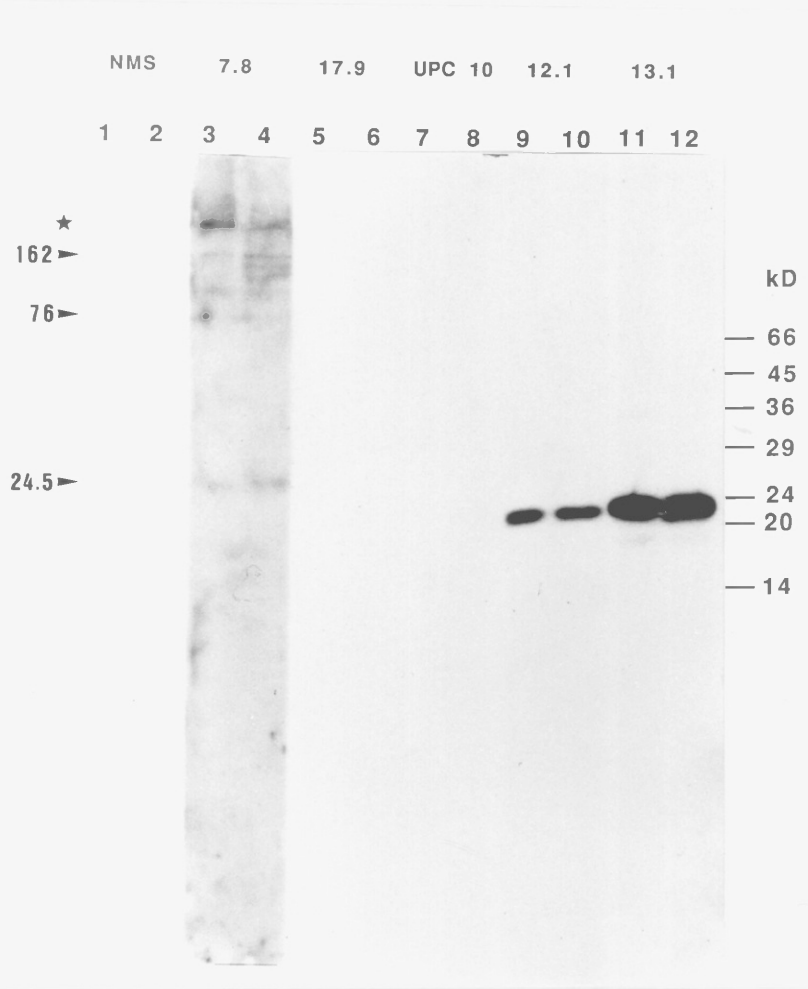


FIGURE 2.4 Immunoblot analysis of 15% non-reducing (lanes 1, 3, 5, 7, 9, 11) and reducing (lanes 2, 4, 6, 8, 10, 12) SDS-PAGE protein profiles of the ammonium chloride-enriched ookinetes of *P. berghei* with normal mouse sera (lanes 1, 2), MAb 7.8 (lanes 3, 4), MAb 17.9 (lanes 5, 6), UPC 10 (lanes 7, 8), MAb 12.1 (lanes 9, 10) and MAb 13.1 (lanes 11, 12). All strips were overlaid with ^{125}I-labelled sheep anti-mouse IgG. Molecular weights are indicated in kilodalton.

* = Boundary between the stacking and separating gels.

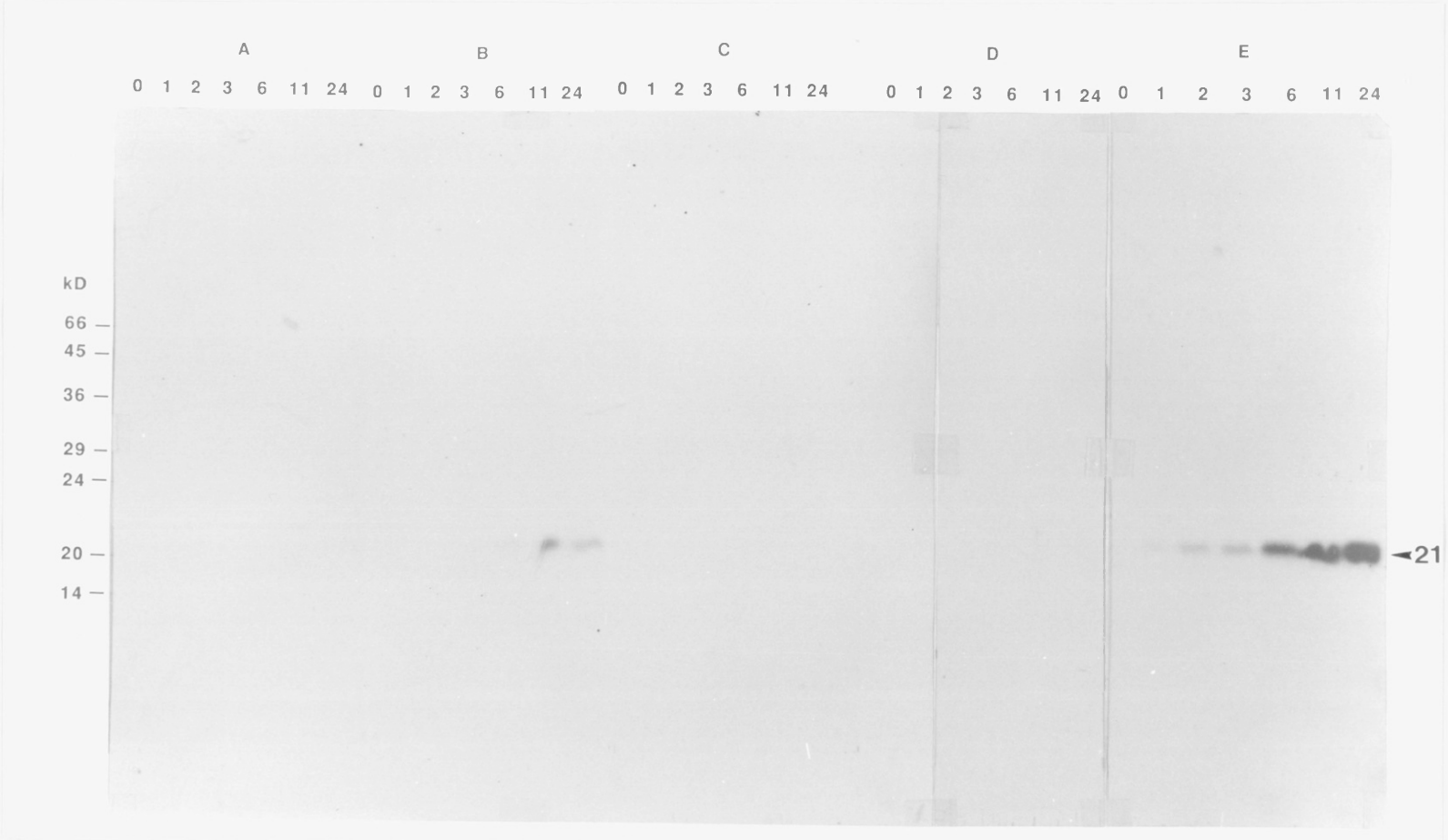


FIGURE 2.5 Immunoblot analysis of 15% reducing (panels A, C and E) and non-reducing (panels B & D) SDS-PAGE protein profiles of the ammonium chloride-enriched ookinetes of *P. berghei* culture at different time course (0, 1, 2, 3, 6, 11 and 24 h post-gametogenesis and fertilisation) with the MAb 17.9 (panels A & B), normal mouse sera (panels C & D) and MAb 13.1 (panel E). All strips were overlaid with ^{125}I-labelled sheep anti-mouse IgG. Molecular weights are indicated in kilodalton.

conditions (Figure 2.5, panel A). The protein was first recognised by the MAb after the parasite was cultured for 6 h post-gametogenesis and fertilisation (Figure 2.5, panel B). Expression of Pbs21 determined from the intensity of the protein band was higher at the 11 h-culture than the 24 h-culture (Figure 2.5, panel B).

The MAb 13.1 was used as a positive control of the same experiment (Figure 2.5, panel E). Parasite proteins were separated on reducing SDS-PAGE. The MAb first recognised Pbs21 after the parasites were cultured for 1 h post-gametogenesis and fertilisation. Expression of the protein was increased thereafter until 24 h (Figure 2.5, panel E). As a negative control, normal mouse sera (NMS) did not react with any proteins under non-reducing and reducing conditions (Figure 2.5, panels C & D respectively).

Thus, it is suggested that the protein epitope(s) recognised by the MAbs 12.1 and 13.1 are not conformation-dependent. In contrast, the epitope recognised by 17.9 is suggested to be conformation-dependent.

The MAb 1.2 did not react with Pbs21 or any other proteins on non-reducing and reducing SDS-PAGE (data not shown). Having established that the MAb reacted with ookinetes in IFA and Pbs21 in ELISA, it is suggested that loss of the immunoreactivity of the protein in immunoblot assay was due to (i) low antibody titre or (ii) the MAb recognised an epitope in Pbs21 which was sensitive to SDS. An alternative approach to characterise the target antigen of the MAb 1.2 was to immunoprecipitate the protein from biosynthetically labelled culture using the MAb prior to separation on SDS-PAGE. This experiment has not been done due to a limitation of the time.

2) Stage-specificity of the target antigen

The expression of Pbs21 is stage-specific. The MAb 13.1 did not react with the gametocyte and asexual stages using IFA (data not shown). Nor did the MAb react with those stages in the immunoblot analysis (Figure 2.6). Preparations of ammonium chloride-enriched asexual stages, gametocytes and ookinetes of *P. berghei* were separated on reducing SDS-PAGE. After Western blot analysis and autoradiography, the MAb 13.1 recognised the protein only in the ookinete preparation (Figure 2.6, panel B, lane 3). Expression of the protein was not detected in any of the asexual or gametocyte stages (Figure 2.6, Panel B, lanes 1 & 2 respectively). As a negative control, NMS did not react with polypeptides in any of those stages (Figure 2.6, panel A, lanes 1-3).

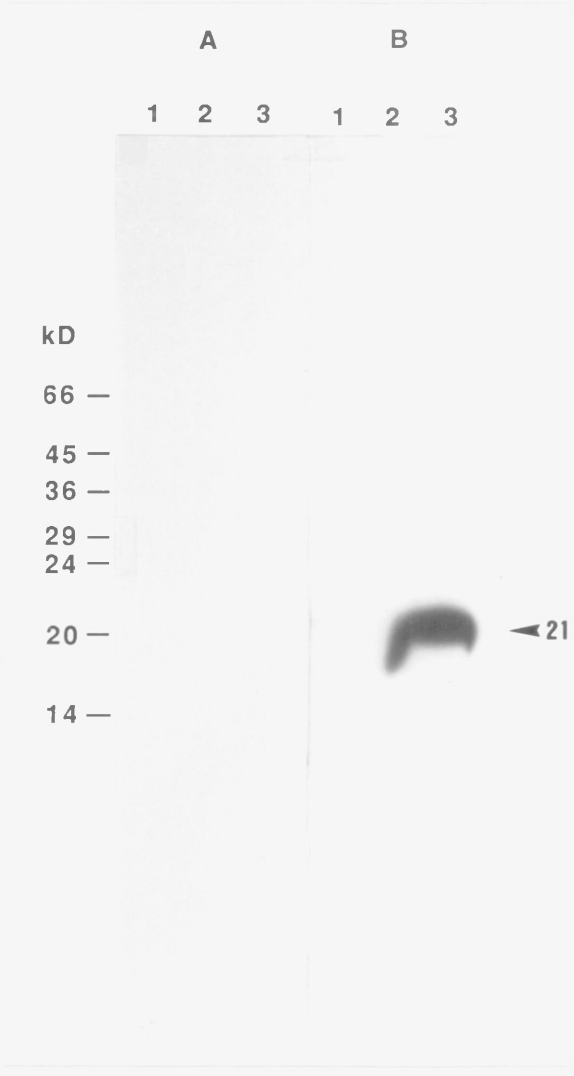


FIGURE 2.6 Immunoblot analysis of 15% reducing SDS-PAGE protein profiles of the ammonium chloride-enriched asexual (lane 1), gametocyte (lane 2) and ookinete (lane 3) preparations of *P. berghei* with normal mouse sera (panel A) and MAb 13.1 (panel B). All strips were overlaid with ^{125}I-labelled sheep anti-mouse IgG. Molecular weights are indicated in kilodalton.

2.4) Discussion

2.4.1) Transmission-blocking immunity in *P. berghei*

Humoral immunity has been reported to confer protection against sporozoite and asexual blood stages of the malaria parasites (Howard, 1986). Mice can be passively protected against sporozoite challenge by inoculation of purified MAbs against CS protein of *P. berghei*, *P. knowlesi*, *P. falciparum* and *P. vivax* (reviewed in Nussenzweig and Nussenzweig, 1988). MAbs against the merozoite surface proteins have also been demonstrated to block merozoite re-invasion e.g. in *P. yoelii*, *P. knowlesi* and *P. falciparum* (reviewed in Howard, 1986).

The principle mechanisms of transmission-blocking immunity are antibody-mediated (Carter *et al.*, 1989). The immunity mediated by antibody against the gamete stages of malaria parasites has been demonstrated by the use of immune animal sera, immune human sera and MAbs against gamete and zygote-ookinete surface antigens e.g. *P. gallinaceum* (Gwadz, 1976 ; Carter *et al.*, 1979b ; Rener *et al.*, 1980 ; Kaushal *et al.*, 1983a ; Grotendorst *et al.*, 1984), *P. knowlesi* (Gwadz and Green, 1978), *P. yoelii* (Mendis and Targett, 1982 ; Harte *et al.*, 1985b), *P. falciparum* (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a ; Quakyi *et al.*, 1987 ; Graves *et al.*, 1988a, b) and *P. vivax* (Munesinghe *et al.*, 1986 ; Mendis *et al.*, 1987a, b ; Peiris *et al.*, 1988).

In the present study, antibody-mediated mechanisms showed an important role in transmission-blocking immunity in *P. berghei* since the ascitic fluids 12.1, 13.1 and 17.9 conferred protection in membrane feed and passive transfer experiments (Tables 2.3 and 2.4 respectively). The hypotheses for the mechanism of transmission-blockade have been proposed (Sinden *et al.*, 1987). (i) The antibody may bind the ookinete within the zygotic aggregates, preventing their invasion of the mosquito gut wall. (ii) The antibody may inhibit recognition and hence the invasion of the midgut epithelium by the ookinete. (iii) The antibody may inhibit directly the subsequent development of the oocyst. Having established that the MAbs 12.1 and 17.9 absolutely prevented *in vitro* ookinete development whilst 13.1 suppressed the parasite development by 91.87%, the mechanism of transmission blockade by 12.1 and 17.9 might be the one described in hypothesis (i). For 13.1, however, the mechanism of transmission blockade would be any of the 3 hypotheses.

Transmission-blocking immunity involves both a variety of target antigens and a number of different immune effector agents (Carter, 1988b). In *P. gallinaceum*, the immunity mediated by anti-Pgs230 MAbs (Kaushal *et al.*, 1983a) functioned in either (i) complement-independent reactions with the antigen to prevent fertilisation or (ii) in complement-fixing reactions which destroyed by lysis both male and female gametes (Carter *et al.*, 1988a ; Carter, 1988b). In *P. falciparum*, anti-Pfs230 (Quakyi *et al.*, 1987) and anti-Pfs48/45 MAbs (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a) also functioned similarly to the MAbs against Pgs230 of *P. gallinaceum* (Carter *et al.*, 1988a). Additionally, the MAbs against Pgs31 and Pfs25 suppressed gametocyte infectivity to mosquitoes at the post-fertilisation stage in a complement-independent fashion (Grotendorst *et al.*, 1984 ; Vermeulen *et al.*, 1985a).

In our system, it is not yet clear whether complement will be involved in function of the MAbs 12.1, 13.1 and 17.9 in suppression of transmission since the experiments described in this chapter i.e. effect of the MAbs on ookinete or oocyst development were carried out in the presence of residual mouse serum which contained active complement. This needs further investigations.

Not only transmission-blocking but also transmission-enhancing effect was observed in *P. vivax* (Mendis *et al.*, 1987b ; Peiris *et al.*, 1988). The anti-Pvs42/36 MAb showed a dual role in transmission-blocking immunity, i.e. a blocking effect at higher concentrations and an enhancing effect at lower concentrations (Mendis *et al.*, 1987b ; Peiris *et al.*, 1988). In *P. falciparum*, the suppressive effect of an anti-Pfs25 MAb (32F81) on gametocyte infectivity to mosquitoes was also studied and no enhancement of transmission was observed at low antibody concentrations (Ponnudurai *et al.*, 1987).

In *P. berghei*, it is not known whether a single anti-Pbs21 MAb would exhibit a dual role in transmission-blocking immunity at different concentrations. However, an enhancement of transmission was observed at a certain dilution of the ascites 1.2. This enhancement of transmission needs further investigations.

2.4.2) Target antigens of the transmission-blocking MAbs

1) Synthesis and expression of Pbs21

Using immunoblot techniques, all the MAbs 12.1, 13.1 and 17.9 were found to recognise Pbs21 antigen (Figures 2.4 and 2.5). The MAbs 12.1 and 13.1 recognised both non-reduced and reduced Pbs21 (Figure 2.4) whilst 17.9 reacted with only the non-reduced protein (Figure 2.5).

Synthesis and expression of Pbs21 were studied using ^{35}S-methionine biosynthetic-labelling synchronous-gametocyte cultures (A. L. Dearsly, pers. comm.). No precursor of Pbs21 was observed and the protein was first detected by the MAb 13.1 in 2 h ookinete culture but not the gametocytes. The trigger of Pbs21 protein synthesis was from activation of the macrogametocyte and not fertilisation of the macrogamete (A. L. Dearsly, pers. comm.). Using EM-immunogold and Fluorescence-Activated Cell Sorter (FACS), Pbs21 was also first detected by the MAb 13.1 in 2 h ookinete culture but not the gametocytes (Sinden *et al.*, 1987 ; Winger *et al.*, 1988). From immunoblot assay in the present study, 13.1 first recognised the antigen after 1 h post-gametogenesis and fertilisation (Figure 2.5, panel E). The discrepancies could be due to the differences in the relative sensitivities of the methods used. Unlike 13.1, the MAb 17.9 first recognised Pbs21 after 6 h post-gametogenesis and fertilisation (Figure 2.5, panel B). This might be due to a relatively lower antibody titre of the MAb 17.9 compared to 13.1.

Persistence of Pbs21 was observed through the maturation of ookinetes i.e. 24 h (Sinden *et al.*, 1987 ; Winger *et al.*, 1988 ; A. L. Dearsly, pers. comm. and Figure 2.5 in the present

study). Reaction of 17.9 to Pbs21 at 11 h culture was relatively higher than 24 h culture (Figure 2.5, panel B). The same phenomenon was not clearly observed with 13.1 in immunoblot assay since the autoradiograph at the 11 and 24 h cultures might be overexposed (Figure 2.5, panel E). Using EM-immunogold, labelling of the MAb 13.1 on the surface of ookinetes showed relatively higher density of the gold particles at 12 h culture compared to 24 h culture (Sinden *et al.*, 1987). It is suggested that Pbs21 was shed after 11-12 h post-gametogenesis and fertilisation. Shed of the surface proteins of zygotes during the transformation of zygotes to ookinetes in immunologically intact and of apparently unchanged molecular weight into the medium was already reported in *P. gallinaceum* (Carter and Kaushal, 1984).

Synthesis and expression of Pgs31 was also examined (Kumar and Carter, 1985a, b). The protein was detected in early zygote development (2 h post-gametogenesis and fertilisation) but not in gametocytes prior to gametogenesis (Kumar and Carter, 1985a, b). It was synthesised onwards for 8-10 h. Thereafter, synthesis of Pgs31 declined and little or none was synthesised in the mature ookinetes (>20 h post-gametogenesis). Instead, Pgs34 which was not the target antigen of transmission-blocking MAbs (Grotendorst *et al.*, 1984) was synthesised throughout the development and it became the predominant protein synthesised by the mature ookinetes. In *P. falciparum*, on the other hand, synthesis of Pfs25 was detected from day 2 of gametocytes onwards until day 6 of oocyst development (Vermeulen *et al.*, 1985a & 1986).

2) Stage-specificity of Pbs21

Using immunoblot techniques, Pbs21 was found to be a stage-specific protein which was not expressed in either asexual or gametocyte stages (Figure 2.6). This result was consistent with IFA which showed that the MAbs reacted with only zygotes-ookinetes but not the asexual or gametocyte stages. Using EM-immunogold, the protein was expressed on macrogametes but not the asexual stages, intracellular macrogametocyte, microgametocyte (either in its intracellular or extracellular activated states), the microgamete or the microgamete residual body (Sinden *et al.*, 1987).

CHAPTER 3

STUDY OF THE TRANSMISSION-BLOCKING IMMUNITY IN *PLASMODIUM BERGHEI*

3.1) Introduction

As described in Chapter 1, transmission-blocking immunity can be induced naturally and artificially. By the use of polyclonal and monoclonal antibodies (MAbs), the immunity has been found to operate against two distinct phases in the life cycle of the malaria parasite (Carter *et al.*, 1988a and Carter, 1988b). Firstly, against the intracellular gametocytes in the vertebrate host e.g. *P. yoelii* (Harte *et al.*, 1985a). Secondly, against the extracellular forms in the mosquito midgut which are

i) Gametes e.g. *P. gallinaceum* (Carter and Chen, 1976 ; Gwadz, 1976 ; Carter *et al.*, 1979a, b ; Rener *et al.*, 1980 ; Kaushal *et al.*, 1983a, b), *P. knowlesi* (Gwadz and Green, 1978 ; Gwadz and Koontz, 1984), *P. falciparum* (Rener *et al.*, 1983 ; Vermeulen *et al.*, 1985a ; Quakyi *et al.*, 1987), *P. vivax* (Munesinghe *et al.*, 1986 ; Mendis *et al.*, 1987a, b ; Ranawaka *et al.*, 1988 ; Peiris *et al.*, 1988).

ii) Newly fertilised zygotes e.g. *P. gallinaceum* (Kaushal *et al.*, 1983a).

iii) Transforming zygotes or ookinetes e.g. *P. gallinaceum* (Grotendorst *et al.*, 1984), *P. falciparum* (Vermeulen *et al.*, 1985a) and *P. berghei* (Sinden *et al.*, 1987 ; Winger *et al.*, 1988).

Using the MAbs, it has been shown that target antigens of the anti-gamete and anti-ookinete antibodies are unrelated and stage-specific. In *P. gallinaceum*, three protein determinants i.e. Pgs230, Pgs48 and 45 were immunoprecipitated by anti-gamete MAbs (Kaushal *et al.*, 1983a ; Kaushal and Carter, 1984 and Carter *et al.*, 1984). Pgs230 was reported to be the sole target antigen of transmission-blocking immunity (Kumar, 1985 ; Kumar and Carter, 1986). During transformation of zygotes into ookinetes, two proteins i.e. Pgs31 and Pgs34 were expressed *de novo* (Carter and Kaushal, 1984) of which only Pgs31 was identified as the target antigen of post-fertilisation transmission-blocking immunity (Carter *et al.*, 1984 ; Grotendorst *et al.*, 1984 ; Carter *et al.*, 1988a).

In parallel, 3 proteins :- Pfs230, Pfs48/45 were identified as target antigens of transmission-blocking MAbs against gametes/zygotes of *P. falciparum* (Rener *et al.*, 1983 ; Carter *et al.*, 1984 & 1988 ; Kumar, 1985 ; Kumar and Carter, 1985b & 1986 ; Vermeulen *et al.*, 1985a ; Quakyi *et al.*, 1987). However, non-blocking MAbs against these antigens were also reported (Meuwissen and Ponnudurai, 1986 ; Vermeulen *et al.*, 1985a & 1986). Furthermore, Pfs25 protein was identified as the target of post-fertilisation transmission-blocking MAbs in *P. falciparum* and non-blocking Mabs against the protein were also reported (Vermeulen *et al.*, 1985a & 1986 ; Meuwissen and Ponnudurai, 1986 & 1988).

Transmission-blocking immunity in rodent malaria species has not been so well-studied though rodent models offer several advantages over avian and simian malaria parasites for many aspects of malaria research (Killick-Kendrick and Peters,1978). Such immunity was first demonstrated in mice immunised with partially-purified gametes of *P. y. nigeriensis* (Mendis and Target ; 1979, 1981 and 1982) and in hamster immunised with irradiated gametes of *P. berghei* (Kaushik *et al.*, 1982).

Persistence of the transmission-blocking immunity in mice immunised with microgametes of murine malaria parasite *P. y. nigeriensis* was reported to be 6 months (Mendis and Targett, 1982). Long-term protection of the transmission-blocking immunity was reported to be at least 12 months for the vaccination with microgametes of *P. y. nigeriensis* (Harte *et al.*, 1985a). Such immunity operated independently from antibody-mediated mechanism. In addition, MAbs against microgametes of *P. y. nigeriensis* have been generated and shown to suppress mosquito infectivity (Harte *et al.*, 1985b). It was extended to a successful vaccination study using purified microgamete antigens (Pys42) (Harte, *et al.*, 1985c).

As described in Chapter 2 and Winger *et al.*, (1988), MAbs against zygotes-ookinetes of *P. berghei* have been produced and shown to block the oocyst development in mosquitoes and ookinete development *in vitro*. Using the antibodies, a transmission-blocking determinant was identified of an M_r 21 kD (under non-reducing and reducing SDS-PAGE). The expression of this protein antigen from gametes to zygotes was also studied using EM immunogold (Sinden *et al.*, 1987).

This chapter will describe the successful vaccination using the affinity-purified ookinete antigen of *P. berghei* (Pbs21). Persistence of the immunity was investigated. ELISA was developed to examine classes and subclasses of the 'anti-Pbs21' antibody in the immune sera. The level of the specific antibody was also determined. In addition, antigens recognised by the immune sera were analysed using SDS-PAGE and immunoblot analysis.

3.2) Materials and Methods

3.2.1) Affinity-chromatography using the monoclonal antibody 13.1

Affinity-chromatography is a method of fractionation which exploits the biospecific binding of a particular molecule to a second molecule, often term 'ligand' (Cuatrecasas, and Anfinsen, 1971). The technique is extremely powerful when combined with the use of monoclonal antibody. It is therefore possible to produce affinity columns of many desired specificities, with high capacity and mild elution conditions (Goding, 1983).

Various methods are available for purification of antibodies from serum, ascites or tissue culture supernatant, depending on the class(es) of antibody, the quantity, the purity required and the starting material involved (Goding, 1983). The methods for IgG purification from the MAb described below are the combination between saturated ammonium sulphate precipitation and anion exchange chromatography. These 2 procedures are often the methods of choice for monoclonal IgG-containing ascites fluid and serum because of their applicability to many classes and subclasses of antibodies.

1) Precipitation of IgG from ascites using saturated ammonium sulphate

This method is gentle, effective and simple. Though it is not possible to purify immunoglobulins to homogeniety, it provides a substantial enrichment, and reduces the protein load on subsequent purification steps.

Two ml of the 13.1 ascites were diluted with an equal volume of PBS. Four ml of cold saturated ammonium sulphate (1 Kg 'dissolved' in 1L of distilled water, pH 6-7) were added dropwise while the solution was continuously stirred on ice for 30 min to precipitate the IgG. At 35-40% concentration of ammonium sulphate, most immunoglobulin will be precipitated (Goding, 1983). After centrifugation at 2, 000g (5, 000 rpm ; Sorvall RC5B), the IgG pellet was redissolved in 2 ml of PBS. The ammonium sulphate precipitation was repeated twice more and the pellet was washed twice with 2 ml of 50% ammonium sulphate before finally being dissolved in 2 ml of PBS. The IgG solution was dialysed extensively against PBS to remove the ammonium ions before it was subsequently purified using an anion exchange chromatography.

2) Purification of IgG using DEAE-anion exchange chromatography

Ion exchange is one of the simplest and most useful ways of purifying immunoglobulins, especially IgG. The most commonly used matrix consists of cellulose or agarose to which is attached to ionisable diethylaminoethyl (DEAE) groups.

Two ml of IgG solution (see section 3.2.1.1) was centrifuged at 13, 000g to remove the fine pellet then diluted to 20 ml with buffer A (0.015 M NaH_2PO_4 pH 6.4). The solution was applied to a Zeta-Prep® disk (LKB) - an anion exchange matrix at a flow rate of 5 ml/min. Following elution of the unbound proteins with 100 ml of buffer A at the flow rate of 20 ml/min, the bound immunoglobulin was then eluted with 60 ml of buffer B (0.1 M NaH_2PO_4 pH 6.8) + 1 M NaCl into 12 fractions (5 ml/fraction). The concentration and immunoactivity of the IgG

were examined by measuring the O.D. at 280 nm ($O.D_{280}$ of 1 mg/ml IgG = 1.4 ; Goding, 1983) and ELISA (see section 2.2.4.6 a) respectively. Fractions at the peak (number 2-6 ; 25 ml) were pooled and dialysed extensively against PBS then concentrated to 10 ml using an Amicon concentrator with PM10 filter (10 kD molecular weight cut off ; Amicon Corp.).

The Zeta-Prep disk is reusable, first by regeneration with 60 ml of 1 M acetic acid (pH 2.7), followed by 60 ml of 0.1 M Na_2HPO_4 pH 8.2 + 1 M NaCl. It was then equilibrated with 75 ml of Buffer B and finally with 100 ml of Buffer A prior to sterilisation at 121°C for 20 min then kept at 4°C.

3) Coupling of the purified IgG to the affinity support matrix

The purified IgG solution (about 10-15 mg protein) was dialysed extensively at 4°C against 0.1 M $NaHCO_3$ pH 8.5 for maximum coupling efficiency to an Affi-Gel 10 (BIO-RAD).

Three ml of the Affi-Gel 10 slurry were transferred to a small Buchner funnel. The supernatant was drained and the gel was washed with 3 bed volumes of cold distilled water. Care was taken, not to allow the gel bed to go dry. For optimum coupling of ligands, the washing procedure was completed and the moist gel cake was transferred to the tube containing the purified IgG solution within 20 min. The gel-IgG mixture was then left at 4°C overnight on an end-to-end rotator.

The gel was transferred to a 5-ml syringe plugged with glass wool to prevent the leakage of the gel beads. The column was washed extensively with TNPB buffer (0.025 M Tris pH 8, 0.1% NP-40 and 0.15 M NaCl). The column was stored in PBS containing 0.005% Merthiolate as a bactericide at 4°C.

3.2.2) Purification of Pbs21 using an affinity-chromatography

The protein was purified *en masse* for 2 main purposes, (i) for the experiments throughout the study i.e. ELISA and transmission-blocking immunity in *P. berghei* using Pbs21 as an immunogen. These studies required the single affinity-purified protein. (ii) For amino acid sequencing (in co-operation with Prof. H. R. Morris, Dept. of Biochemistry, Imperial College) which required the double affinity purified-protein.

1) Solubilisation of the ammonium chloride-enriched ookinetes of *P. berghei*

Membrane proteins can be solubilised by detergents. Hence, the pellet of the ammonium chloride-enriched zygotes-ookinetes of *P. berghei* from 20 mice was subjected to solubilisation with 20 ml of TNPA buffer (0.025 M Tris pH 8, 0.5% NP-40, 0.15 M NaCl, 0.001 M PMSF and 2 µg/ml of DNase and RNase). The reaction was left at 4°C for an hour before centrifugation at 13, 000g for 15 min at 4°C to remove the residual unsolubilised materials.

2) Column chromatography

After centrifugation, the supernatant described as above was loaded onto the affinity column with the rate of 1 ml/min. The gel was washed with 100 ml of TNPB buffer to remove the unbound proteins. At the elution step, 12 fractions containing phosphate buffer (0.004 M NaH_2PO_4, 0.25 M Na_2HPO_4 pH 7.5) in the ratio of 2 ml of the eluate to 3 ml of phosphate buffer were collected. Ten ml of the elution buffer (0.05 M diethylamine, 0.1% NP-40 and 0.15 M NaCl) was used to elute the bound protein into the first 5 fractions then TNPB buffer was used to wash the column further. The eluate was also collected into fractions number 6-12. Fractions at the peak (number 3-6) were pooled and dialysed extensively against PBS at 4°C overnight.

Double affinity purification was developed in an attempt to purify Pbs21 for amino acid sequencing. Fractions at the peak (after extensive dialysis against PBS) were reapplied to the column and the affinity-purification procedure repeated. The purified protein, after dialysis against distilled water, was either kept frozen at -80°C or lyophilised and kept at 4°C until use.

3) Quality assessment of the affinity-purified Pbs21

a) ^{125}I-labelling of the protein antigen using the Iodogen method

Quality of the single and double affinity-purified Pbs21 was assessed using ^{125}I-labelling. Radioiodination with ^{125}I is by far the commonest method of labelling soluble proteins. It is inexpensive, simple to perform and results in protein of high specific activity. The main disadvantage of radioiodination is that it may occasionally damage the protein, resulting in loss of antigenic determinants or other biological activity (Goding, 1983).

Fraker and Speck (1978) described a novel method of protein iodination which used the water-insoluble compound 1, 3, 4, 6-tetrachloro-3α, 6α-diphenyl glycoluril (Iodogen). The method which has achieved considerable acceptance labels at tyrosine residue of the protein (Salacinski *et al.*, 1981). Damage to proteins is minimal, because they are not exposed to soluble oxidising agents as in Chloramine-T method (Greenwood *et al.*, 1963). Moreover, Iodogen labelling is simpler and less expensive compared to lactoperoxidase catalysis which requires the addition of hydrogen peroxide that may damage cell membrane (Marchalonis, 1969) or Bolton-Hunter reagent which is very gentle for protein iodination, but suffers from yielding relatively low levels of protein radioactivity (Bolton and Hunter, 1973). The Iodogen method has also been applied successfully to study the zygote surface antigens of *P. gallinaceum* (Howard *et al.*, 1982 ; Kaushal *et al.*, 1983b). In the present study, purity of the affinity-purified Pbs21 especially the double affinity purification was a major concern as a supply for amino acid sequencing. Quality of the purified-protein was, therefore, assessed by ^{125}I-labelling using the Iodogen method.

Iodogen (Pierce Chemicals) was dissolved in methylene chloride at a concentration of 1 mg/ml then 200 µl of the solution was pipetted into 1.5 ml Eppendorf tubes.

The solvent was allowed to evaporate and the tubes were stored sealed and kept in the dark at room temperature.

Prior to use, the Iodogen tube was rinsed twice with PBS to remove any loose flakes. Fifty µl of the single or double affinity-purified protein (at least 0.5 µg) was pipetted into the Iodogen tube in the presence of a cocktail of protease inhibitors (at final concentration ; 0.001 M of EDTA, EGTA, N-ethylmaleimide, p-hydroxymercuribenzoate, O-phenanthroline and PMSF, 0.00135 M TLCK and 0.0001 M TPCK ; see Appendix 2). All chemicals were obtained from Sigma. To reduce exposure to volatile radioactive iodine or aerosols, 100 µCi of $Na^{125}I$ (Amersham ; IMS.30, 100 mCi/ml) was then added into the tube in a well-ventilated fume cupboard. The reaction tube was kept on ice for 10 min before 5 µl of saturated tyrosine (0.1 g of tyrosine was dissolved in 20 ml of distilled water) was added to stop the reaction.

The labelled protein was separated from free iodine by gel filtration. The solution was therefore loaded onto Sephadex G-25 column (Pharmacia ; fractionate range of peptides 1-5 kD) then eluted with 0.5% Triton X-100 in PBS (the addition of the detergent was to reduce the loss of protein on the column) into 10 fractions (250 µl/fraction). Total radioactivity and TCA precipitability were determined. The first radioactive peak of the fractions (number 5-8) which contained the labelled protein was retained, aliquoted and kept at -20°C.

b) Trichloroacetic acid (TCA) precipitation

After radiolabelling, the incorporation of radioactive material into the protein is measured by TCA precipitation. If the percentage of total radioactivity in the protein is high, a liquid-phase precipitation is adequate. However, if the fraction of total counts in the protein is low (for example, after cell surface iodination or biosynthetic labelling), more accurate results will be obtained by collection and washing of the precipitate on a filter (Goding, 1983). As described below, the liquid-phase procedure was used to determine the incorporation of ^{125}I into Pbs21.

Five µl of NGS was pipetted into a 1.5 ml Eppendorf tube as a protein carrier. The tube was added with 1-5 µl and subsequently with 500 µl of cold 10% TCA then left on ice for 1/2 h. The protein was pelleted at 13, 000g for 5 min. Radioactivity in both supernatant and the remaining pellet was finally counted using a γ-counter (LKB 1275).

c) SDS-Polyacrylamide Gel Electrophoresis

The ^{125}I-labelled single or double affinity-purified Pbs21 was subjected to 8-20% gradient SDS-PAGE (2 x 10^4 cpm/lane). The recipe for SDS-PAGE and electrophoresis conditions were as described in Appendix 1 and section 2.2.5.3 a. After electrophoresis, the gel was dried down at 80°C (BIO-RAD slab gel drier) and autoradiographed (section 2.2.5.3 b).

d) Two-dimensional gel electrophoresis

The separation of proteins in SDS-PAGE is based on their size, thus there is a probability that more than one polypeptide will co-migrate as a single band. Two-dimensional gel electrophoresis is one of the most powerful tools in the separation of most proteins from each other (O' Farrell, 1975). The proteins are separated firstly by charge (isoelectric focussing : IEF or non-equilibrium pH gradient electrophoresis : NEPHGE) and secondly by molecular size (SDS-PAGE). Using IEF, the method is suitable for acidic proteins (O' Farrell, 1975). A later modification of the technique (NEPHGE) allows the analysis of basic proteins (O' Farrell *et al.*, 1977). To assess the purity of the double affinity-purified Pbs21, two-dimensional separation was therefore performed.

Not only is NEPHGE faster (5 h) compared to IEF (16 h) but also the analysis is capable of resolving all but the most acidic and basic proteins (Goding, 1983). Hence, the ^{125}I-labelled the double affinity-purified Pbs21 was subjected to NEPHGE (the recipe was described in Appendix 1) as the first dimensional electrophoresis and subsequently with 8-20% SDS-PAGE as the second dimensional electrophoresis. Molecular weight markers (BDH) with known isoelectric point (pI) values were also included in the first and second dimensional electrophoresis. The markers were :- hen egg Ovotransferrin (76 kD), bovine serum albumin (66.2 kD), hen egg Ovalbumin (45 kD ; pI = 4.63), Carbonic anhydrase (30 kD ; pI = 5.68), equine Myoglobin (17.2 kD ; pI = 7) and equine Cytochrome C (12.3 kD ; pI = 10.6).

3.2.3) Transmission-blocking study using Pbs21 as an immunogen

Early immunisation trials (Groups A-M) were done in close collaboration with Dr. L. A. Winger and the results are summarised in Appendices 3 and 4 . Initially, my role was that of antigen preparation. In later experiments (numbered as groups N and O so as to correspond to the initial studies in Appendix 3), all steps were carried out by me using the methods modified from Harte *et al.*, (1985c). Oocyst counting was necessarily carried out by numerous numbers of the research group.

1) Animals

Groups of 3 T. O. female mice (Harlan Olac Ltd.), aged between 4-6 weeks were used as controls and immunised animals. All were healthy at the commencement of the trials.

2) Immunisation trials

An acetone-precipitated single affinity-purified Pbs21 was used as an immunogen. The protein at appropriate concentration was precipitated by the addition of 3 volumes of anhydrous acetone (BDH) and left at -20°C for 1/2 h before centrifugation at 13, 000g for 15 min. Acetone precipitation was suggested to improve the immunogenicity of the protein (P. G. Harte pers. comm.). The supernatant was discarded and the pellet was air-dried before the

protein was resuspended in 100 µl of PBS. The immunisation trials of only groups N and O will be presented in this Chapter.

Group N were divided into 2 subgroups : N 1 and N 2 (3 mice/subgroup). The mice subgroup N 2 were immunised intramuscularly (I. M.) with 5 µg of the antigen without adjuvant. A month later, each mouse was re-inoculated I. M. with 20 µg of the antigen. As controls, 3 mice (subgroup N 1) were injected I. M. with PBS.

Group O were divided into 2 subgroups : O 1 and O 2 (3 mice/group). The mice subgroup O 2 were immunised twice with 20 µg of the antigen without adjuvant I. M. The intervening period between the 2 inoculations was 1 month. As controls, 3 mice (subgroup O 1) was injected I. M. with PBS.

3) Parasite Inoculation

On day 20 after the boost, each mouse was infected intraperitoneally (I. P.) with 10^7 *P. berghei*-infected erythrocytes. Throughout the immunisation experiments, parasites at the passage P3 were used to intiate the infection. During the course of infection, parasitaemias were examined daily by taking thin film blood smears from each mouse then Giemsa stained (see section 2.2.5.2 b).

4) Mosquito feed

Three days after the infection, the mice were anaesthetised before offering to mosquitoes as described in section 2.2.1.2.

Duration of the protective immunity in the vaccinated mice subgroup O 2 (that were inoculated twice with 20 µg of the antigen) was also studied. After the first feed (at week 7 post-immunisation), the mice were cured from infection by chloroquine treatment. Five doses of the drug were injected I. P. on daily basis (125 µg/mouse or ~ 5 µg/gm body weight ; A. L. Dearsly, pers. comm.). At week 34 after the first immunisation, the mice were reinfected with *P. berghei*. Another batch of mosquitoes were fed on to the mice and the number of oocysts was determined.

5) Determination of the oocysts

Number of oocysts was observed in mosquitoes that administered to the immunised mice compared to the ones that fed to the controls as described in section 2.2.5.2. The number of oocysts was reported as the arithmetic mean from 3 mice/subgroup. %Transmission (%Tm) and %Blockade (%Bl) were calculated from the following equations.

$$\%\,Tm = \frac{\text{Arithmetic mean number of oocysts/gut in the experimental group}}{\text{Arithmetic mean number of oocysts/gut in the control group}} \times 100$$

$$\%\,Bl = 100 - \%Tm$$

6) Sera collection

Sera from both control and immunised mice subgroups N 1 and N 2 respectively were collected individually after the mice were fed on the mosquitoes for blockade assessment (week 7 after the first immunisation). In the mice group O, sera were collected as appropriate from week 7 until week 39 after the first immunisation. The mice were tail-bled and the blood was allowed to coagulate at 4°C overnight. Sera were removed after centrifugation at 13, 000g for 5 min, aliquoted and kept at -80°C.

3.2.4) Determination of the level and subclasses of specific antibody in the immune sera

Quantitative measurements of specific antibody in ELISA are usually determined by comparison to a standard curve produced using a purified antibody. The isolation and purification of specific antibodies in sufficient quantities to run a standard curve is time-consuming and often not practical for low titre antibodies or for antibodies from animals with a small serum volume such as the mouse. In addition, the preparation of immunosorbents for antibody purification requires a substantial amount of purified antigen. This is a very laborious process and not possible in many circumstances. An alternative approach for the standardisation of a positive control was developed in this Chapter using the method modified from Beatty *et al.*, (1987). This method of comparison of unknown serum with non-specific IgG of known quantity as the standard did not require the isolation and purification of a specific antibody or antigen.

Antibody-mediated immunity in the mice immunised with Pbs21 was investigated using ELISA. From preliminary observation, IgG was the class of immunoglobulin detected in the immune sera. The following experiments were, therefore, designed to determine the quantity of the specific IgG in the immune sera based on using a commercially available heterologous mouse IgG as a standard of known concentrations in ELISA. The correlation between the optical density and the concentration of the standard immunoglobulin was then calibrated.

Since class and subclass of an immunoglobulin dictate its biological functions, subclasses of the 'anti-Pbs21' IgG were also studied. The mice subgroups N 2 (that were immunised with 5 and boosted with 20 μg of the antigen) and O 2 (that were immunised twice with 20 μg of the antigen) were selected for this study as a very high blockade of mosquito infectivity was achieved.

1) Standard Immunoglobulin G

The affinity-purified goat anti-mouse IgG (Sigma M-8642) at a concentration of 10 µg/ml was used to pre-coat the wells of microtitre plates (100 µl/well) as a capture agent in sandwich ELISA (from preliminary observations, this amount of capture antibody was in excess). Residual binding sites of the plates were blocked as described before the purified mouse IgG (Sigma I-5381 ; containing approximately 80% protein and 95% pure by electrophoresis) was subsequently titrated in a doubling dilution series (10.000-0.079 µg/ml) as a standard immunoglobulin in an individual plate. Dilution of the standard IgG was always performed in 2.5% NGS-PBS. The following steps were as described in section 2.2.4.6 a.

A standard curve in individual microtitre plate was, therefore, calibrated so as to avoid errors from variation between plates. Using BIO-RAD EIA software®, curve-fitting facilities automatically produced best-fit calibration curves which were then used to calculate results.

2) Experimental sera

Wells of the microtitre plates were pre-coated with 50 µl/well of an excess amount of the affinity-purified Pbs21 (2 µg/ml). After blocking the residual binding sites of the plates, 100 µl of individual serum from both control and immunised mice was subsequently titrated in a doubling dilution series (1:125-1:16, 000). Since titration of the serum was time-consuming, 3 plates were set up at a time so as to keep the incubation period (37°C, 1 h) constant between plates. The following steps were as described in section 2.2.4.6 a.

The $O.D_{414}$ was measured after 1/2 h incubation at 37°C using the EIA reader (BIO-RAD 2550). All $O.D_{414}$ readings were converted to µg IgG/ml serum using the standard curve described as above. The data was analysed using the BIO-RAD EIA software®.

As has been suggested by Catty *et al.*, (1983), the O.D values which lie the closest to the centre of the linear part of the standard curve were selected to determine the IgG level for individual serum i.e. the $O.D_{414}$ at dilutions of 1:2000,1:1000 and 1:250 were selected to calculate the IgG concentrations for the mice subgroups N 2 and O 2 (at week 7 and 34 post-immunisation i.e. first and second blockade assessments) respectively.

Subclasses of the immunoglobulin in the immune sera were also investigated. Wells of the microtitre plates pre-coated with the antigen were incubated with 100 µl/well of 1:125 dilution of the sera. As the second antibody-enzyme conjugate, a panel of different anti-immunoglobulin subclasses conjugated to the enzyme Alkaline phosphatase i.e. goat anti-mouse IgG1, 2a, 2b and 3 (Serotec) was added. The following steps were as described above.

3.2.5) Analysis of antigens recognised by the immune sera

To further understand the immune response of the host in the vaccination study, the ammonium chloride-enriched ookinetes of *P. berghei* was subjected to SDS-PAGE (under reducing conditions with 2-ME) and immunoblot techniques. Pooled control and immune sera

collected at week 7 post-immunisation from the mice subgroups N 2 (that were immunised twice with 5/20 µg of Pbs21) and O 2 (that were immunised twice with 20/20 µg of Pbs21) were used at a dilution of 1:100 as first antibody. After the addition of ^{125}I-labelled sheep anti-mouse IgG as second antibody, the nitrocellulose was autoradiographed as described in section 2.2.5.3 b.

In parallel, *B. divergens* merozoites were subjected to SDS-PAGE and immunoblot techniques under the same conditions as a control antigen. The ascitic fluid 13.1 was used as a positive control of the assay.

3.3) Results

3.3.1) Quality assessment of the affinity-purified Pbs21

Quality and quantity of a protein antigen depend greatly on the purification step. Here, affinity chromatography was developed to purify Pbs21 antigen *en masse*. The protein was double affinity-purified for amino acid sequencing. To ensure of the quality of the purified Pbs21, both single and double affinity-purified proteins were ^{125}I-labelled using the Iodogen method. The labelled-protein was separated from free iodine by gel filtration. Ten fractions were collected, radioactivity of each fraction was counted by TCA precipitation (Table 3.1). The elution profile from gel filtration is shown in Figure 3.1. Specific activity of ^{125}I-labelled Pbs21 was ~ 1.13-2.59 x 10^7 cpm/µg protein.

Fractions at the peak (5-8) from gel filtration of both single and double affinity column purifications were subsequently subjected to SDS-PAGE (20,000 cpm/lane) and autoradiography (Figure 3.2). Although Pbs21 was the most prominent protein in the single column purification, trace contaminant polypeptides with apparent M_r of 66, 45, 24 and 14 kD (indicated by arrows) and some minor bands at M_r between 45-66 and 24-45 kD were co-eluted with Pbs21 antigen (Figure 3.2, lanes 2-5). Pbs21 was the prominent protein in the double column purification (Figure 3.2, lanes 6-9). Thus, the results implied that quality of the affinity-purified protein was adequate for further studies.

Since quality of the purified Pbs21 was a major concern for amino acid sequencing, purity of the protein was further assessed. The ^{125}I-labelled double affinity-purified Pbs21 (fraction 8 from gel filtration ; 20, 000 cpm) was subjected to 2-dimensional gel electrophoresis (2-D gel) (Figure 3.3). One smeary spot of the protein with an M_r of 21 kD was observed which also indicated homogeniety of the double affinity-purified antigen. The smeary effect might be, however, correlated to glycosylation which is a characteristic of most membrane proteins (Goding, 1983). This antigen preparation was, therefore, applied for amino acid sequencing. The isoelectric point (pI) of Pbs21 was also estimated to be ~ 7 in 2-D gel (Figure 3.3).

Quantity of the affinity-purified protein was improved from 3-5 µg/mouse (with 60-70% parasitaemia ; L. A. Winger, pers. comm.) in earlier preparations to 15-20 µg/mouse (after double purification) routinely. It was also observed that the affinity column could be reused at least 14 times.

3.3.2) Transmission-blocking study using the affinity-purified Pbs21 as an immunogen

Details of the previous immunisation trials (groups A-M) including the arithmetic mean number of oocysts/mosquito gut, % Transmission (Tm) and % Blockade (Bl) are summarised in Appendix 3. %Transmission of the immunisation trials groups A-M is illustrated in Appendix 4.

The two latest immunisation trials were coded as groups N and O so as to correspond to the previous trials in Appendix 3. Group N mice were divided into 2 subgroups.

TABLE 3.1 Radioactivity of the fractions from gel filtration containing ^{125}I-labelled single and double affinity column purification of Pbs21 using the Iodogen method.

Fraction numbers from Sephadex G-25	Radioactivity of single affinity column purification (cpm)	Radioactivity of double affinity column purification (cpm)
1	0	0
2	0	0
3	24, 330	3, 330
4	579, 810	373, 290
5*	949, 710	1, 001, 640
6*	1, 584, 570	1, 802, 130
7*	2, 942, 610	3, 355, 170
8*	5, 259, 750	4, 927, 290
9	691, 470	5, 849, 370
10	1, 409, 970	976, 890

*Peak fractions (5-8) were subjected to SDS-PAGE (as shown in Figure 3.2).

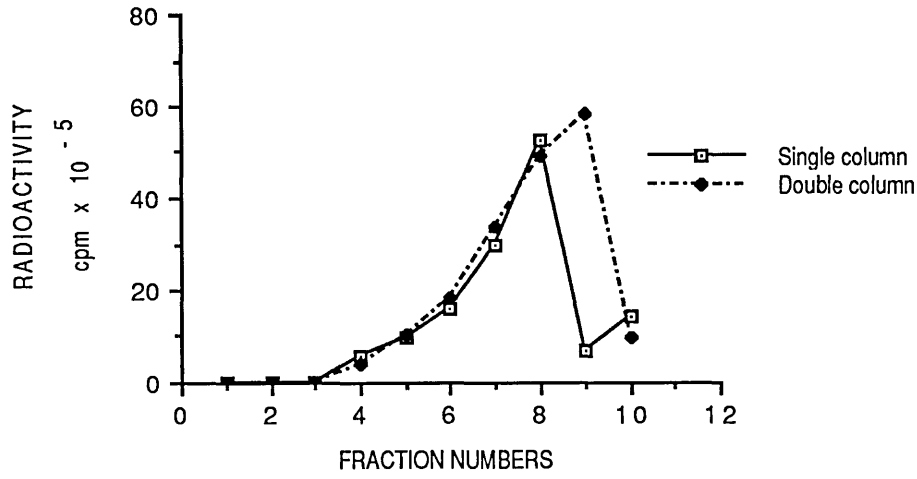


FIGURE 3.1 Elution profile of ^{125}I-labelled single (illustrated in solid line) and double (illustrated in dotted line) affinity-purified Pbs21 labelled by the Iodogen method. Both preparations of the protein, after the ^{125}I-labelling were subsequently separated from free iodine by gel filtration. Ten fractions were collected and the radioactivity of each fraction was counted as presented in Table 3.1.

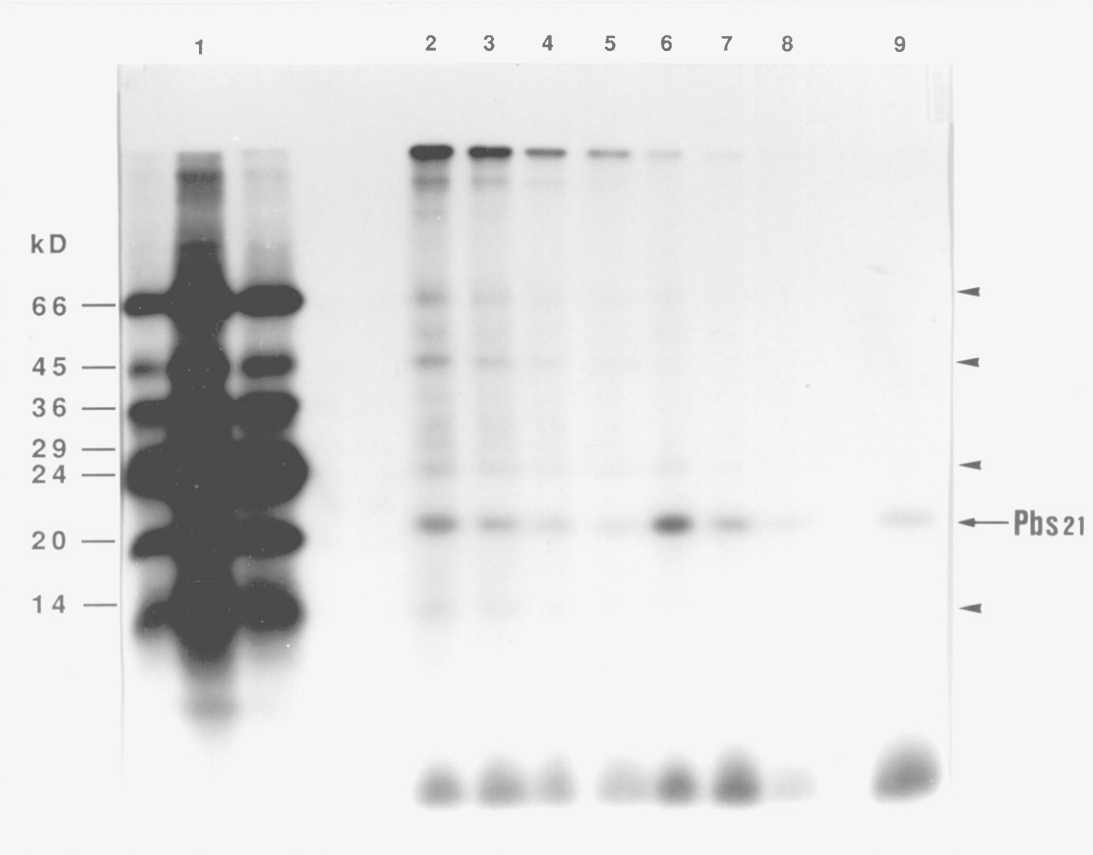


FIGURE 3.2 An autoradiograph of the peak fractions from gel filtration (5-8 ; Table 3.1) of ^{125}I-labelled the single (lanes 2-5 for fractions 5-8 respectively) and double (lanes 6-9 for fractions 5-8 respectively) affinity-purified Pbs21 antigen on 8-20% gradient SDS-PAGE. Molecular weight markers are indicated in kilodalton (lane 1).



FIGURE 3.3 An autoradiograph of ^{125}I-labelled the double affinity-purified Pbs21 antigen on two-dimensional electrophoresis.

N 1 indicated the control mice (3/subgroup) and N 2 (the vaccinated mice that were immunised twice with 5/20 μg of Pbs21). Similarly, Group O mice were divided into 2 subgroups ; O 1 indicated the control mice (3/subgroup) and O 2 indicated the vaccinated mice that were immunised twice with 20/20 μg of Pbs21 (3/subgroup).

1) Differential parasite counting

The mice were infected with 10^7 *P. berghei*-infected erythrocytes to determine the effect of the immunisation on the transmission-blocking immunity. During the course of infection, bloodsmears from the control subgroups N 1 and O 1 and vaccinated mice subgroups N 2 and O 2 were made daily then Giemsa stained.

Persistence of the transmission-blocking immunity in the mice group O was studied. The second blockade was assessed at week 34 post-immunisation. The mice were re-infected with 10^7 *P. berghei*-infected erythrocytes and re-offered to mosquitoes. Unfortunately, bloodsmears taken from the mice subgroups O 1 and O 2 during the course of infection were lost due to the relocation of our lab from the Field Station to South Kensington. Therefore, differential parasite counting for group O mice at the second blockade assessment was not available.

The parasites were counted from at least 10 fields on the slides of which the cells were equally distributed (at least 1, 000 non-infected red blood cells were counted). Differential parasite counting for groups N and O mice is reported as percent total cells (Tables 3.2 & 3.3 respectively). %Parasitaemia and gametocytaemia from the mice groups N and O during the course of infection are illustrated in Figures 3.4 A and B respectively. By day 3 after the infection, %parasitaemia and gametocytaemia reached between 3.43-4.74 and 0.38-0.63 respectively. There was no significant protection ($p < 0.5$) from the immunisation with Pbs21 against the asexual erythrocytic and gametocyte stages in any of the vaccinated animals.

2) Assessment of the transmission-blocking immunity

After blockade assessment, numbers of oocysts/mosquito gut are expressed in the arithmetic mean from 3 mice per each subgroup of the control and vaccinated animals. The results are summarised in Tables 3.4 and 3.5 for groups N and O respectively.

In the mice subgroup N 2, mosquito infectivity was suppressed by greater than 98% (Table 3.4) and full blockade of oocyst development was observed in subgroup O 2 (Table 3.5). Persistence of the transmission-blocking immunity in the mice subgroup O 2 was also studied. At week 34 after the first immunisation, such immunity was still demonstrable with ca.90% blockade of oocyst development in mosquitoes.

TABLE 3.2 Differential parasite counting during the course of infection for blockade assessment in the mice subgroups N 1 (control) and N 2 (vaccinated mice that were immunised twice with 5/20 µg Pbs21).
Values, expressed as percent total cells (at least 1, 000 non-infected red blood cells were counted), are the arithmetic mean (± S. D.) of three mice/subgroup.

Day of infection	Parasitaemia		Non-infected RBC*		Ring		Trophozoite		Schizont		Gametocyte	
	N 1	N 2	N 1	N 2	N 1	N 2	N 1	N 2	N 1	N 2	N 1	N 2
1	0.79 (0.13)	1.08 (0.45)	99.21 (0.13)	98.92 (0.45)	0.24 (0.23)	0.37 (0.27)	0.51 (0.17)	0.71 (0.35)	0 0	0 0	0.03 (0.05)	0 0
2	2.07 (1.42)	2.08 (0.6)	97.93 (1.42)	97.92 (0.6)	0.14 (0.13)	0.26 (0.13)	1.24 (0.71)	1.76 (0.44)	0 0	0.06 (0.05)	0 0	0 0
3	3.86 (0.69)	3.63 (0.07)	96.14 (0.69)	96.37 (0.07)	0.31 (0.21)	0.6 (0.24)	2.43 (0.46)	1.96 (0.21)	0.56 (0.26)	0.57 (0.38)	0.56 (0.14)	0.49 (0.03)

*RBC = Red blood cell
N 1 = Control mice (3/subgroup)
N 2 = Vaccinated mice (3/subgroup)

TABLE 3.3 Differential parasite counting during the course of infection for blockade assessment in the mice subgroups O 1 (control) and O 2 (vaccinated mice that were immunised twice with 20/20 µg Pbs21).

Values, expressed as percent total cells (at least 1, 000 non-infected red blood cells were counted), are the arithmetic mean (± S. D.) of three mice/subgroup.

Day of infection	Parasitaemia		Non-infected RBC*		Ring		Trophozoite		Schizont		Gametocyte	
	O 1	O 2	O 1	O 2	O 1	O 2	O 1	O 2	O 1	O 2	O 1	O 2
1	1.45 (0.34)	1.87 (0.71)	98.55 (0.34)	98.13 (0.71)	0.09 (0.09)	0.08 (0.09)	1.36 (0.43)	1.76 (0.60)	0 0	0.03 (0.05)	0 0	0 0
2	4.1 (0.79)	3.22 (1.31)	95.90 (0.79)	96.78 (1.31)	0.09 (0.10)	0 0	3.67 (1.10)	4.17 (1.05)	0.30 (0.27)	0.05 (0.09)	0.03 (0.05)	0 0
3	4.74 (1.59)	3.43 (1.28)	95.26 (1.59)	96.57 (1.28)	0.24 (0.09)	0.24 (0.21)	4.04 (1.66)	2.30 (1.10)	0.08 (0.09)	0.21 (0.20)	0.38 (0.04)	0.63 (0.13)

*RBC = Red blood cell

O 1 = Control mice (3/subgroup)

O 2 = Vaccinated mice (3/subgroup)

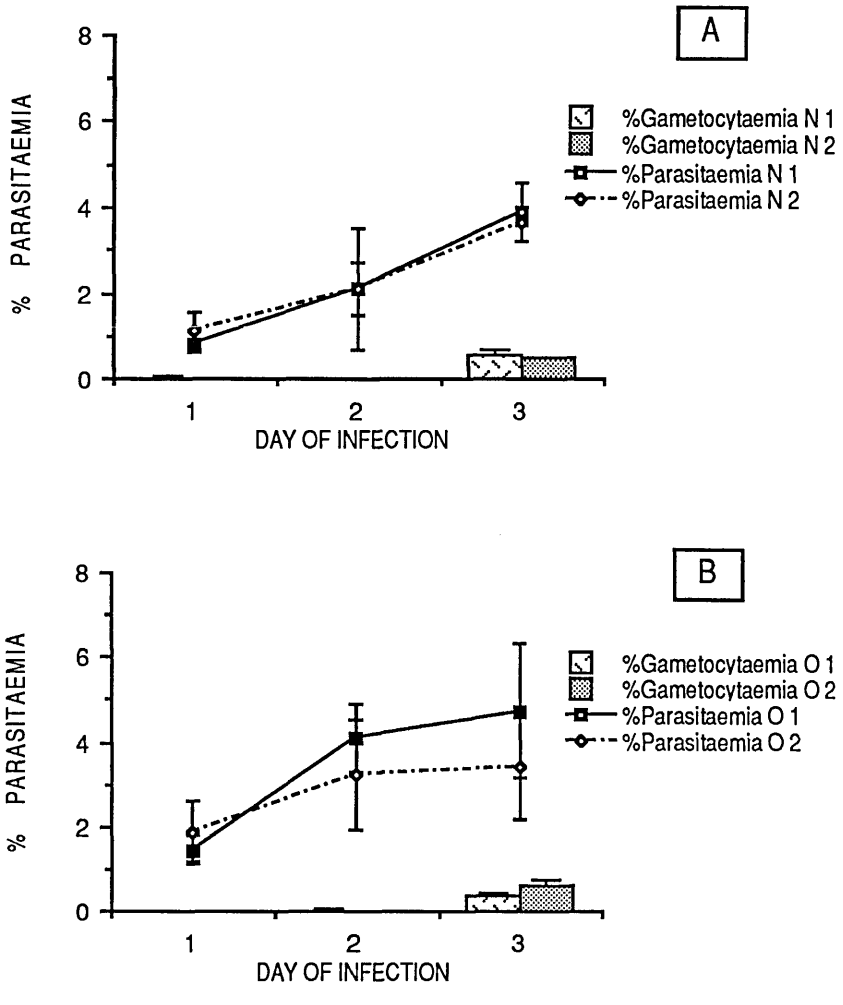


FIGURE 3.4 %Parasitaemia (illustrated in line graphs) and gametocytaemia (illustrated in bar graphs) in the mice groups N (Figure 3.4 A) and O (Figure 3.4 B) during the course of infection for blockade assessment.

Group N ; subgroups N 1 and N 2.

Three mice were imunised twice with 5 and 20 µg Pbs21, I. M. (subgroup N 2). As the control, another set of 3 mice were injected twice with PBS (subgroup N 1).

Group O ; subgroups O 1 and O 2.

Three mice were immunised twice with 20 and 20 µg Pbs21, I. M. (subgroup O 2). As the control, another set of 3 mice were injected twice with PBS (subgroup O 1).

All values are the arithmetic mean of percent total cells (described in Tables 3.2 and 3.3 for groups N and O mice respectively) ± S. D. of 3 mice in individual subgroup.

TABLE 3.4 Immunisation summary of the mice subgroups N 1 (control) and N 2 (vaccinated mice that were immunised twice with 5/20 µg Pbs21).

Blockade assessment	Mice subgroup	Treatment*** Primary	Secondary	Mean oocysts/ mosquito gut♦ ± S. D. (Range)	%Transmission	%Blockade	No.infected mosquitoes/ Total
week 7 post-immunisation	N 1* (3 mice)	PBS	PBS	107.24 ± 58.22 (0 - 626)	100	0	53/66
week 7 post-immunisation	N 2** (3 mice)	5 µg acetone-antigen (Ac.Ag) I. M.	20 µg Ac.Ag I. M.	2.09 ± 2.85 (0-20)	**1.95**	**98.05**	32/102

* = N 1 ; Control mice

** = N 2 ; Vaccinated mice

*** = The intervening period between primary and secondary treatments was 4 weeks.

♦ = The arithmetic mean number of oocysts/gut from total mosquitoes obtained from an indicated number of mice.

TABLE 3.5 Immunisation summary of the mice subgroups O 1 (control) and O 2 (vaccinated mice that were immunised twice with 20/20 µg Pbs21).

Blockade assessment	Mice subgroup	Treatment*** Primary	Secondary	Mean oocysts/ mosquito gut♦ ± S. D. (Range)	%Transmission	%Blockade	No.infected mosquitoes/ Total
First at Week 7 post-immunisation	O 1* (3 mice)	PBS	PBS	45.11 ± 37.24 (0 - 620)	100	0	24/48
	O 2** (3 mice)	20 µg acetone-antigen (Ac.Ag) I. M.	20 µg Ac.Ag I. M.	0	**0**	**100**	0/45
Second at Week 34 post-immunisation	O 1 (2 mice)	as above	as above	16.54 ± 18.09 (0 - 59)	100	0	7/13
	O 2 (2 mice)	as above	as above	1.6 ± 0.89 (0 - 3)	**9.67**	**90.33**	5/32

* = O 1 ; Control mice

** = O 2 ; Vaccinated mice

*** = The intervening period between primary and secondary treatments was 4 weeks.

♦ = The arithmetic mean number of oocysts/gut from total mosquitoes obtained from an indicated number of mice.

3.3.3) Determination of the level and subclasses of specific antibody in the immune sera

The level of 'anti-Pbs21' IgG in the control and immune sera was determined using ELISA. Sera from the mice subgroups N 1 (control) & N 2 that were immunised twice with 5/20 μg of Pbs21 were collected at the time of blockade assessment. Sera from the mice subgroups O 1 (control) & O 2 that were immunised twice with 20/20 μg of Pbs21 were also collected from week 7-39 post-immunisation. Individual serum was titrated against Pbs21 in ELISA. The antibody titration from the control and immune sera is illustrated in Appendix 5. Using the heterologous mouse IgG to calibrate the standard curve in ELISA, the level of the 'anti-Pbs21' IgG in individual mice in the control subgroups N 1 and O 1 was, therefore, determined. The arithmetic mean of the specific IgG in the control sera varied between 1-35 μg/ml. In parallel, the level of the 'anti-Pbs21' IgG in the immune sera subgroups N 2 and O 2 (from week 7 to 39 post-immunisation) was also determined and the level of the specific IgG in the immune sera is presented individually in Appendix 6. The arithmetic mean of the specific IgG from an indicated number of mice/subgroup after subtraction from the control IgG values are summarised in Table 3.6.

In the immune sera subgroup N 2 and O 2 (at the first blockade assessment i.e. week 7 post-immunisation), the 'anti-Pbs21' IgG level was determined to be 277.33 and 182.45 μg/ml serum respectively.

Distribution of the 'anti-Pbs21' IgG level in the immune sera subgroup O 2 from week 7-39 post-immunisation is illustrated in Figure 3.5. The specific IgG level was at the maximum at week 7 (first blockade assessment i.e. 182.45 μg/ml). It was dropped dramatically to 55.38 μg/ml at week 9 since this figure was the arithmetic mean of the specific IgG level from 2 mice with lower level of the IgG (the other mouse with the highest level of the IgG was dead). The level of the specific IgG remained in the range of 23.25-48.34 μg/ml until week 39. At week 34 (second blockade assessment) where 90% blockade was achieved, the specific IgG was still elevated (38 μg/ml).

Subclass of the specific IgG in the immune sera that block transmission of gametocyte infectivity to mosquitoes was investigated using ELISA. Sera from the mice subgroups N 2 and O 2 (only at the first and second blockade assessments i.e. week 7 and 34 post-immunisation) were subjected to ELISA in order that prediction of the biological function of the IgG subclass in the transmission-blocking immunity might be possible. IgG1 was found to be the only subclass of the 'anti-Pbs21' IgG in the immune sera (O. D_{414} values of the positive reaction were between 0.6-0.78 while they were between 0.002-0.03 for the negative reaction) . However, this subclass of IgG was the predominant immunoglobulin isotype in a normal mouse serum (6.5 mg IgG1/ml serum of a Balb/C mouse) (Hudson and Hay, 1980).

TABLE 3.6 Level of the 'anti-Pbs21' IgG in the immune sera from the mice subgroups N 2 (that were immunised with 5/20 µg Pbs21) and O 2 (that were immunised with 20/20 µg Pbs21) determined using the heterologous mouse IgG as the standard of known concentrations in ELISA.

Subgroup of Mice	Immunisation regime	Sera collection (week post-immunisation)	Level of 'anti-Pbs21' IgG (µg/ml) ± S.D. determined by ELISA
N 2	5/20 µg Pbs21	7	**277.33 ± 2.83***
O 2 (1st Feed)	20/20 µg Pbs21	7	**182.45 ± 92.13***
		9	55.38 ± 23.98**
		11	27.88 ± 23.16
		15	44.33 ± 15.56
		16	48.34 ± 30.64
		27	31.07 ± 10.34
		28	29.25 ± 13.08
		30	23.25 ± 18.38
O 2 (2nd Feed)		34	**38 ± 0****
		39	27.12 ± 0***

* = Arithmetic mean from 3 mice after subtraction from the IgG value of the control sera.

** = Arithmetic mean from 2 mice after subtraction from the IgG value of the control sera (this criterion was also applied to the IgG values of week 11-34).

*** = The value was obtained from 1 mouse that was alive after subtraction from the IgG value of the control sera.

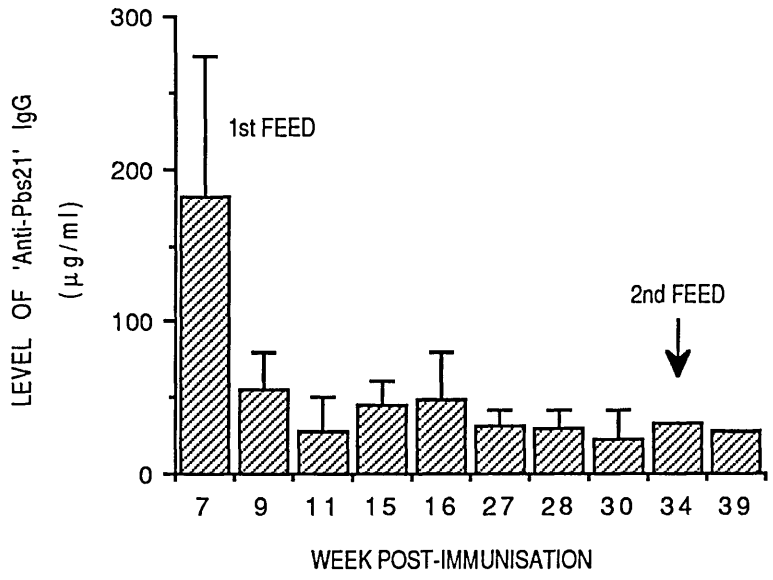


FIGURE 3.5 Distribution of the 'anti-Pbs21' IgG levels (µg/ml) in the immune sera from the mice subgroup O 2 (that were immunised twice with 20/20 µg Pbs21) during the study of transmission-blocking immunity (week 7-39 post-immunisation). All IgG values (described in Table 3.6) were the arithmetic mean (from 3 mice, 2 mice and 1 mouse at week 7, 9-34 and 39 post-immunisation respectively) after subtraction from the IgG values of the control subgroup O 1 ± S. D.

3.3.4) Analysis of antigens recognised by the immune sera

P. berghei antigens

Immune sera N 2 and O 2 (Figure 3.6, lanes 8, 10 respectively)

Using Western blot analysis, Pbs21 antigen was recognised by the immune sera from the mice subgroups N 2 and O 2 (that were immunised with 5/20 and 20/20 μg of Pbs21 respectively). This protein was similar to the major polypeptide in the affinity-purified ookinete antigen preparation (Figure 3.2) and thus confirming the immunogenicity of the purified protein.

Other polypeptides were also visualised by the immune sera. A protein of an $M_r \geq 66$ kD was revealed by the sera subgroup N 2 (Figure 3.6, lane 8). The sera subgroup O 2 interacted with proteins between the M_r of 29-66 kD (Figure 3.6, lane 10).

Control sera N 1 and O 1 (Figure 3.6, lanes 4 and 6 respectively)

Using the control sera from the mice subgroups N 1 and O 1, protein of high $M_r \geq 66$ kD was detected (the nitrocellulose strip lanes 5 & 6 was placed at a slightly higher position than others during the autoradiography).

The ascitic fluid 13.1 (Figure 3.6, lane 2)

As a positive control of the assay, the MAb 13.1 recognised Pbs21 which was the most prominent protein and also polypeptides of M_r 41, 17 kD and the minor bands between the M_r range of 45-66 kD.

B. divergens merozoite antigens

The protein at M_r 21 kD was not recognised by the ascitic fluid 13.1 or any of the control and immune sera when merozoites of *B. divergens* were used as the antigen (Figure 3.6, lanes 1, 3, 5, 7 and 9 respectively). Instead, high M_r polypeptide at ≥ 66 kD was recognised by all of the antibodies and the protein of M_r range between 36-45 kD was recognised by the control sera subgroup N 1 (Figure 3.6, lane 3). However, the reaction to these proteins of *B. divergens* merozoites might be 'non-specific'.

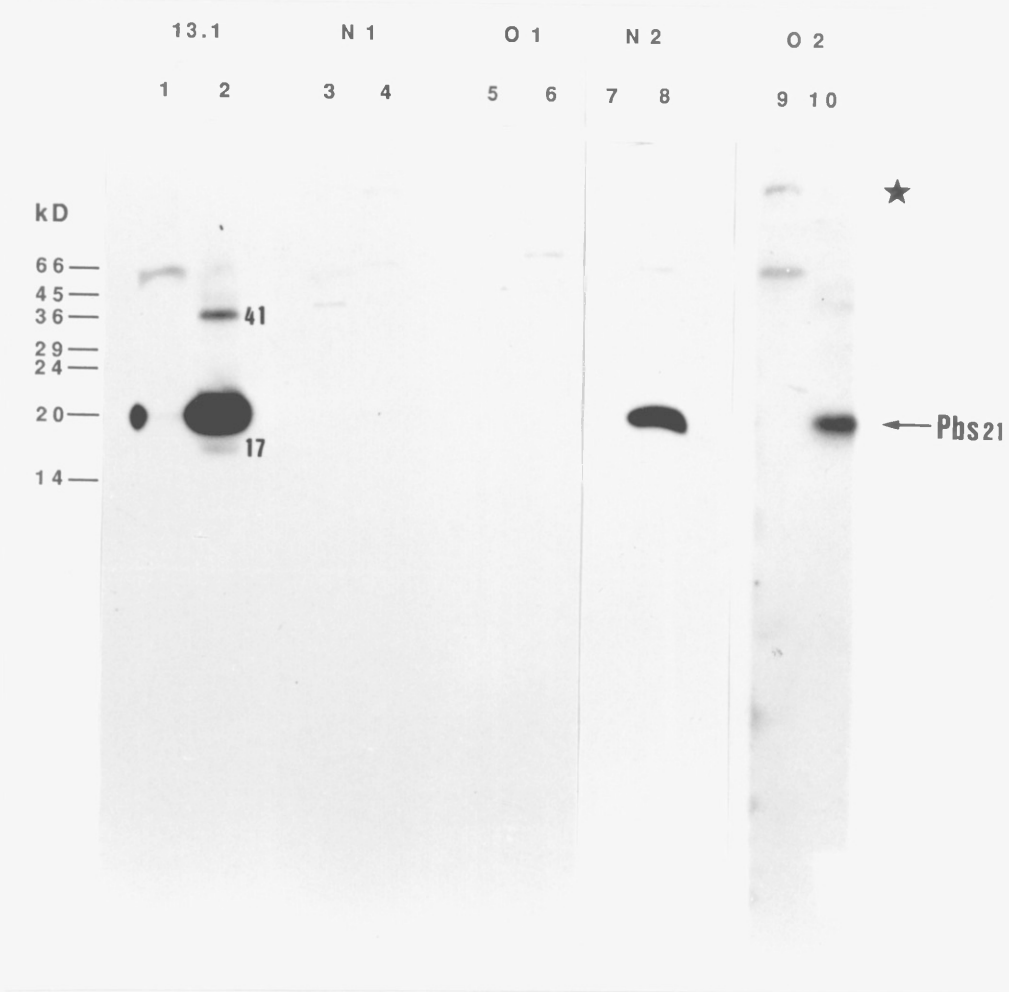


FIGURE 3.6 Immunoblot analysis of 15% reducing SDS-PAGE protein profiles of the ammonium chloride-enriched ookinetes of *P. berghei* (lanes 2, 4, 6, 8, 10) and merozoites of *B. divergens* (lanes 1, 3, 5, 7, 9) with the ascitic fluid 13.1 (lanes 1, 2), pooled control sera subgroups N 1 (lanes 3, 4), O 1 (lanes 5, 6), pooled immune sera subgroups N 2 (lanes 7, 8) and O 2 (lanes 9, 10). All strips were overlaid with ^{125}I-labelled sheep anti-mouse IgG. Molecular weights are indicated in kilodalton.

* = The boundary between the stacking and separating gels.

3.4) Discussion

Transmission-blocking immunity in murine malaria parasites has been studied in *P. berghei* (Kaushik *et al.*, 1982) and *P. y. nigeriensis* (Mendis and Targett, 1979, 1981, 1982 ; Harte *et al.*, 1985b). A successful vaccination study using purified microgamete antigen of *P. y. nigeriensis* (Pys42) was reported (Harte *et al.*, 1985c). Vaccination with 2 doses of the affinity-purified microgamete antigens (40 & 30 µg) by the I. V. or I. M. route was equally effective and did not require the co-administration of an adjuvant.

Transmission-blocking immunity in *P. berghei* was demonstrated above using the MAbs against ookinetes (Chapter 2). That immunisation with Pbs21 elicits transmission-blocking immunity in the host has already been demonstrated in the previous studies in this laboratory (Appendices 3 and 4). It could be summarised that (i) there was no considerable blockade of the transmission when the vacinees were immunised with low amount of the antigen (subgroups A 2-F 2, H 2, J 2 and L 2). Neither was the blockade significant when higher doses of the antigen were applied once (subgroups G 2 and M 2). On the contrary, the enhancement of transmission was observed (subgroups A 2, C 2, E 2, H 2 and L 2). (ii) antigenicity of Pbs21 antigen was improved by acetone precipitation (subgroup G 2 compared to I 2). (iii) induction of the immunity was considerably more effective when the vaccine was given as double doses of the acetone-precipitated antigen (subgroup I 2 compared to K 2 ; Appendix 3). (iv) route of immunisation might have an effect on blocking of transmission (subgroups K 2 compared to O 2 in the present study).

Thus, in the latest immunisation trial reported in the present study, the animals were given two doses of the vaccine I. M. without an adjuvant. The mice subgroup O 2 that were immunised twice with 20 µg of Pbs21 were found to be totally uninfective to mosquitoes. The average 'anti-Pbs21' IgG was calculated to be 182.45 µg/ml.

Persistence of transmission-blocking immunity in the mice subgroup O 2 was observed to be at least 34 weeks after the first immunisation with the final 'anti-Pbs21' IgG level of 38 µg/ml. Subsequent challenge did not boost the transmission-blocking immunity. This was determined from the level of the 'anti-Pbs21' IgG at week 30 and 34. There was no significant difference between these 2 IgG levels (using student's t-test of the transformed data ; $p < 0.5$).

Using Western blot analysis, Pbs21 was the most prominent protein detected by the immune sera from the mice subgroups N 2 and O 2. The sera also reacted with Pbs21 in ELISA. Thus, the mechanism by which the host immune system mediates suppression of transmission may be explained by antibody-mediated mechanisms which have already been shown to have an important role in suppression of transmission by the MAbs 12.1, 13.1 and 17.9 in membrane feed and passive transfer experiments (the results of Chapter 2 and Winger *et al.*, 1988).

The antibody-mediated mechanism has been suggested to be involved in the suppression of mosquito infectivity by immunising with gametes of *P. gallinaceum* (Gwadz, 1976 ; Carter *et al.*, 1979b ; Rener *et al.*, 1980). In *P. vivax*, anti-gamete rabbit sera showed suppression of gametocyte infectivity to mosquitoes (Munesinghe *et al.*, 1986). Naturally-acquired

transmission-blocking antibodies were detected in immune human sera from patients infected with *P. vivax* (Mendis *et al.*, 1987a, b and Ranawaka *et al.*, 1988). In *P. falciparum*, the sera immunoprecipitated Pfs230, Pfs48 and Pfs45 proteins from ^{125}I surface-labelling *P. falciparum* gametes (Graves *et al.*, 1988a, b ; Carter *et al.*, 1989).

In *P. yoelii*, the specific mechanism by which host immunity mediated suppression of gametocyte infectivity to mosquitoes could be by agglutinating male gametes in the mosquito guts by the immune sera and consequently preventing fertilisation (Mendis and Targett, 1982). A similar mechanism was also demonstrated using the MAbs against microgametes of *P. yoelii* (Harte *et al.*, 1985b). It is suggested that immune sera from the mice vaccinated with Pys42 could act to prevent mosquito infectivity by agglutination of microgametes (Harte *et al.*, 1985c).

In our system, it is not known which specific mechanisms would be involved in suppression of transmission or whether complement is an essential component of antibody action. Having demonstrated that IgG1 was the predominant immunoglobulin isotype in the immune sera from the mice subgroups N 2 and O 2, it may be speculated that complement-dependent antibody-mediated mechanism is not involved in the suppression of transmission. Murine IgM, IgG2a, IgG2b have been reported to be able to activate the classical pathway of complement, whereas IgA, IgE and most IgG1 antibodies cannot (Ey *et al.*, 1979 ; Hirayama *et al.*, 1982).

Transmission-blocking immunity is suggested to be stage-specific (Carter *et al.*, 1988). Although partial protection against the asexual stages induced by the immunisation with gametes of *P. knowlesi* (Gwadz and Green, 1978) and *P. yoelii* (Mendis and Targett, 1979) was reported, it was due to the presence of the asexual forms rather than a cross-protection induced by the gametes (Mendis and Targett, 1982). The results presented here also showed that there was no significant protection (using proportion analysis ; $p < 0.5$) against the asexual erythrocytic forms nor gametocytes in any of the vaccinated animals.

It is noteworthy that a transmission-enhancing effect of mosquito infectivity was also observed in our previous studies (Appendices 3 & 4 ; subgroups C 2, E 2, H 2 and L 2). Using ELISA, the 'anti-Pbs21' IgG level was determined to be in the range of 31-36 µg/ml (L. A. Winger, pers. comm.). The enhancement of transmission in our study is not yet clear and needs further investigation.

Implicit evidence of antibody-mediated enhancement of plasmodial development has been described in a simian malaria system. *P. cynomolgi* infections from Rhesus monkeys with a past history of *P. knowlesi* were significantly more infectious to mosquitoes than from immunologically naive animals (de Arruda-Mayr *et al.*, 1979). A comparable phenomenon has been described in another phase of the life cycle of the malaria parasite, viz., antibody-mediated enhancement of asexual blood infections of *P. berghei* in mice following immunisation (Jerusalem *et al.*, 1971). Enhancement of the number of exoerythrocytic schizont by low concentrations of

the MAbs against circumsporozoite protein of *P. yoelii* was also observed (Mazier *et al.*, 1988). However, the mechanism of enhancement in these reports might be different from our system.

Enhancement of transmission has also been noted in *P. falciparum* and *P. vivax* by some of the immune human sera (Graves *et al.*, 1988a and Ranawaka *et al.*, 1988). Recently, it is reported that both blocking and enhancing of transmission could be mediated by a single MAb against the same target epitope on gametes of *P. vivax* (Mendis *et al.*, 1987b ; Peiris *et al.*, 1988). In the human sera, however, such effects might be mediated by antibodies against the same or different epitopes (Mendis *et al.*, 1987b and Peiris *et al.*, 1988).

An alternative type of immune effector mechanism mediated independently by T-cells was shown to operate in the transmission-blocking immunity of *P. y. nigeriensis*. Passive transfer of immune T cells from the mice vaccinated with microgametes of *P. y. nigeriensis* into naive recipients was reported to reduce transmission of a subsequent infection of the parasite by 95% (Harte *et al.*, 1985a). Such immunity also reduced the numbers of circulating gametocytes during the infection.

Antibody-independent cellular mechanisms of transmission-blocking immunity may also have an important role in human malaria *P. falciparum*. T-cell proliferation could be induced *in vitro* from peripheral blood of non-exposed donors by gametes of *P. falciparum* (Good *et al.*, 1987). However, this mechanism of transmission-blocking immunity in *P. falciparum* remains to be further investigated.

Unfortunately, knowledge of cell-mediated immunity against the sexual stages of *P. berghei* is still very limited and needs further investigation.

CHAPTER 4

EPITOPE ANALYSIS OF Pbs21

4.1) Introduction

As described in chapter 2, Mabs against ookinetes of *P. berghei* were produced (Winger *et al.*, 1988) and three such antibodies (12.1, 13.1 and 17.9) were extensively studied. In membrane feed and passive immunisation experiments, the Mabs were demonstrated to block parasite development in the mosquito vector *Anopheles stephensi* at a certain dilution by greater than 93%. In addition, all the Mabs also blocked parasite development *in vitro*. Using SDS-PAGE and immunoblot techniques, the Mabs 12.1 and 13.1 recognised Pbs21 under both non-reducing and reducing conditions. By contrast, the Mab 17.9 recognised only the non-reduced Pbs21 protein.

Transmission-blocking immunity in the host elicited by the immunisation with Pbs21 was demonstrated in Chapter 3. In this Chapter, the immunological and biochemical structures of the antigen will be characterised i.e. the number of target epitope(s) recognised by the MAbs, the conformational structure of the epitopes and the role of carbohydrate in the immunogenicity of the epitopes.

The number of epitopes in a protein molecule can be investigated using MAbs in a two-site immunoradiometric assay (IRMA). This method was successfully used to study the number of epitopes in the circumsporozoite protein (CSP) of *P. falciparum* (Zavala *et al.*, 1982 & 1983) and was extended for field work study to detect the infection rate in mosquitoes by Collins *et al.*, (1984). A similar assay was also applied to study the number of epitopes on the gamete surface antigens (Pfs48/45) of *P. falciparum* (Carter *et al.*, 1985).

Burkot *et al.*, (1984a, b) developed a two-site Enzyme-Linked Immunosorbent Assay (ELISA) to detect the number of *P. falciparum* sporozoites in infected mosquitoes. The method was proved to be as useful, sensitive, specific and reliable as IRMA or even more advantageous for field work in terms of the use of non-radioactive materials. It was then widely applied to detect and identify the sporozoite-infected mosquitoes from different species of *Plasmodium* e.g. *P. falciparum* (Burkot *et al.*, 1984a, b ; Ferreira *et al.*, 1986 ; Beier *et al.*, 1987), *P. vivax* (Ferreira *et al.*, 1986) and *P. malariae* (Collins *et al.*, 1988). The advantage of the use of nonradioactive materials to detect the number of sporozoites in *P. falciparum* was further modified by the use of biotin-streptavidin system in two-site ELISA by Verhave *et al.*, (1988).

Not only two-site ELISA but also the competitive inhibition analysis can be applied to study the immunological structure of a protein. The method using ^{125}I-labelling antibodies was successfully shown to detect the multiple epitopes on the murine leukemia virus protein (Stone and Nowinski, 1980). It was further developed by the use of enzyme-immunoassays to measure natural immune response to particular epitopes on protein antigens (Cruise *et al.*, 1981 ;

Budkowska *et al.*, 1987). Recently, a similar assay was applied to the study of epitope recognised by naturally occurring antibodies on Pfs48/45 proteins (Graves *et al.*, 1988b).

Since murine antibodies have a reputation for being easily damaged by radioiodination (Goding, 1983) and the use of radioactive material is hazardous, two-site and competitive ELISA were developed to investigate the number of epitopes recognised by the transmission-blocking MAbs in Pbs21. The involvement of post-translational modification i.e. glycosylation to transmission-blocking epitopes in Pbs21 was further described using two-site ELISA.

4.2) Materials and Methods

4.2.1) Epitope analysis of Pbs21

The number of epitopes recognised by the transmission-blocking MAbs in Pbs21 was studied using competitive ELISA. Two-site ELISA was further assessed to demonstrate whether an epitope is repeated. Throughout the study for both competitive and two-site ELISA, double affinity-purified Pbs21 was used as a matter of convenience (since it was prepared *en masse* for amino acid sequencing in co-operation with Prof. H.R. Morris ; Department of Biochemistry, Imperial College).

The methods used to study the number of epitopes in Pbs21 was modified from Burkot *et al.*, (1984a, b), Zavala *et al.*, (1982 & 1983) and Carter *et al.*, (1985).

1) Precipitation of the immunoglobulin G from the monoclonal antibodies using saturated ammonium sulphate and demonstration of its immunogenicity

IgG from one ml of each MAb (12.1, 13.1 and 17.9) was enriched using saturated ammonium sulphate (as previously described in section 3.2.1.1). At the final step, the IgG pellet was dissolved in one ml of 0.9% NaCl and dialysed extensively overnight at 4°C against 0.9% NaCl. After precipitation, the immunological activity of the IgG was assessed using indirect one-site ELISA (as described in section 2.2.4.6 a)

Wells of microtitre plates were pre-coated with 50 µl of the affinity-purified Pbs21 in a doubling dilution series (2.000-0.125 µg/ml). Residual binding sites of the wells were blocked with 5% NGS-PBS before the ammonium sulphate-precipitated IgG (12.1, 13.1 and 17.9) at 20 µg/ml was added. After 3 washings in PBS-Tween, 100 µl of 1:1000 dilution of goat anti-mouse IgG conjugated to the enzyme alkaline phosphatase was pipetted into each well. Following washing as described, the substrate was added and the $O.D_{414}$ was measured. It should be noted here that the incubation time before the O.D. measurement in individual experiments was assessed as appropriate so as to give a significant reading of the specific to non-specific signal ratio. In the present assay, the O.D. was measured after 10 min incubation at 37°C.

2) Conjugation of the enzyme Horseradish Peroxidase to the antibodies

The enzyme is coupled to the protein via its carbohydrate moiety by the two-step glutaraldehyde procedure (Voller and Bidwell, 1986). Peroxidase activity is normally detected with O-phenylenediamine (Mayer and Walker, 1987). An alternative substrate is 2, 2'-azino-di [3-ethyl-benzthiazoline sulphonate] (ABTS) which is reported to have many advantages over O-phenylenediamine in terms of the stability of the substrate solution and of the bright green reaction product (Mayer and Walker, 1987).

For each MAb-HRP conjugate, 5 mg of the enzyme HRP (Sigma ; P-8357) was dissolved in 200 µl of 0.1 M NaH_2PO_4/Na_2HPO_4 pH 6.8 buffer containing 1.25% glutaraldehyde

(Sigma ; G-5882) to activate the enzyme. The mixture was left at room temperature overnight in the dark and subsequently dialysed against 0.9% NaCl at 4°C for 18 h. After dialysis, the total volume of the enzyme solution was made up to one ml with 0.9% NaCl.

The concentration of the IgG solution (see section 4.2.1.1) was determined by measuring the optical density at 280 nm ($O.D_{280}$ of 1 mg/ml IgG = 1.4 in a cell with 10 mm path length; Goding, 1983) and 2.5 mg of the antibody (in 1 ml of 0.9% NaCl) was added to the HRP solution (5 mg of the enzyme in 1 ml solution). Following the addition of 100 µl of 1 M carbonate/bicarbonate buffer pH 9.6, the mixture was left in the dark at 4°C overnight on an end-to-end rotator.

After the addition of 200 µl of 0.2 M L-lysine (Sigma ; L-5626) to stop the HRP conjugation, the solution was left at room temperature for 2 h before dialysis against PBS at 4°C overnight. The conjugate was subsequently precipitated using saturated ammonium sulphate as previously described (section 3.2.1.1) before dissolving in 0.5 ml of PBS and extensive dialysis against PBS at 4°C. For long-term storage, the conjugate was mixed with an equal volume of glycerol as cryoprotectant then kept at -20°C.

3) Assessment of the antibody-HRP conjugates

Direct one-site ELISA was used to assess the activity of the antibody-enzyme conjugates. Duplicate wells of the microtitre plates (FLOW Lab., Cat. no. 76-381-04) were pre-coated with 50 µl of a doubling dilution series of Pbs21 (4.000-0.008 µg/ml for 12.1 and 17.9 assays and 2.000-0.008 µg/ml for 13.1 assay). The plates were left overnight at room temperature in a moist chamber. Residual binding sites of the plates were saturated with 200 µl/well of 5% NGS-PBS at 37°C for an hour. The wells were then washed 3 times with 200 µl of PBS-Tween before they were incubated with 100 µl of antibody-HRP conjugates (at 1:1000 dilution for 12.1 & 17.9 conjugates and 1: 100 for 13.1 conjugate in PBS-Tween). After an hour at 37°C, the wells were washed as described, before the addition of 100 µl of the substrate (Kirkegaard & Perry Labs) which was a mixture of 2,2'-azino-di [3-ethyl-benzthiazoline sulphonate] (ABTS ; reagent A) and hydrogen peroxide (reagent B) in the ratio of 1:1. The reaction was left at 37°C for 15-60 min before the optical density at 405 nm was read.

As negative controls, wells in which the antigen was omitted were incubated with the enzyme-antibody conjugates and subsequently the substrate.

4) Competitive Enzyme-Linked Immunosorbent assay

The number of different epitopes in Pbs21 was demonstrated using competitive ELISA (illustrated in Figure 4.1). The plates were coated with an excess amount of the antigen. Unlabelled antibody is allowed to bind before the labelled antibody is added to increase the sensitivity of the assay. Binding of the enzyme-labelled antibody to the antigen will be prevented only by other antibodies which recognise the same epitope in the protein. The signal in the assay is then inversely related to the concentration of the antibody competitor. The method had been assessed in chequerboard titration so as to obtain the optimum conditions for the present study.

Having established that the antigen at the concentration of 2 µg/ml was in excess (from the experiment described in section 4.2.1.3), wells of the microtitre plates were pre-coated with 50 µl of 2 µg/ml of Pbs21 and left overnight at room temperature in a moist chamber. The wells were washed and non-specific binding sites were saturated as described above.

As an antibody competitor, 50 µl of the ammonium sulphate-precipitated IgG in 1% NGS-PBS was pipetted into each well in a doubling dilution series (0-10 µg/ml). Following an incubation at 37°C for 1 1/2 h, 50 µl of the HRP-conjugates at a dilution of 1:500 for 12.1, 17.9 and Pb 3.28.1 and 1:50 for 13.1-HRP were added. An anti-circumsporozoite protein of *P. berghei*, Pb 3.28.1 (a kind gift from Dr. R. A. Wirtz ; Walter Reed Army Institute of Research) was used as a negative control of the assay (the suggested working dilution of the enzyme-antibody conjugate was 1:1000). Therefore, the final concentration of the conjugates was 1:1000 for 12.1, 17.9 & Pb 3.28.1 and 1:100 for 13.1. The plates were incubated for another 1 1/2 h at 37°C before the wells were washed, the substrate was added and the $O.D_{405}$ was measured after 5-35 min incubations at 37°C. As negative controls, (i) wells without the antigen were incubated with the competitors and the HRP-conjugated to Pbs21-specific MAbs or (ii) wells pre-coated with the antigen were incubated with the competitors and Pb 3.28.1-HRP.

5) Two-site Enzyme-Linked Immunosorbent Assay

The number and the repetitive nature of each epitope in Pbs21 was further demonstrated using two-site ELISA (illustrated in Figure 4.2). Plates were coated with capture antibody. Following the addition of antigen, the enzyme-conjugated homologous or heterologous Mab was then applied. The addition of heterologous antibody was to examine the number of unique epitopes while the homologous antibody was to examine the repeated nature of an epitope.

The method modified from Burkot *et al.*, (1984a, b) and Carter *et al.*, (1985) had been assessed in chequerboard titration so as to obtain the optimum conditions for the present study. Wells of the microtitre plates were pre-coated with 50 µl of a 20 µg/ml solution of the ammonium sulphate-precipitated 12.1, 13.1 and 17.9 proteins. The plates were left overnight

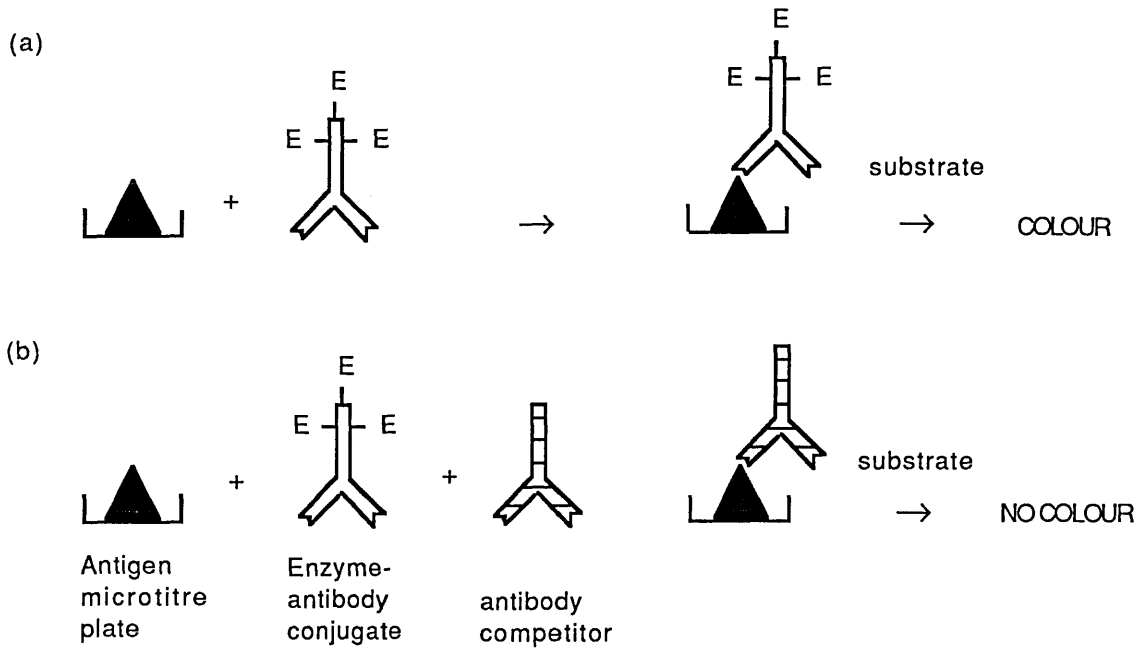


FIGURE 4.1 Competitive Enzyme-Linked Immunosorbent Assay for detection of the number of epitopes in Pbs21.

Competition of binding to the antigen can be carried out using the antigen-coated microtitre plate where a fixed level of enzyme-antibody conjugate competes with varying levels of antibody competitor directed against the same epitope :- (a) in the absence of the competitor, the enzyme-antibody conjugate binds, (b) in the presence of the competitor, binding of the enzyme-antibody conjugate is inhibited. If the 2 antibodies recognise different epitopes, binding of the enzyme-antibody conjugate to the antigen will not be inhibited.

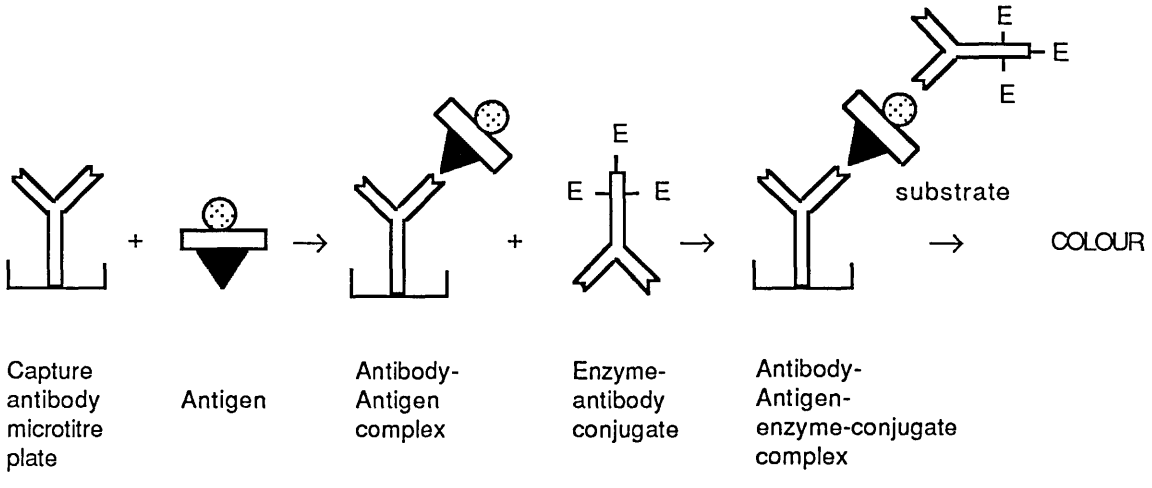


FIGURE 4.2 Two-site Enzyme-Linked Immunosorbent Assay for detection of the number of epitopes in Pbs21.

Antibody on the solid phase binds the antigen in the sample which is subsequently detected using another MAb (that recognises different epitope on the antigen) conjugated to the enzyme.

at room temperature in a moist chamber. The wells were washed 3 times with 200 µl of 0.5% NGS-PBS and the residual binding sites of the plates were then saturated with 200 µl/well of 5% NGS-PBS at 37°C for an hour. Following washing as described, the wells were incubated with 50 µl of a doubling dilution series of Pbs21 in 1% NGS-PBS. For 12.1-HRP and 17.9-HRP assays, the antigen was varied from 4.000-0.008 µg/ml. For 13.1-HRP assay, the antigen was varied from 2.000-0.063 µg/ml. This modification was due to shortage of the enzyme conjugate (as the optimum dilution was 1:100). The plates were then left at 37°C for 2 h.

After washing with PBS-Tween, the wells were incubated with 100 µl of either homologous or heterologous HRP-conjugates (1:1000 dilution for 12.1 and 17.9 HRP conjugates and 1:100 for 13.1 HRP conjugate in PBS-Tween). Following an incubation at 37°C for 2 h, the wells were washed as above before the substrate was added. The $O.D_{405}$ was read after the plates were left at 37°C for 20 min.

As negative controls, (i) wells pre-coated with 50 µl of 20 µg/ml of an irrelevant MAb Pb 3.28.1 were incubated with 50 µl/well of a doubling dilution series of the antigen (4.000-0.008 µg/ml for 12.1-HRP and 17.9-HRP or 2.000-0.063 µg/ml for 13.1-HRP assays) and following with the HRP-conjugated to Pbs21-specific MAbs or (ii) the antibody-coated wells were loaded with the antigen and subsequently with Pb 3.28.1-HRP.

4.2.2) Study of the conformational structure of the transmission-blocking epitopes

The preliminary results in Chapter 2 have already suggested that the MAbs 12.1 and 13.1 recognised Pbs21 on both non-reducing and reducing SDS-PAGE. The MAb 17.9 reacted with only the non-reduced form of the antigen. Here in this Chapter, the conformational structure of the epitopes was extensively studied. To demonstrate whether the epitopes recognised by the transmission-blocking MAbs in Pbs21 were conformation-dependent, the protein was subjected to reduction using 2-mercaptoethanol (2-ME) compared to dithiothreitol (DTT) which was more advantageous than 2-ME in that DTT was odourless and did not tend to auto-oxidise (Hames and Rickwood, 1981). Following alkylation with iodoacetamide to prevent renaturation of the polypeptide chain, the immunological reactivity of Pbs21 was examined using both immunoblot techniques and indirect one-site ELISA.

1) Immunoblot assay

The ammonium chloride-enriched ookinete parasite pellet was resuspended with an equal volume of double strength of modified non-reducing Laemmli buffer (Laemmli, 1970) (the final concentration of the reagents was 0.004% bromophenol blue, 0.001 M EDTA, 10% glycerol, 0.02 M iodoacetamide, 0.002 M PMSF, 5% SDS and 0.01 M Tris pH 6.8). Reduction was performed with either 0.05 M DTT or 5% 2-ME. The solution was boiled for 3 min and the proteins were subjected to electrophoresis using 15% SDS-PAGE with 5% stacking gel before blot-transfer to nitrocellulose membrane as previously described (section 2.2.5.3).

The nitrocellulose was cut off into strips before the non-specific binding sites were blocked. As a primary antibody, the ascitic fluids 12.1, 13.1 and pooled polyclonal monospecific mouse sera (PMS) subgroup N 2 that were immunised twice with 5/20 µg of Pbs21 (described in section 3.2.3.2) at 1:100 were incubated with the nitrocellulose strips. As negative control, NMS was also included. Following washing off the unbound first antibody, ^{125}I-labelled sheep anti-mouse IgG was then added as a second antibody and the autoradiography was finally performed as described above.

2) Enzyme-Linked Immunosorbent Assay (ELISA)

In this Chapter, indirect one-site ELISA was developed in an attempt to establish a quicker and simpler assay (compare to SDS-PAGE and immunoblot analysis) for the study of conformational structure of Pbs21. The protein was subjected to reduction and alkylation and following with the ELISA. Binding efficacy of the reduced-alkylated protein to the microtitre plate was also investigated compared to the control preparation.

a) Reduction and carboxymethylation of the protein antigen

Reduction of the protein was performed using either 0.01 M dithiothreitol (DTT ; Fries, H. C. W. pers. comm.) or 5% 2-mercaptoethanol (2-ME ; modified from Laemmli, 1970). To prevent the reassociation of the SH-groups formed during the reaction, carboxymethylation was then performed using 0.02 M iodoacetamide.

Double affinity-purified Pbs21 at the concentration of 4 µg/ml in 200 µl of PBS was added to 20 µl of 0.1 M DTT (Sigma ; D-9779) or 10 µl of 2-ME (Sigma ; M-3148). After incubation at room temperature for an hour, 22 µl of 0.2 M iodoacetamide (Sigma ; I-6125) was added and the mixture was incubated in the dark at room temperature for another hour. An equal amount of iodoacetamide was also included in the control preparation. Excess reagents were then removed by overnight dialysis against PBS at 4°C.

b) The assay

Using ELISA (as described in section 2.2.4.6 a), the immunological reactivity of Pbs21 after reduction and alkylation was examined compared to the control preparation (non-reduced form of Pbs21).

The reduced-alkylated Pbs21 (described as above) and the control preparations of the protein were each diluted with an equal volume of PBS to produce a final concentration of proteins to 2 µg/ml. Wells of the microtitre plates were pre-coated with a doubling dilution series of the antigen (2.000-0.016 µg/ml) and left overnight at room temperature. Residual binding sites of the plates were blocked as above before the ascitic fluid 12.1, 13.1 and 17.9 was added at 1:500 dilution. The plates were left at 37°C for 2 h. Following the addition of goat anti-mouse IgG-alkaline phosphatase conjugate and the substrate p-nitrophenyl phosphate, the $O.D_{414}$ was finally measured.

Pooled polyclonal monospecific sera obtained from 3 mice each immunised with 5 µg and boosted once with 20 µg of Pbs21 (subgroup N 2 mice described in Chapter 3) has

already been shown to recognise both non-reduced and reduced forms of Pbs21 in immunoblot assay (section 4.2.2.1). As a positive control of ELISA, the wells containing a doubling dilution series of either the contol or reduced-alkylated preparations of Pbs21 (2.000-0.016 µg/ml) were incubated with 1:500 dilution of the sera and the rest of the assay was as described above. Normal mouse sera were used as a negative control of ELISA.

3) Determination of the binding efficiency of the reduced-alkylated protein to the microtitre plate

To examine the efficiency of the reduced-alkylated preparations of Pbs21 in binding to the microtitre plates compared to the control protein, 200 µl of the ^{125}I-labelled double affinity-purified antigen (at 44,766 cpm/µg of TCA-precipitable protein) was subjected to reduction and alkylation as described in section 4.2.2.2 a. After overnight dialysis against PBS, 50 µl of the protein solution was added at a doubling dilution series (1.150-0.018 µg/ml) into duplicate wells of microtitre plates then left at room temperature overnight.

Following 3 washings with PBS, an individual well was cut off and the radioactivity was measured using a γ - counter (Mini-instruments Company, Mini-assay Type 6-20).

4.2.3) Study of the post-translational modification of the transmission-blocking epitopes in Pbs21

Mild periodate oxidation at acid pH modifies the carbohydrate moiety of a protein by cleaving the vicinal hydroxyl groups without affecting the polypeptide backbone (Woodward *et al.*, 1985). This method is carried out in combination with two-site ELISA to study the role of carbohydrate for the immunoactive epitopes in Pbs21.

1) Study of the 12.1 epitope

Wells of the microtitre plates were pre-coated with 50 µl of 20 µg/ml of the ammonium sulphate-precipitated 13.1 protein. The plates were washed before non-specific binding sites were saturated and the antigen was added in a doubling dilution series (2.000-0.063 µg/ml) as described.

Following 5 washings with PBS-Tween, the wells were washed twice more with 0.05 M sodium acetate pH 4.5. Bound protein was then exposed to 100 µl/well of 0.01 M sodium periodate (Aldrich Chem.) in 0.05 M sodium acetate for one hour at room temperature in the dark. As a negative control, the protein antigen was incubated with 100 µl/well of sodium acetate buffer. After washing the wells twice with sodium acetate and once with PBS, the aldehyde groups formed during the reaction were blocked by incubation with 1% glycine in PBS for 1/2 h at room temperature. Both periodate and glycine solutions were freshly prepared prior to use.

The wells were washed 5 times with PBS-Tween before 100 µl of 1:1000 dilution of the enzyme 12.1-HRP was added. The plates were left at 37°C for 2 h. Following 3 washings

with PBS-Tween, the wells were incubated with the substrate and the $O.D_{405}$ was measured after 20 min incubation at 37°C.

2) Study of the 13.1 epitope

Wells of the microtitre plates were pre-coated with 20 µg/ml of the ammonium sulphate-precipitated 12.1 protein. Processing of the assay was as described above for the study of 12.1 epitope. As an antibody-enzyme conjugate, 1:100 dilution of 13.1-HRP was added. Following washing as described, the substrate was added and the $O.D_{405}$ was finally measured.

3) Study of the 17.9 epitope

Using direct one-site ELISA, the effect of periodate oxidation on the 17.9 epitope was also determined. Wells of the microtitre plates were pre-coated with 50 µl of a doubling dilution series of Pbs21 (2.000-0.063 µg/ml) before the residual binding sites were saturated.

The following assay was processed as described above before the wells were incubated with 100 µl of 1:1000 of 17.9-HRP conjugate. After the addition of the substrate, the $O.D_{405}$ was finally measured.

4.3) Results

4.3.1) Assessment of the immunological activity of the ammonium sulphate-precipitated IgG and the activity of the enzyme-antibody conjugates

Before enzyme conjugation, the immunological activity of the ammonium sulphate-precipitated IgG was determined using indirect one-site ELISA (Figure 4.3). A dose-response curve was observed when the antigen was varied from 2.000-0.125 µg/ml using 20 µg/ml of the 12.1, 13.1 and 17.9 IgG.

After enzyme conjugation, activity of the HRP conjugates was assessed by chequerboard titration in direct one-site ELISA (data not shown). Having established that the optimum dilution of 12.1-HRP and 17.9-HRP was 1:1000 and 13.1-HRP was 1:100, titration of the enzyme-antibody conjugates using the optimum dilutions of the conjugates against Pbs21 in ELISA is shown (Figures 4.4 A-C). A concentration-dependent curve is described when the antigen was varied from 0.008-4.000 µg/ml using 12.1-HRP and 17.9-HRP (Figure 4.4 A and C respectively). The activity of 13.1-HRP against Pbs21 was relatively lower than 12.1-HRP and 17.9-HRP (Figure 4.4 B). These dilutions of the HRP-conjugates were used in all of the following assays.

4.3.2) Epitope study of Pbs21

1) Competitive Enzyme-Linked Immunosorbent Assay

The effect of competition between homologous and heterologous MAbs in a one-site assay was investigated to determine the number of epitopes recognised by the transmission-blocking MAbs in Pbs21 antigen. The results are summarised in Table 4.1.

a) Heterologous monoclonal antibody assays

The MAbs 12.1, 13.1 and 17.9 at concentrations of 0-10 µg/ml were added in competition with the heterologous HRP-conjugates in a criss-cross assay. The results of each reciprocal competitive assay are shown in Figures 4.5 A (for 12.1-13.1), B (for 12.1-17.9) and C (for 13.1-17.9) respectively. As negative controls, wells without the antigen were incubated with competitors and enzyme-antibody conjugates. The results are indistinguishable from zero in all of the assays, therefore, they are not included in the illustrations.

MAbs 12.1-13.1

It was clearly demonstrated that 12.1 competitor at a concentration as high as 10 µg/ml did not inhibit the binding of 13.1-HRP to Pbs21 (Figure 4.5 A ; denoted as 12.1-13.1HRP in dotted line). The result was also confirmed in the reciprocal situation (Figure 4.5 A ; denoted as 13.1-12.1HRP in solid line). In fact, binding of both enzyme conjugates to the antigen in the presence of heterologous competitors above 0.625 µg/ml was enhanced. This is suggested that binding of an antibody to its epitope could induce conformational changes in the antigen resulting in the enhancement of binding to a second antibody. Thus, it is concluded that these 2 MAbs recognised 2 distinct epitopes in Pbs21 antigen.

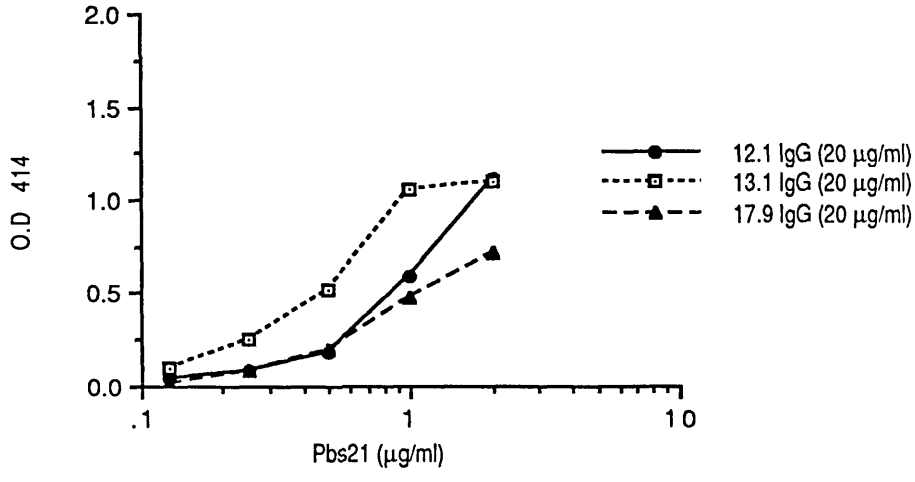


FIGURE 4.3 Indirect one-site ELISA demonstrating the immunological acitivity of 12.1, 13.1 and 17.9 IgG against Pbs21 before the enzyme HRP conjugation. Wells pre-coated with a doubling dilution series of the antigen (2.000-0.125 µg/ml) were incubated with 20 µg/ml of the ammonium sulphate-precipitated MAbs, subsequently with goat anti-mouse IgG-alkaline phosphatase conjugate and finally with the substrate as described in section 4.2.1.1.

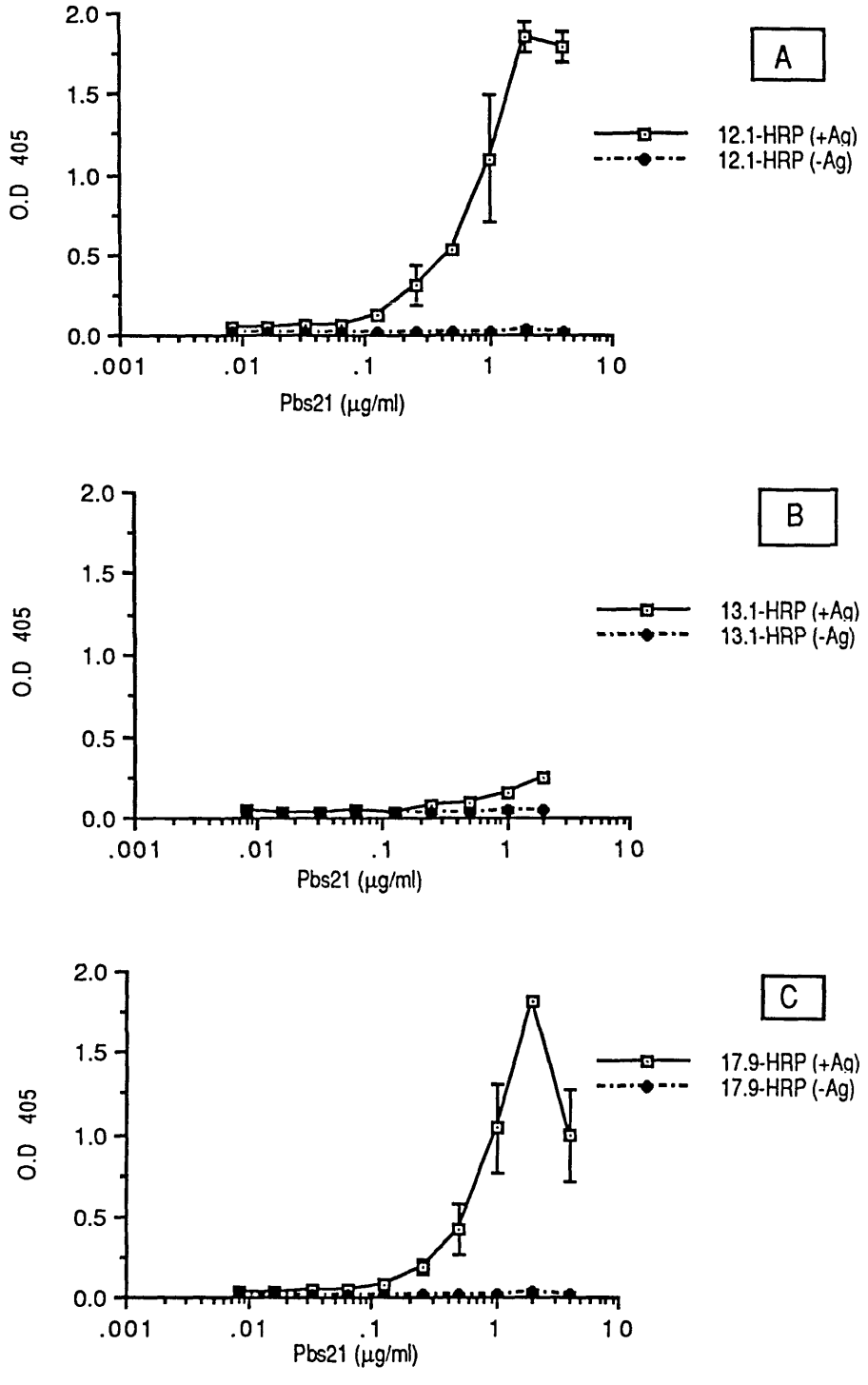


FIGURE 4.4 Direct one-site ELISA demonstrating the activity of enzyme-antibody conjugates against Pbs21. Wells pre-coated with a doubling dilution series of the antigen (4.000-0.008 µg/ml for 12.1 & 17.9 or 2.000-0.008 µg/ml for 13.1 assays) were incubated with a certain dilution of the antibody-HRP conjugates :- (A) 12.1-HRP at 1:1000, (B) 13.1-HRP at 1:100 and (C) 17.9-HRP at 1:1000. All values given are the mean of duplicate samples ± S. D. which is shown by error bars. The antigen is omitted for the negative controls.

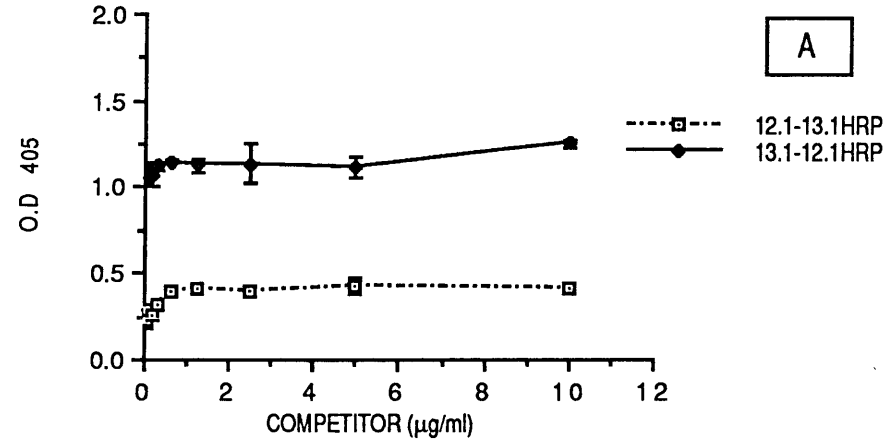
TABLE 4.1 Summary of competitive Enzyme-Linked Immunosorbent Assay.

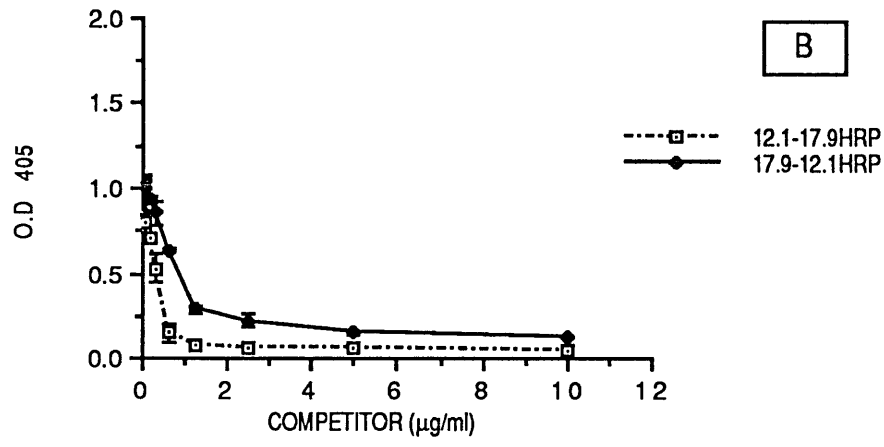
Competitor / Enzyme-antibody conjugate	12.1	13.1	17.9
12.1 - HRP	+	-	+
13.1 - HRP	-	+	+
17.9 - HRP	+	+	+

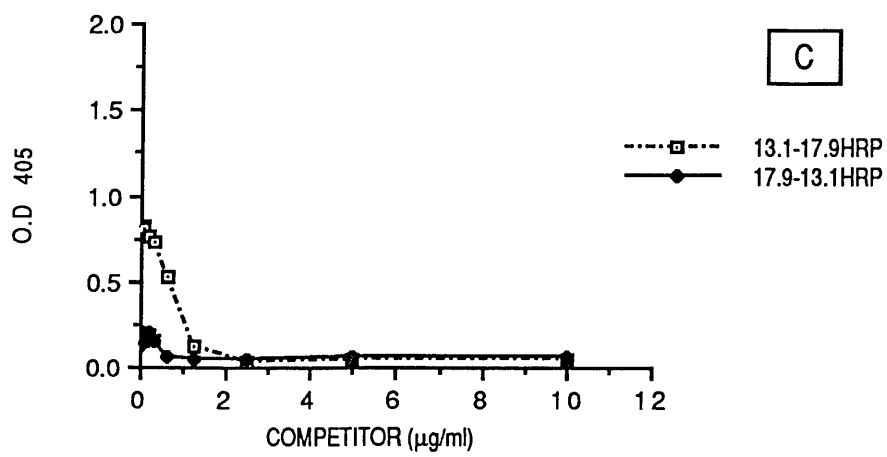
\+ = A competitive curve is demonstrated i.e. the 2 MAbs recognise **the same** or closely located epitope(s) in the protein molecule.

\- = A competitive curve is not demonstrated i.e. the 2 MAbs recognise **different** epitopes in the protein molecule.

FIGURE 4.5 Competitive ELISA demonstrating the number of epitopes recognised by heterologous MAbs in Pbs21. Wells pre-coated with 2 µg/ml of the antigen were incubated with 0-10 µg/ml of competitors and subsequently with heterologous enzyme-antibody conjugates (1:1000 dilution for 12.1-HRP and 17.9-HRP and 1:100 dilution for 13.1-HRP) :- (A) 12.1 was used as a competitor and 13.1 as an enzyme-antibody conjugate (denoted as 12.1-13.1HRP in dotted line) and their reciprocal assay (denoted as 13.1-12.1HRP in solid line), (B) 12.1 was used as a competitor and 17.9 as an enzyme-antibody conjugate (denoted as 12.1-17.9HRP in dotted line) and their reciprocal assay (denoted as 17.9-12.1HRP in solid line), (C) 13.1 was used as a competitor and 17.9 as an enzyme-antibody conjugate (denoted as 13.1-17.9HRP in dotted line) and their reciprocal assay (denoted as 17.9-13.1HRP in solid line). All values given are the mean of duplicate samples ± S. D.


A
12.1-13.1HRP
13.1-12.1HRP
O.D 405
COMPETITOR (µg/ml)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12


B
12.1-17.9HRP
17.9-12.1HRP
O.D 405
COMPETITOR (µg/ml)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12


C
13.1-17.9HRP
17.9-13.1HRP
O.D 405
COMPETITOR (µg/ml)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12

MAbs 12.1-17.9

In contrast to 12.1-13.1 assays, binding of 17.9-HRP to Pbs21 was inhibited by the presence of 12.1 IgG as the competitive curve showed 50% inhibition (determined from 50% reduction of the $O.D_{405}$ in the absence of 12.1 competitor) when the concentration of 12.1 competitor was between 0.313-0.625 µg/ml (Figure 4.5 B ; denoted as 12.1-17.9HRP in dotted line). Binding of 17.9-HRP to the antigen was totally abolished when the concentration of 12.1 competitor was above 2.5 µg/ml (Figure 4.5 B ; dotted line). The same phenomenon was also observed in the reciprocal situation (Figure 4.5 B ; denoted as 17.9-12.1HRP in solid line). The competitive curve showed 50% inhibition when the concentration of 17.9 competitor was between 0.625-1.25 µg/ml. Binding of 12.1-HRP to the antigen was totally inhibited when the concentration of 17.9 IgG was above 5 µg/ml (Figure 4.5 B ; solid line).

MAbs 13.1-17.9

Similar competitive results were obtained with the MAbs 13.1 and 17.9 assays. Binding of 17.9-HRP to the antigen was competed by 13.1 IgG (Figure 4.5 C ; denoted as 13.1-17.9HRP in dotted line). At a concentration of 13.1 competitor between 0.625-1.25 µg/ml, 50% inhibition of the binding of 17.9-HRP to the antigen was recognised. At the concentration of 13.1 IgG above 2.5 µg/ml, binding of 17.9-HRP to the antigen was successfully abolished. The results were confirmed in the reciprocal assay (Figure 4.5 C ; denoted as 17.9-13.1HRP in solid line). The competitive curve also showed similar characteristics as described above.

b) Homologous monoclonal antibody assays

The MAbs 12.1, 13.1 and 17.9 at the concentrations of 0-10 µg/ml were added in competition with its homologous enzyme-antibody conjugates (at 1:1000 dilution for 12.1 and 17.9-HRP and 1:100 for 13.1-HRP).

Competition with its own homologue was clearly observed in all of the assays (Figures 4.6 A, B and C for 12.1-12.1HRP, 13.1-13.1HRP and 17.9-17.9HRP assays respectively). It is common in all of the assays that at the concentration of between 0.313-0.625 µg/ml of the IgG competitor, binding of the enzyme-conjugates to Pbs21 was inhibited by 50%. At the concentration of competitors above 2.5 µg/ml, binding of the enzyme conjugates to the antigen was sucessfully abolished.

c) Assay specificity

To test the specificity of the assay, an irrelevant enzyme-antibody conjugate i.e. Pb 3.28.1-HRP was used in competition with 12.1, 13.1 and 17.9 IgG. The results are shown in Figures 4.7 A (for 12.1-Pb 3.28.1 HRP), B (for 13.1-Pb 3.28.1 HRP) and C (for 17.9-Pb 3.28.1 HRP) respectively. The results are indistinguishable from zero in all of the assays.

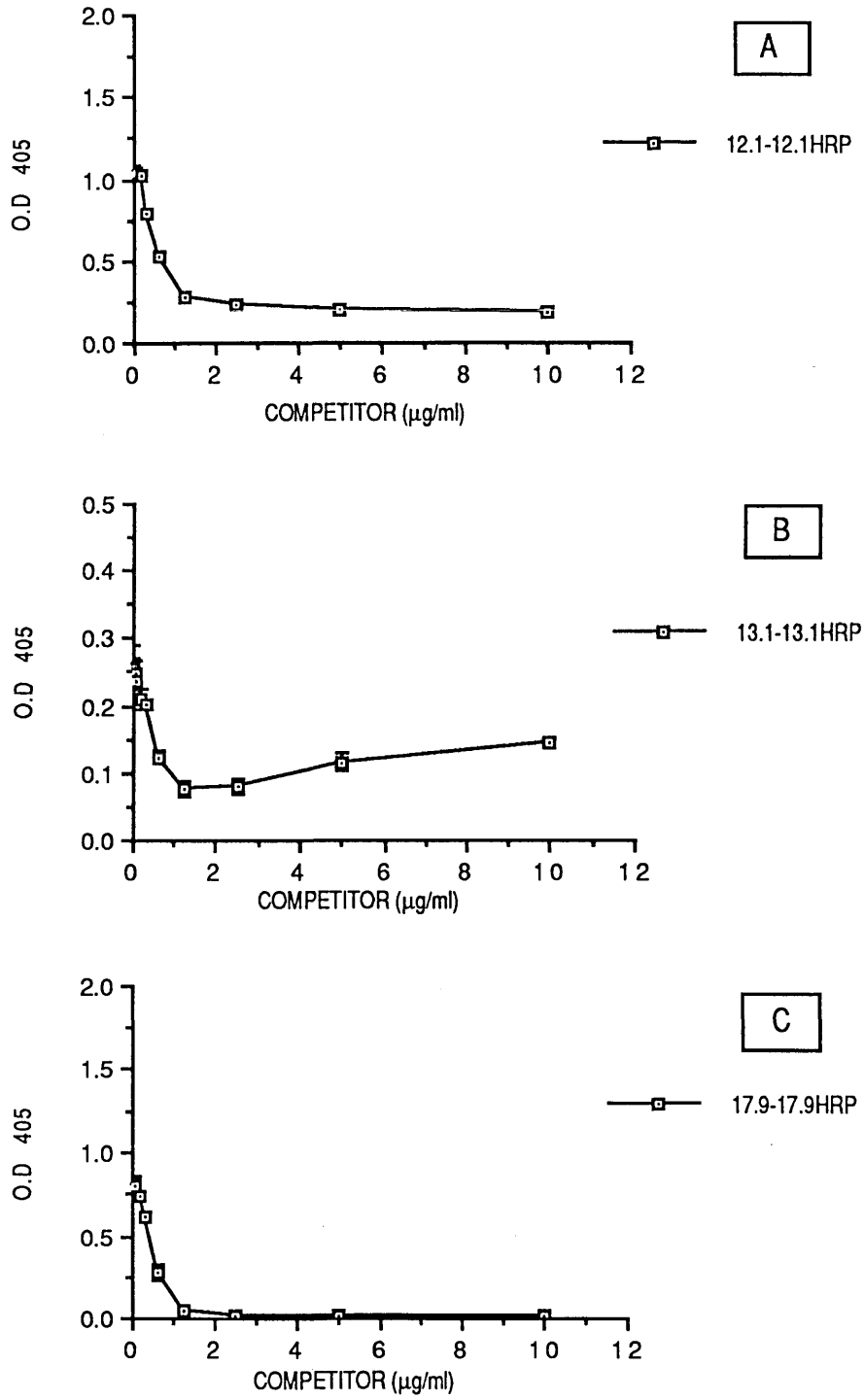


FIGURE 4.6 Competitive ELISA demonstrating the number of epitopes in Pbs21 antigen using the homologous MAbs. Wells pre-coated with 2 µg/ml of the antigen were incubated with 0-10 µg/ml of competitors and subsequently with enzyme-antibody conjugates (1:1000 dilution for 12.1 and 17.9-HRP and 1:100 dilution for 13.1-HRP) :- (A) MAb 12.1 and its homologue, (B) MAb 13.1 and its homologue, (C) MAb 17.9 and its homologue. All values given are the mean of duplicate samples ± S. D.

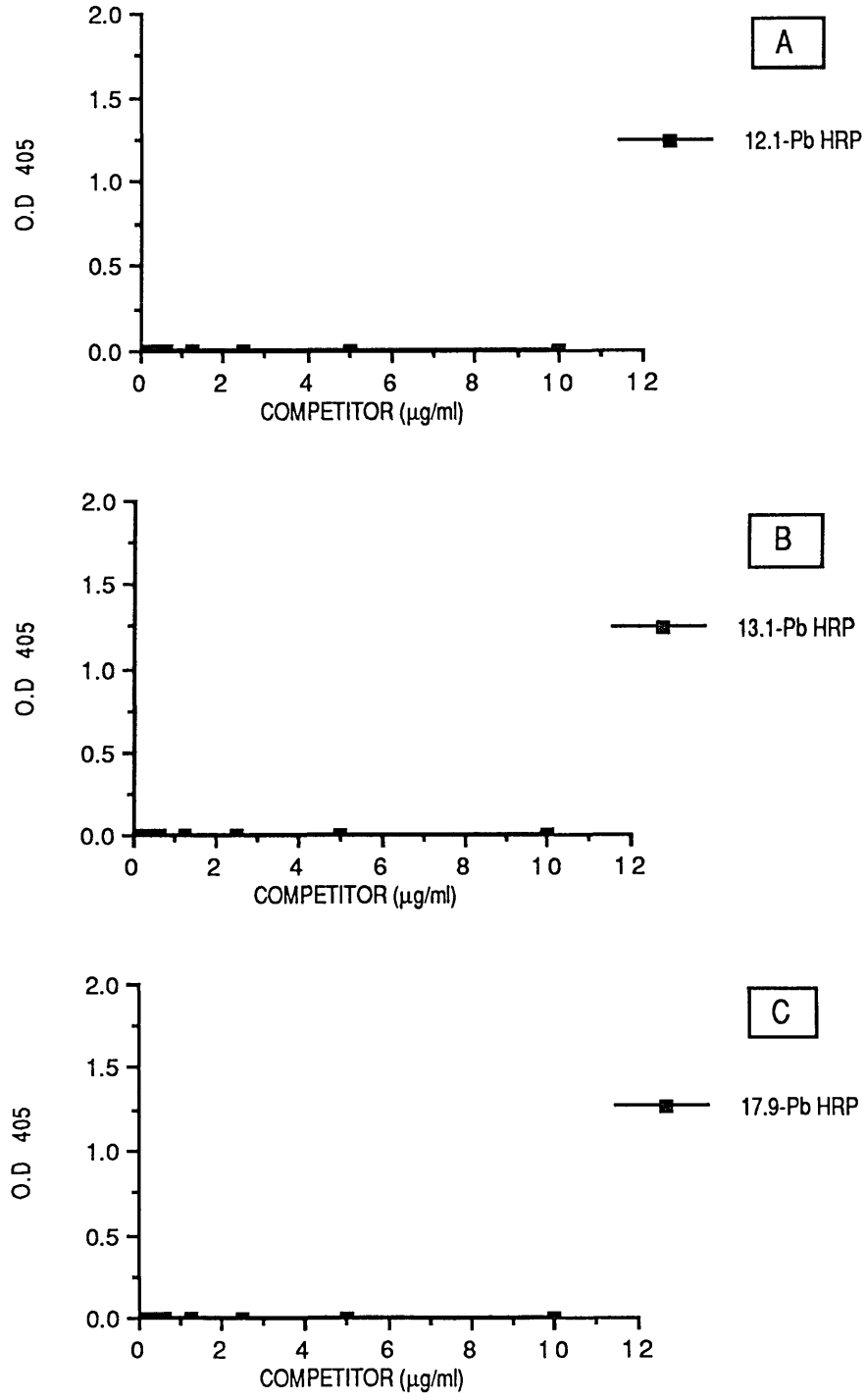


FIGURE 4.7 Assay specificity of competitive ELISA against Pbs21. Wells pre-coated with 2 µg/ml of the antigen were incubated with 0-10 µg/ml of competitors :- (A) 12.1, (B) 13.1, (C) 17.9 and subsequently with an irrelevant enzyme-antibody conjugate Pb 3.28.1-HRP at 1:1000 dilution. All values given are the mean of duplicate samples ± S. D.

It is, therefore, concluded from the results of competitive ELISA that the MAbs 12.1 and 13.1 recognised 2 distinct epitopes (namely epitope I and II respectively) in Pbs21 antigen. A third MAb 17.9 behaved in an analogous manner to both 12.1 and 13.1 thus suggested that it recognised another epitope (namely epitope III) which was closely located to the epitopes I and II.

2) Two-site Enzyme-Linked Immunosorbent Assay

The MAbs 12.1, 13.1 and 17.9 were used as a capture antibody and an enzyme-antibody conjugate in a criss-cross assay. The results are summarised in Table 4.2.

a) Heterologous monoclonal antibody assays

The number of epitopes detectable in Pbs21 was further investigated using different capture and enzyme-antibody conjugate in two-site ELISA. The results of each reciprocal experiment are shown in Figures 4.8 A & B (for 12.1-13.1), 4.9 A & B (for 12.1-17.9), 4.10 A & B (for 13.1-17.9). As negative controls in individual experiment, the antigen was omitted from the wells pre-coated with capture antibody which were subsequently incubated with enzyme-antibody conjugate. The results of the negative control were illustrated in dotted lines in individual assay.

Mabs 12.1-13.1

The concentration-dependent curve was well-defined when the MAb 12.1 was used as a capture antibody and 13.1 was used as an enzyme-antibody conjugate (Figure 4.8 A). The results which confirmed that these 2 MAbs recognised separate epitopes in Pbs21 are shown in the reciprocal situation (Figure 4.8 B). In this study, sensitivity of the two-site ELISA was also recognised. The antigen at a concentration of 0.016 µg/ml was readily detected (Figure 4.8 B). At this point, the $O.D_{405}$ value was twice as much of the background.

MAbs 12.1-17.9

Concentration-dependent curve was not well-established when 12.1 was used as a capture antibody and 17.9 was used as an enzyme-antibody conjugate (Figure 4.9 A). The same phenomenon was also observed in the reciprocal assay (Figure 4.9 B). At the antigen concentration above 1 µg/ml, binding of the enzyme-antibody conjugates to the antigen was observed (Figure 4.9 A and B). It is speculated that such binding might be due to non-specificity and the specificity was assessed as described in section 4.3.2.2 c.

MAbs 13.1-17.9

Similar characteristic of the curves described as above was recognised in 13.1-17.9 and their reciprocal assays (Figure 4.10 A and B respectively). At the concentration of the antigen above 0.5 µg/ml, binding of the conjugates to the antigen was observed. The specificity was assessed as described in section 4.3.2.2 c.

TABLE 4.2 Summary of two-site Enzyme-Linked Immunosorbent Assay

Capture antibody / Enzyme-antibody conjugate	12.1	13.1	17.9
12.1 - HRP	+	-	+
13.1 - HRP	-	+	+
17.9 - HRP	+	+	+

\+ = A concentration-dependent curve is not demonstrated i.e. the 2 MAbs might recognise **the same** epitope in the protein molecule.

\- = A concentration-dependent curve is demonstrated i.e. the 2 MAbs recognise **different** epitopes in the protein molecule.

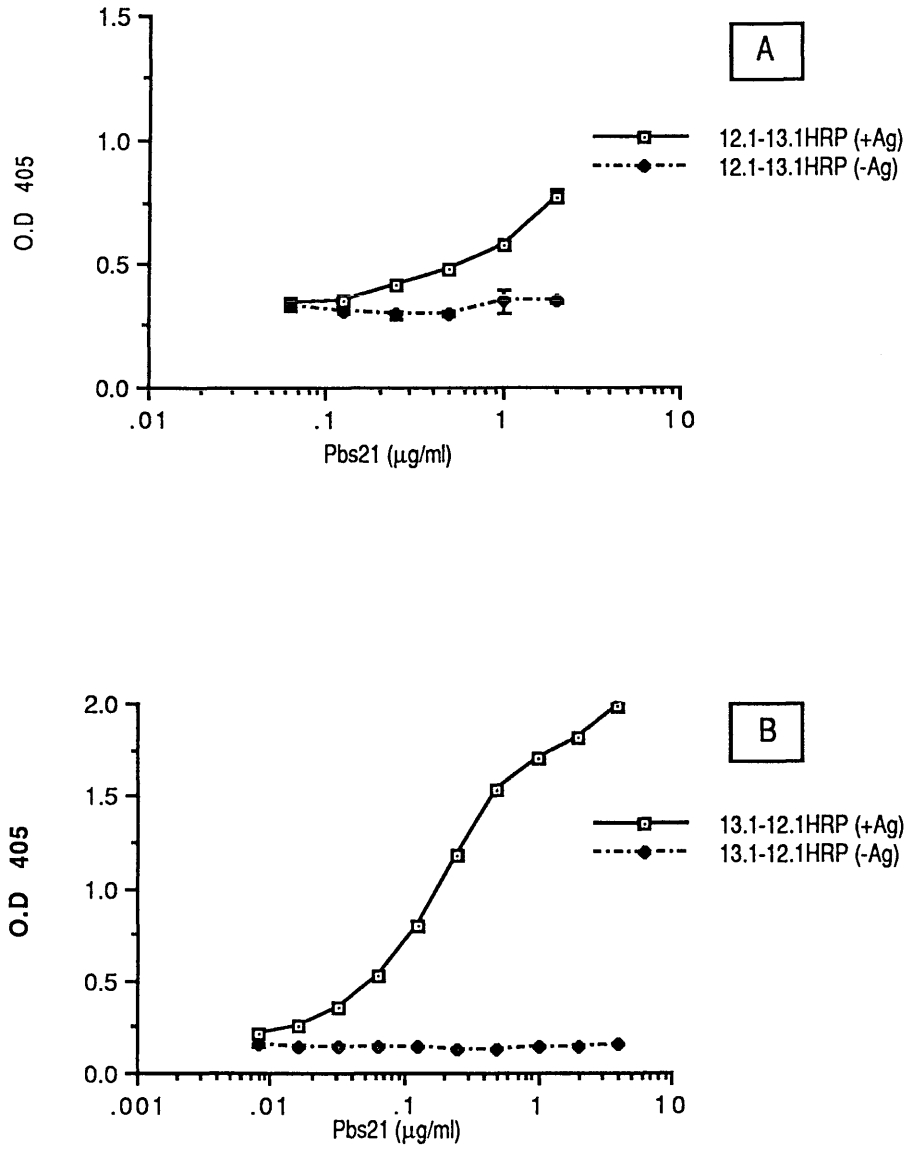


FIGURE 4.8 Two-site ELISA demonstrating 2 distinct epitopes recognised by the MAbs 12.1 and 13.1 in Pbs21. Wells pre-coated with 20 µg/ml of the ammonium sulphate-precipitated 12.1 IgG were incubated with a doubling dilution series of the antigen (2.000-0.063 µg/ml) and subsequently with 1:100 dilution of 13.1-HRP (A). The reciprocal assay is shown in (B) with slight modifications. Wells pre-coated with 13.1 IgG were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of 12.1-HRP. All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

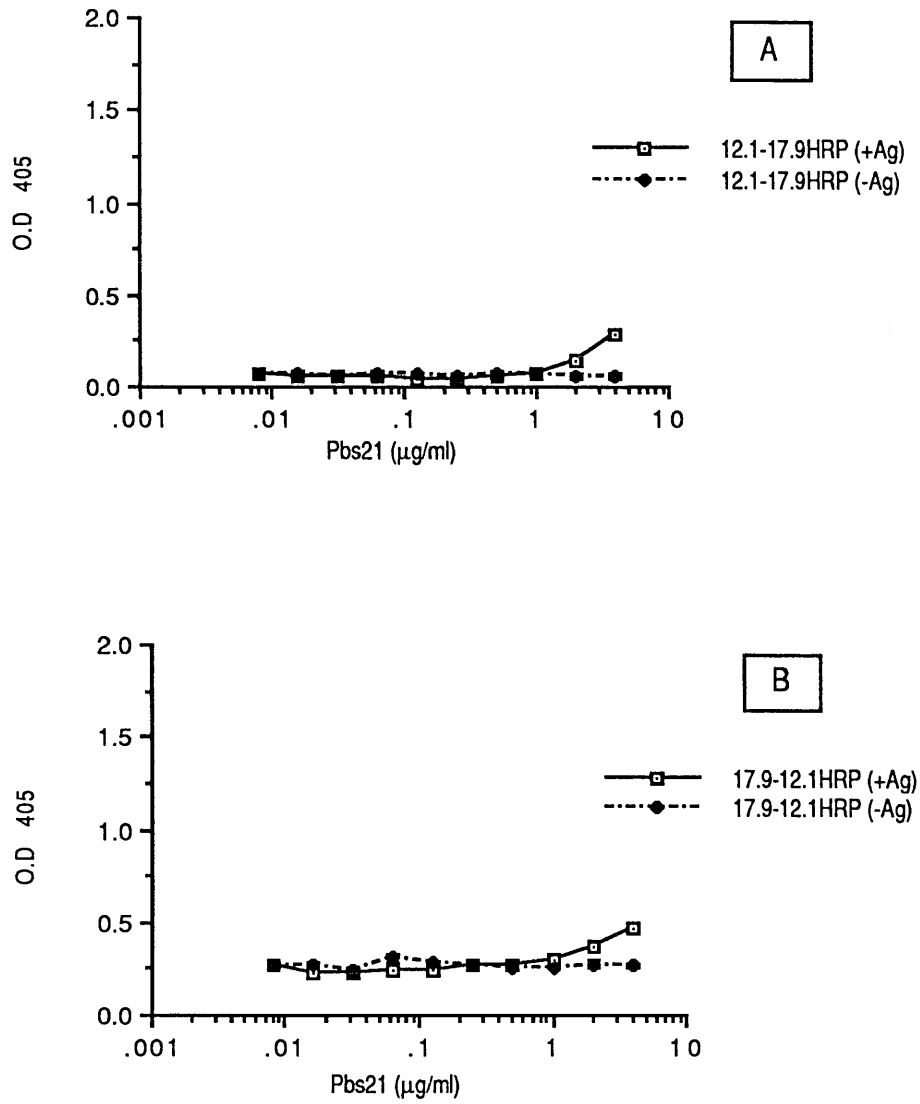


FIGURE 4.9 Two-site ELISA demonstrating the number of epitopes recognised by the MAbs 12.1 and 17.9 in Pbs21. Wells pre-coated with 20 µg/ml of the ammonium sulphate-precipitated 12.1 IgG were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of 17.9-HRP (A). The reciprocal assay is shown in (B). All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

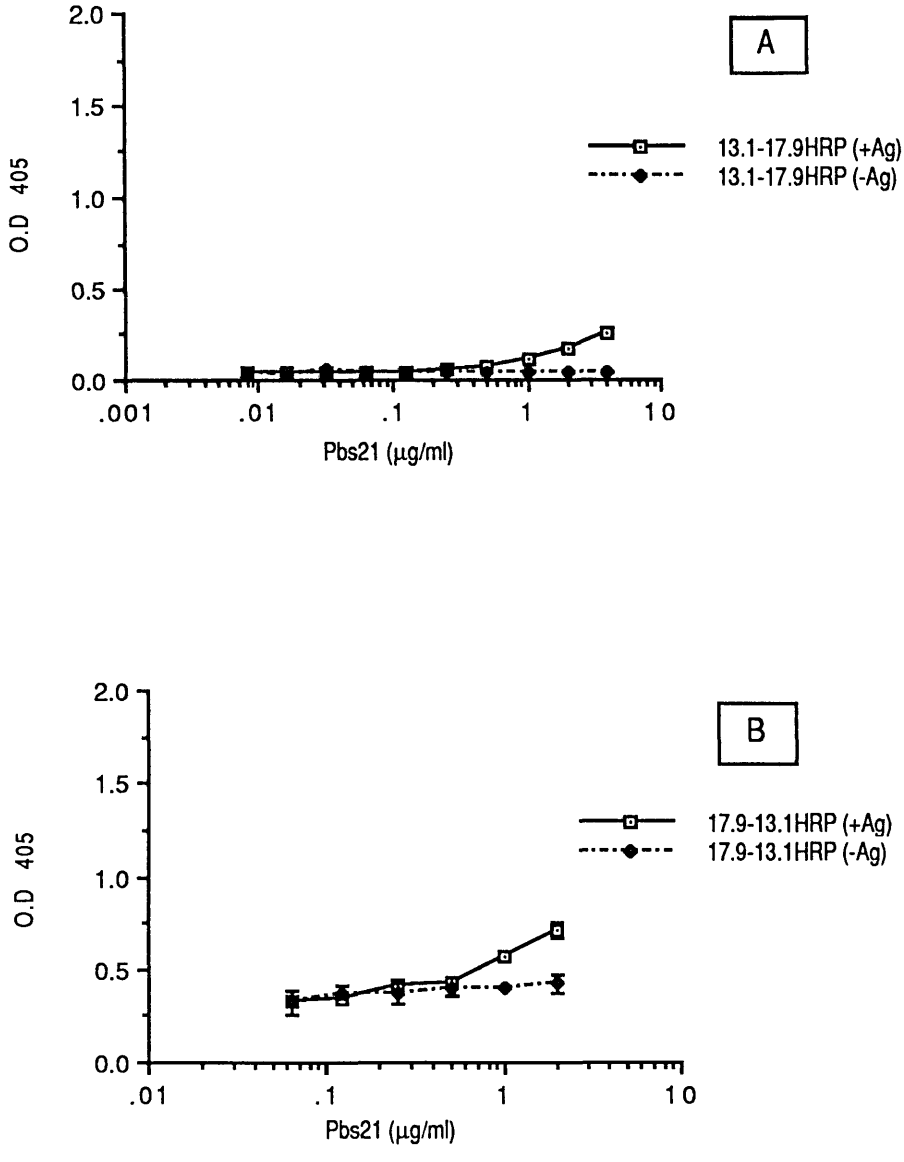


FIGURE 4.10 Two-site ELISA demonstrating the number of epitopes recognised by the MAbs 13.1 and 17.9 in Pbs21. Wells pre-coated with 20 µg/ml of the ammonium sulphate-precipitated 13.1 IgG were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of 17.9-HRP (A). The reciprocal assay is shown in (B) with slight modifications. Wells pre-coated with 17.9 IgG were incubated with a doubling dilution series of the antigen (2.000-0.063 µg/ml) and subsequently with 1:100 dilution of 13.1-HRP. All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

b) Homologous monoclonal antibody assays

To study the number of repeated epitopes in Pbs21, MAb and its homologue were used as a capture antibody and an enzyme-antibody conjugate. The results in which the MAbs 12.1, 13.1 and 17.9 were used are shown in Figures 4.11 A, B and C respectively.

A curve with characteristic similar to that already described for 12.1-17.9 and 13.1-17.9 assays was recognised with 12.1-12.1HRP assay (Figure 4.11 A). Binding of 12.1-HRP to the antigen was observed at antigen concentrations above 1 µg/ml. This observation highlighted a complication of the assay and it is speculated that such binding might be due to non-specificity. Assay specificity was, therefore, assessed as described below.

By contrast, concentration-dependent curve was not observed in 13.1 and 17.9 assays (Figures 4.11 B and C, respectively).

c) Assay specificity

Specificity of two-site ELISA was assessed, (i) wells pre-coated with an irrelevant MAb Pb 3.28.1 were incubated with the antigen and subsequently with enzyme-antibody conjugates (Figures 4.12 A-C), (ii) wells pre-coated with anti-Pbs21 MAbs were incubated with the antigen and subsequently with an irrelevant enzyme conjugate (Pb 3.28.1) (Figures 4.13 A-C).

From 12.1-17.9 & 13.1-17.9 and their reciprocal assays, activity of the enzyme conjugates at the antigen concentration above 0.5 µg/ml was observed (Figures 4.9 and 4.10 respectively). To assess the specificity of 12.1-HRP, and 17.9-HRP, wells pre-coated with 20 µg/ml of an irrelevant MAb Pb 3.28.1 were incubated with the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of either 12.1-HRP or 17.9-HRP (Figures 4.12 A and C respectively). Enzyme activity was recognised at the antigen concentration above 1 µg/ml. This effect was regarded as non-specific binding of the enzyme and, therefore, excluded from 12.1-17.9 assays (Figures 4.9 A, B).

Specificity of 13.1-HRP was also assessed with little modifications. Wells pre-coated with 20 µg/ml of Pb 3.28.1 were incubated with the antigen (2.000-0.063 µg/ml) and subsequently with 1:100 dilution of 13.1-HRP (Figure 4.12 B). At the concentration of the antigen above 0.5 µg/ml, non-specific binding was observed. This reaction was also disregarded from 13.1-17.9 assays (Figures 4.10 A, B).

A similar phenomenon was observed in the assay of 12.1 and its homologue (Figure 4.11 A). Activity of 12.1-HRP was observed at the antigen concentration above 1 µg/ml. This effect which might be due to aggregation of the antigen at high concentration was regarded as non-specific reaction and ignored from the assay since the enzyme activity was also observed when the wells were pre-coated with Pb 3.28.1 (Figure 4.12 A).

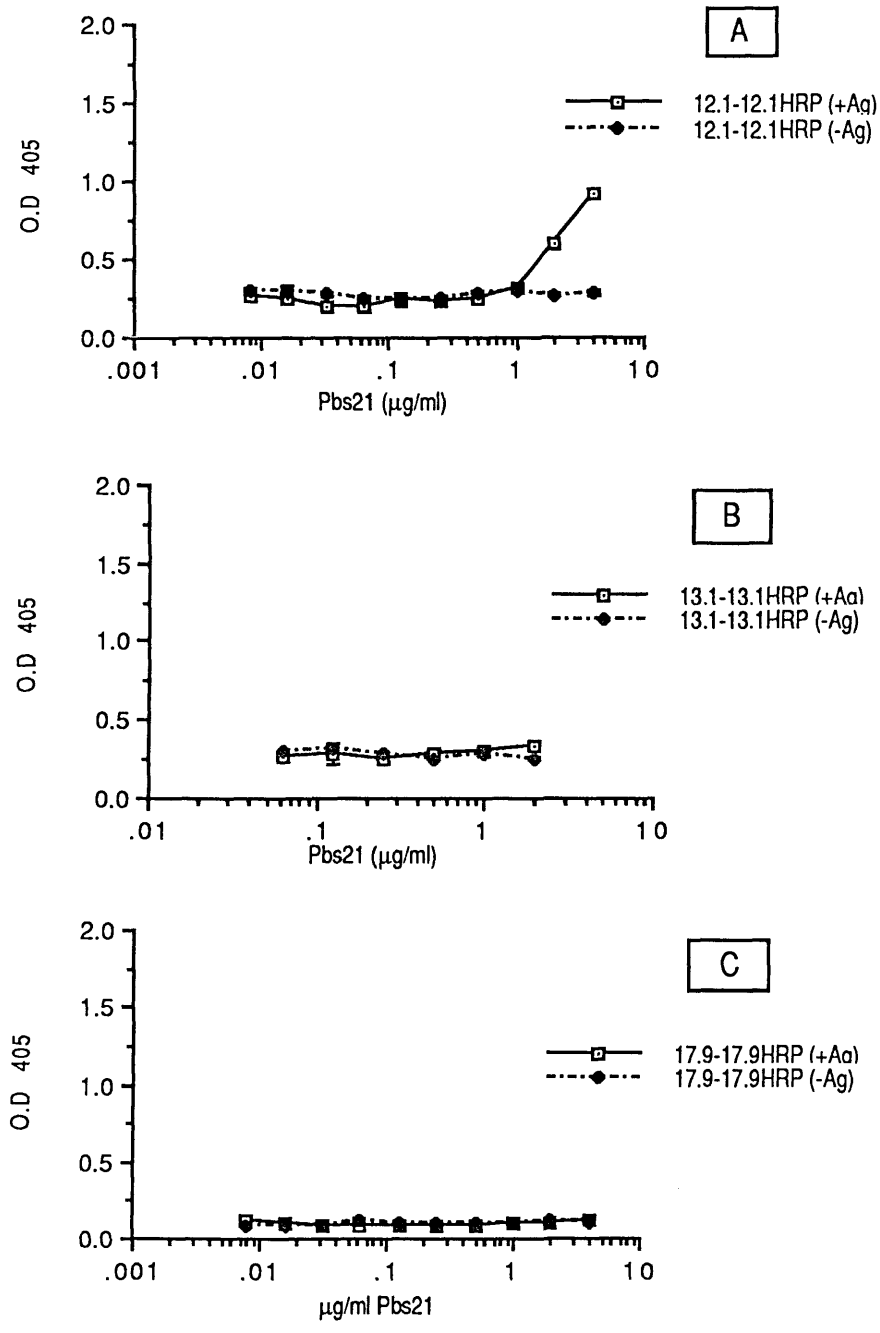


FIGURE 4.11 Two-site ELISA demonstrating non-repeated nature of the epitopes recognised by the MAbs in Pbs21. Wells pre-coated with 20 µg/ml of the ammonium sulphate-precipitated IgG were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml for 12.1 & 17.9 assays and 2.000-0.063 µg/ml for 13.1 assay) and subsequently with the homologous enzyme-antibody conjugates (at 1:1000 dilution for 12.1 and 17.9-HRP and 1:100 dilution for 13.1-HRP :- (A) 12.1 and its homologue, (B) 13.1 and its homologue, (C) 17.9 and its homologue. All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

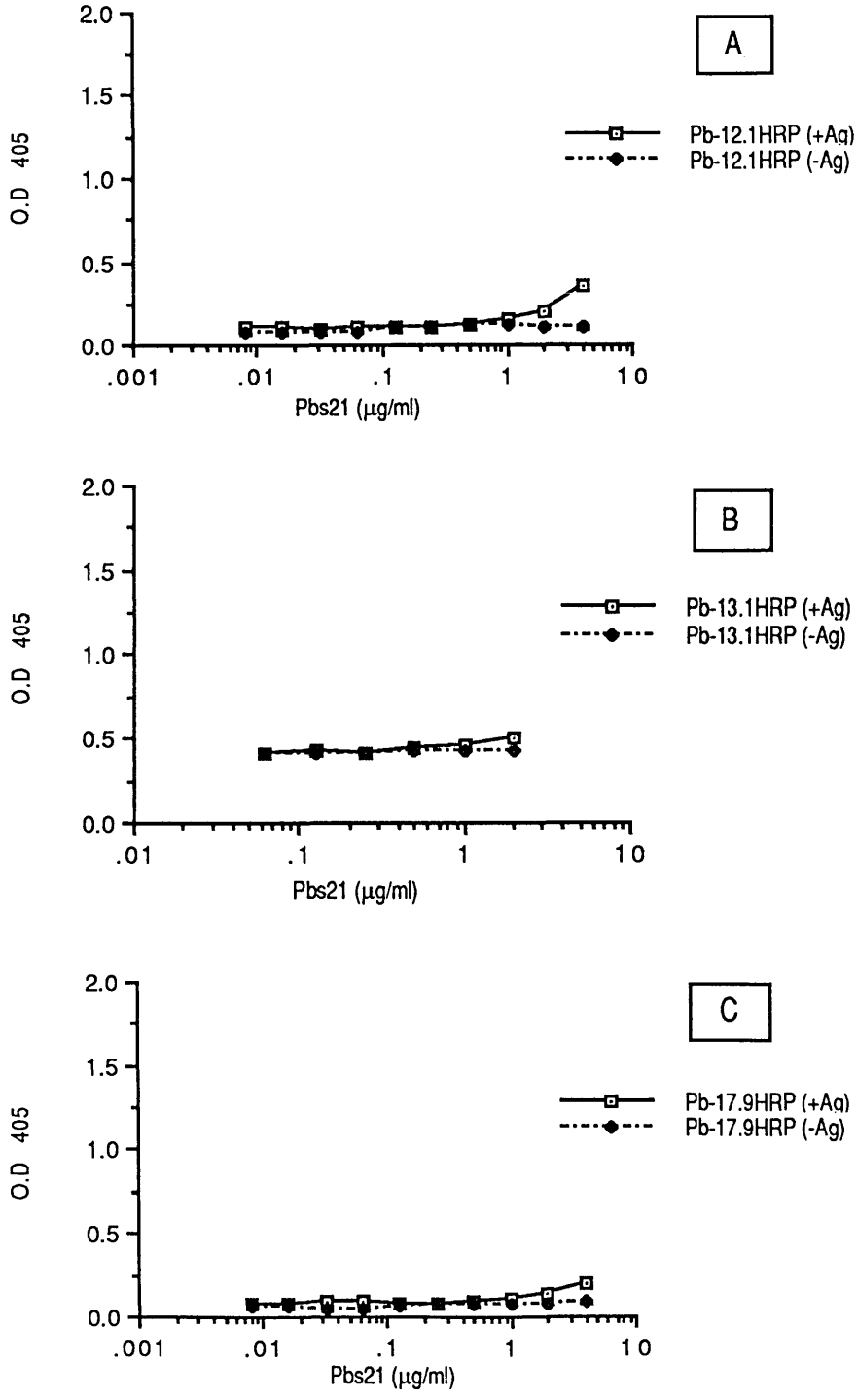


FIGURE 4.12 Assay specificity of two-site ELISA against Pbs21 using an irrelevant MAb as a capture antibody. Wells pre-coated with 20 µg/ml of Pb 3.28.1 were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml for 12.1 and 17.9 assays or 2.000-0.063 µg/ml for 13.1 assay) and subsequently with (A) 12.1-HRP at 1:1000 dilution, (B) 13.1-HRP at 1:100 dilution, (C) 17.9-HRP at 1:1000 dilution. All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

An alternative approach to assess specifity of two-site ELISA was carried out. Wells pre-coated with 12.1, 13.1 and 17.9 IgG were incubated with the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of Pb 3.28.1-HRP (Figures 4.13 A, B and C respectively). The results showed negligible binding of the conjugate to the antigen.

Thus, the results from two-site ELISA which were consistent with competitive ELISA indicated that there were at least 3 epitopes (designated epitopes I-III) recognised by the MAbs 12.1, 13.1 and 17.9 respectively in Pbs21. Epitopes I and II were at a sufficiently distant sites on Pbs21 since the MAbs 12.1 and 13.1 showed a concentration-dependent curves in two-site ELISA and non-competitive curves in competitive ELISA. Epitope III recognised by the MAb 17.9 topologically overlapped to epitopes I and II since the MAb did not show the concentration-dependent curve in two-site ELISA. In competitive ELISA, the MAb 17.9 showed competition with the binding of 12.1-HRP and 13.1-HRP to the antigen. In addition, none of the epitopes were found to be repeatedly expressed since the concentration-dependent curve was not well-defined using homologous MAbs in two-site ELISA.

4.3.3) Study of the conformational structure of the transmission-blocking epitopes in Pbs21

1) Determination of the immunological activity of the reduced and alkylated Pbs21 protein using immunoblot techniques

a) Visualisation of the parasite proteins

Equal amount of the non-reduced and reduced (with either 0.05 M DTT or 5% 2-ME) & alkylated (with 0.02 M iodoacetamide) parasite proteins were subjected to SDS-PAGE and immunoblot techniques. After SDS-PAGE, the protein profile was visualised using Coomassie blue staining (recipe in Appendix 1). The gel was photographed and Coomassie blue staining pattern of the parasite proteins is shown in Figure 4.14.

From the Coomassie blue staining pattern, major differences between native and reduced & alkylated proteins were observed with the proteins of Mr ~ 159, 85, 70 and 63 kD (indicated by arrows). However, the proteins at the molecular weight ~ 21 kD were not intensely stained with Coomassie blue.

The protein of M_r 159 kD was more prominent under non-reducing conditions (Figure 4.14, Lane B) than the protein under DTT-treatment (figure 4.14, Lane C), whereas under 2-ME treatment (Figure 4.14, Lane D) the protein was less intense. It is, therefore, suggested that the 159 kD protein was more sensitive to the reduction by 2-ME than DTT. The same phenomenon was also observed with the protein of M_r ~ 85 kD.

The 63 kD protein was the most prominent under non-reducing conditions (Figure 4.14, lane B) compared to the protein under DTT-treatment (Figure 4.14, lane C) and 2-ME treatment (Figure 4.14, lane D).

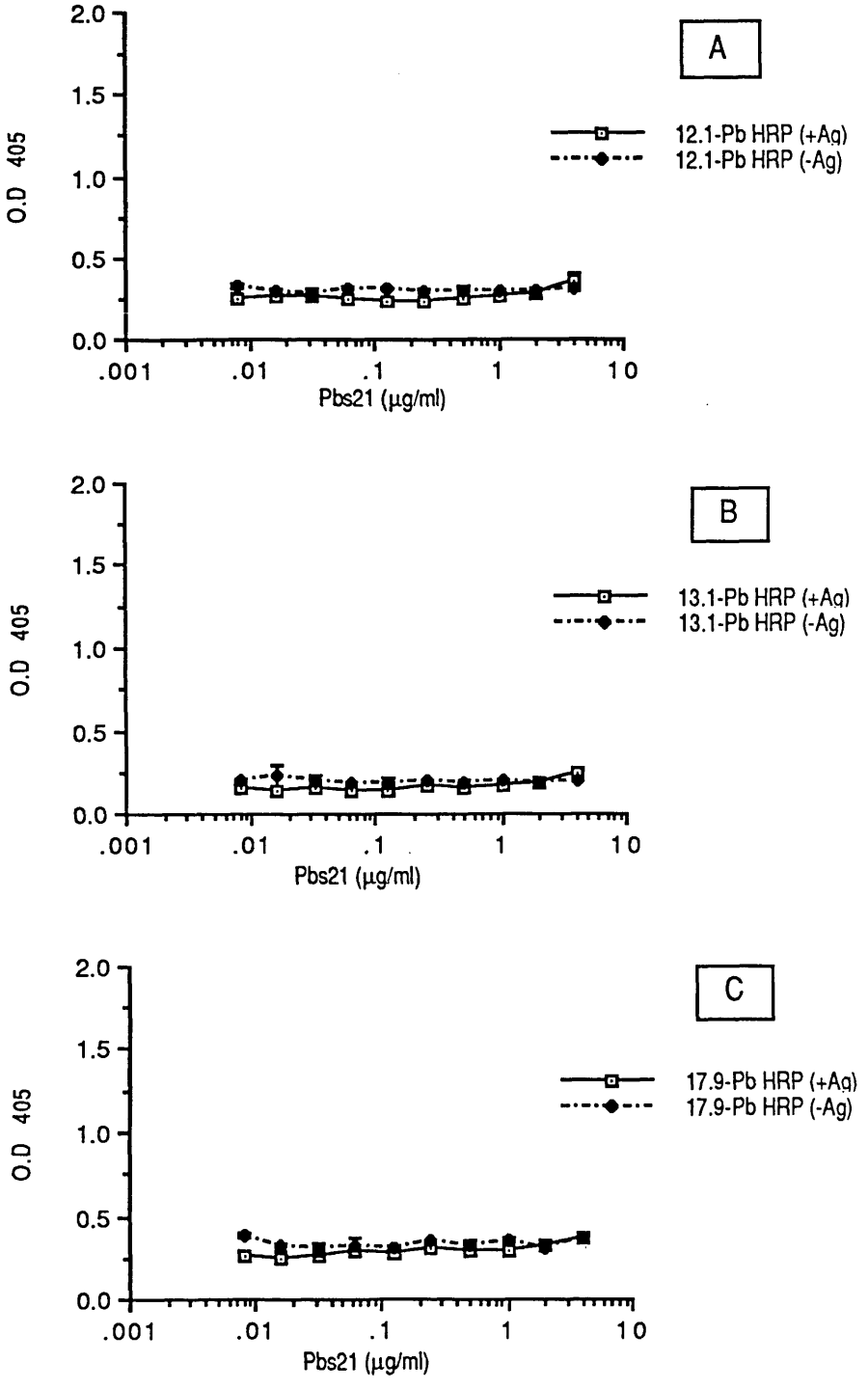


FIGURE 4.13 Assay specificity of two-site ELISA against Pbs21 using an irrelevant enzyme-antibody conjugate. Wells pre-coated with 20 µg/ml of the ammonium sulphate-precipitated IgG :- (A) 12.1, (B) 13.1, (C) 17.9 were incubated with a doubling dilution series of the antigen (4.000-0.008 µg/ml) and subsequently with 1:1000 dilution of Pb 3.28.1-HRP. All values given are the mean of duplicate samples ± S. D. The antigen is omitted for the negative controls (dotted lines).

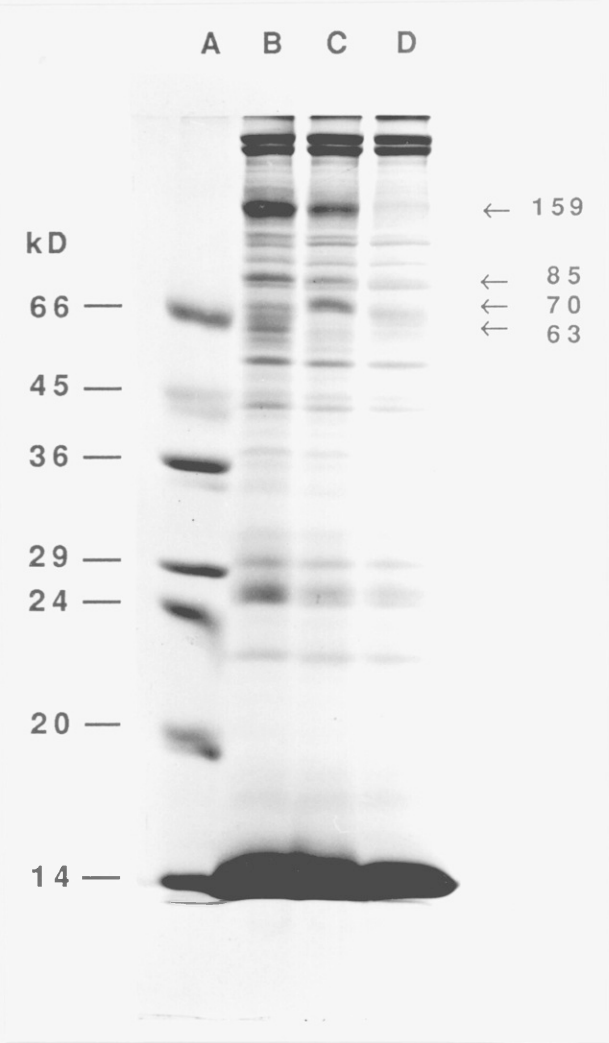


FIGURE 4.14 Coomassie blue staining protein profiles of 15% SDS-PAGE on the ammonium chloride-enriched ookinetes of *P. berghei* under non-reducing conditions (lane B), DTT-reducing (lane C) and 2-ME reducing (lane D). The molecular weight markers are indicated in kilodalton (lane A).

In contrast, the protein of M_r ~ 70 kD was more prominent when the protein was treated with DTT (Figure 4.14, Lane C) compared to the non-reduced (Figure 4.14, Lane B) and 2-ME reduced protein (Figure 4.14, Lane D).

b) Immunoblot analysis

Using immunoblot techniques, the conformational structure of the epitopes recognised by the transmission-blocking MAbs 12.1, 13.1 in Pbs21 was further investigated (Figure 4.15). The study using the MAb 17.9 was not included due to an unfortunate shortage of the antibody. However, the antibody was clearly shown to recognise only non-reduced Pbs21 (Chapter 2, Figure 2.5).

As a positive control of the assay, pooled polyclonal monospecific sera from the mice subgroup N 2 that immunised twice with 5/20 µg of Pbs21 (described in Chapter 3, section 3.2.3.2) was used. Normal mouse sera (NMS) was also included as a negative control of the assay.

Both MAbs 12.1 and 13.1 clearly recognised non-reduced Pbs21 (Figure 4.15, lanes 1 and 4 for 12.1 and 13.1 respectively) as well as the protein under DTT & 2-ME reduction (Figure 4.15, lanes 2 & 3 and 5 & 6 for 12.1 and 13.1 respectively) which implied that reacitivity of the antibodies to the protein was not influenced by the reducing agents that had been used. However, by visual judgement, the relative intensity of the protein band was slightly lower when the protein was reduced with DTT compared to 2-ME before an incubation with the MAb 12.1 (Figure 4.15, lane 2 and 3) and 13.1 (Figure 4.15, lanes 5 and 6). No significant difference of the intensity of the protein band was observed when the 2-ME reduced protein was compared with non-reduced protein for the MAbs 12.1 (Figure 4.15, lanes 1 & 3) and 13.1 (Figure 4.15, lanes 4 & 6).

Similar results were obtained using polyclonal monospecific mouse sera (Figure 4.15, lanes 10-12). Binding of the sera to the protein was not affected by the reducing agents that had been used since the proteins under DTT (Figure 4.15, lane 11) or 2-ME (Figure 4.15, lane 12) reductions were still reacted with the sera. However, less intensity of the protein band was observed in the DTT-treated sample (Figure 4.15, lane 11) compared to 2-ME treated sample (Figure 4.15, lane 12).

Using NMS, no protein band was detected (Figure 4.15, lanes 7-9). Thus, the results from immunoblot analysis implied that Epitopes I and II recognised by the MAbs 12.1 and 13.1 were not likely to be conformation-dependent. However, the degree of the linearity of the epitopes was not known. From visual judgement of the intensity of Pbs21 band, hierarchy of the relative intensity was non-reducing ≥ 2-ME reduction ≥ DTT-reduction.

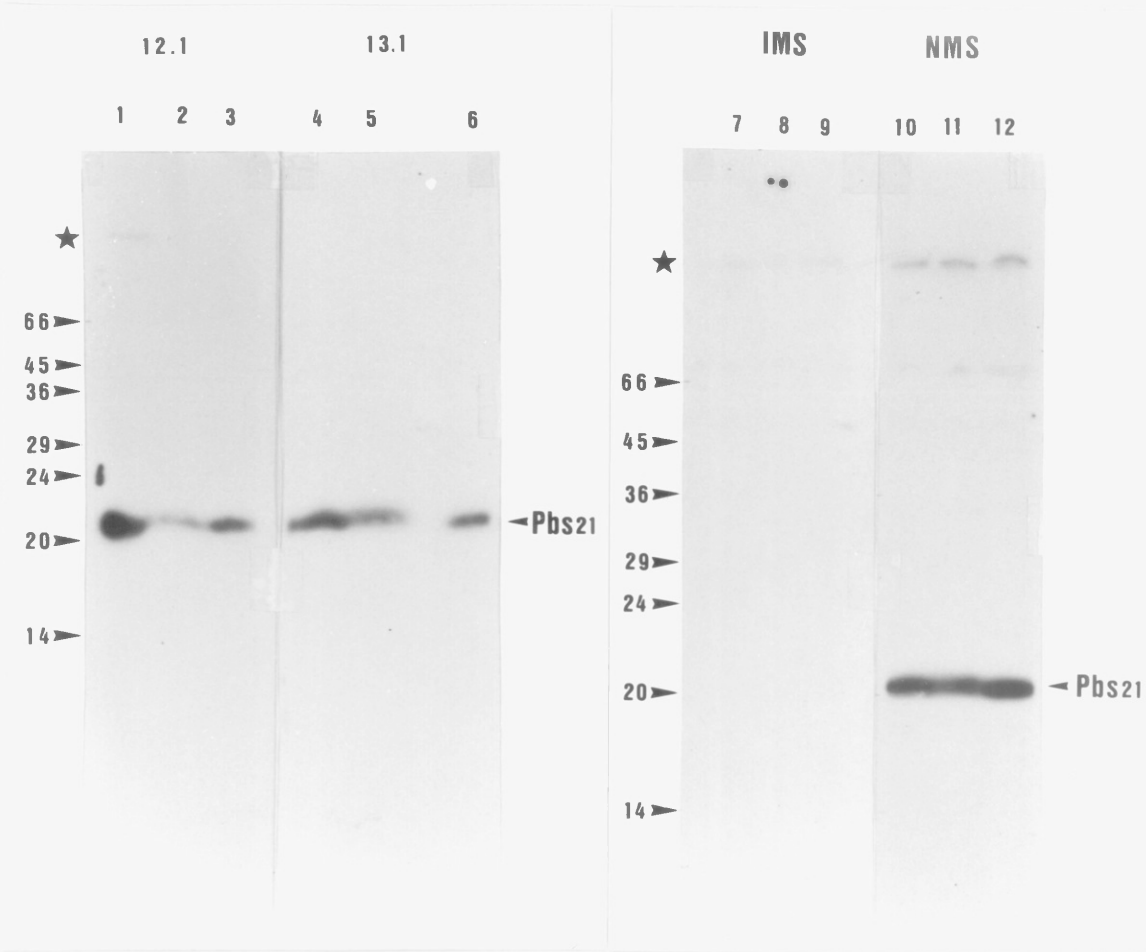


FIGURE 4.15 Immunoblot analysis of 15% non-reducing (lanes 1, 4, 7, 10), DTT-reducing (lanes 2, 5, 8, 11) and 2-ME reducing (lanes 3, 6, 9, 12) SDS-PAGE protein profiles of the ammonium chloride-enriched ookinetes of *P. berghei* with the MAbs 12.1 (lanes 1-3), 13.1 (lanes 4-6), normal mouse sera (NMS) (lanes 7-9) and polyclonal monospecific mouse sera (PMS) (lanes 10-12). All strips were overlaid with ^{125}I-labelled sheep anti-mouse IgG. Molecular weight are indicated in kilodalton.

* = Boundary between the stacking and separating gels.

2) Determination of the immunological activity of the reduced and alkylated Pbs21 protein using ELISA

The conformational structure of the epitopes recognised by the transmission-blocking MAbs was further studied using indirect one-site ELISA. After reduction (with either DTT or 2-ME) & alkylation, the immunological reactivity of Pbs21 was determined using the MAbs 12.1, 13.1 and 17.9. As positive control of the assay, the polyclonal monospecific sera from the mice subgroup N 2 (as described above) was included since the recognition of non-reduced, DTT-reduced and 2-ME reduced proteins by the sera was already observed in the immunoblot assay (Figure 4.15, lanes 10-12). Normal mouse sera was used as a negative control of the assay. The results are shown in Figures 4.16 A, B, C and D for the MAbs 12.1, 13.1, 17.9 and polyclonal monospecific sera respectively.

All 3 MAbs recognised the protein in a similar manner. Concentration-dependent curves with a saturation point at 2 µg/ml of the antigen were observed. Hierarchy of the antibody activity was 13.1 > 12.1 > 17.9. Different levels of binding of the MAbs to Pbs21 might be due to different binding affinity of the MAbs (J. E. Lillywhite, pers. comm.).

Following DTT reduction, all antibodies showed a significant reduction of response to Pbs21 in ELISA (determined from the $O.D_{414}$ values) by 70.8-100%. Hierarchy of the antibody activity was also 13.1 > 12.1 > 17.9. The immunological reactivity of the protein after 2-ME reduction was abolished in all of the assays.

Unexpectedly, similar results were obtained with the polyclonal monospecific sera which was used as a control of the assay (Figure 4.16 D). The sera showed a significant reduction of response to the DTT-reduced protein by 57.58-100%. As negative control of the assay, normal mouse serum gave negligible reactivity with Pbs21 in ELISA ($O.D_{414} < 0.01$) (results not shown).

Thus, in ELISA, hierarchy of the reaction was the untreated native antigen preparation > DTT-reduction > 2-ME reduction.

Discrepancies between ELISA and immunoblot techniques were observed. In immunoblot assay, the MAbs 12.1, 13.1 and the polyclonal monospecific sera showed the reactivity with 2-ME reduced Pbs21 (Figure 4.15, lanes 3, 6 and 12 respectively). Reactivity of the antibodies to 2-ME reduced protein was not detected in ELISA (Figure 4.16 A, B and D). It would seem unlikely that the nonresponsiveness in ELISA was due to a failure of the binding of the antibodies to 2-ME reduced-alkylated protein. It was, therefore, decided to assess the binding efficiency of the control and reduced-alkylated proteins to the microtitre plate.

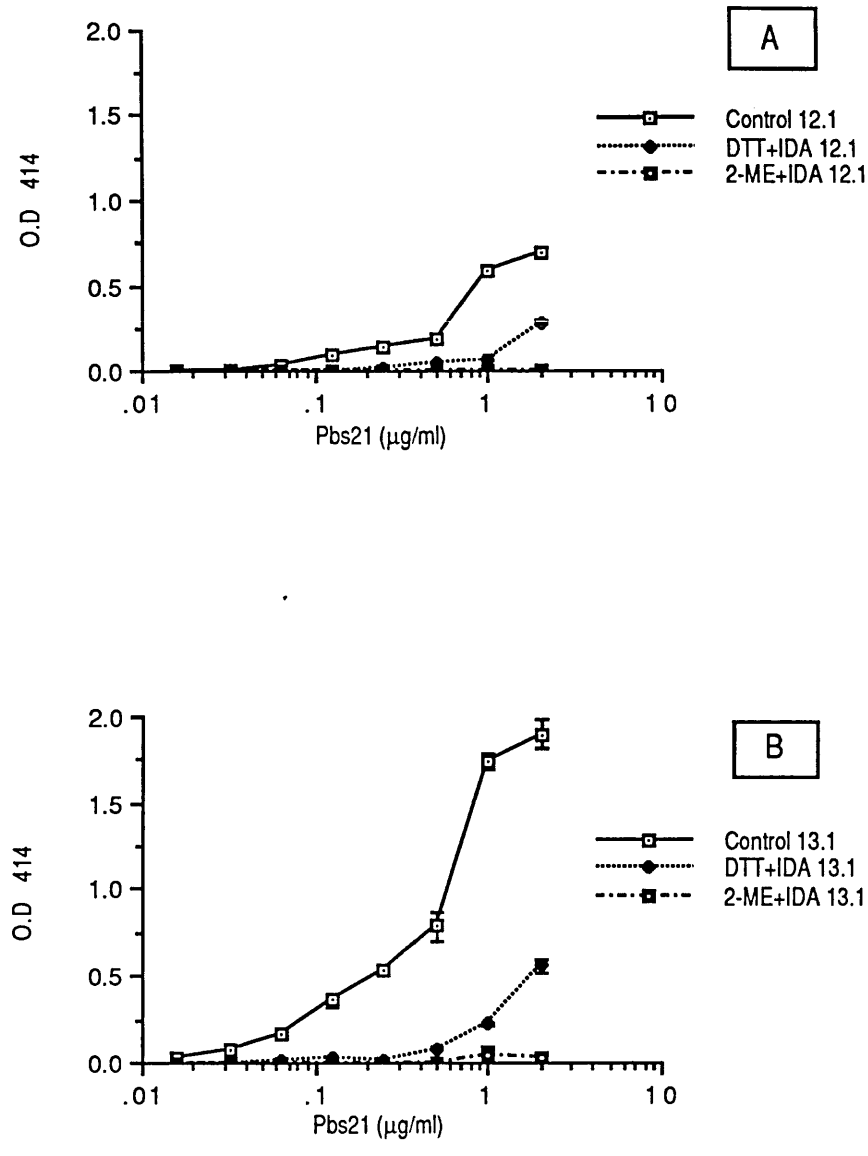


FIGURE 4.16 Indirect one-site ELISA testing the immunological activity of Pbs21 after reduction with either 0.01 M DTT (denoted as DTT+IDA in fine dotted lines) or 5% 2-ME (denoted as 2-ME+IDA in course dotted lines) and alkylation with 0.02 M iodoacetamide (IDA) compared to the control preparation of the protein (denoted as Control in solid line). Wells pre-coated with a doubling dilution series of control, DTT-reduced and 2-ME reduced Pbs21 (2.000-0.016 µg/ml) were incubated with 1:500 dilution of the ascitic fluids 12.1 (A), 13.1 (B), 17.9 (C) and polyclonal monospecific sera (or PMS) (D) and subsequently with goat anti-mouse IgG-alkaline phosphatase conjugate. All values are the means of duplicate samples ± S. D. (the results continued in page 131).

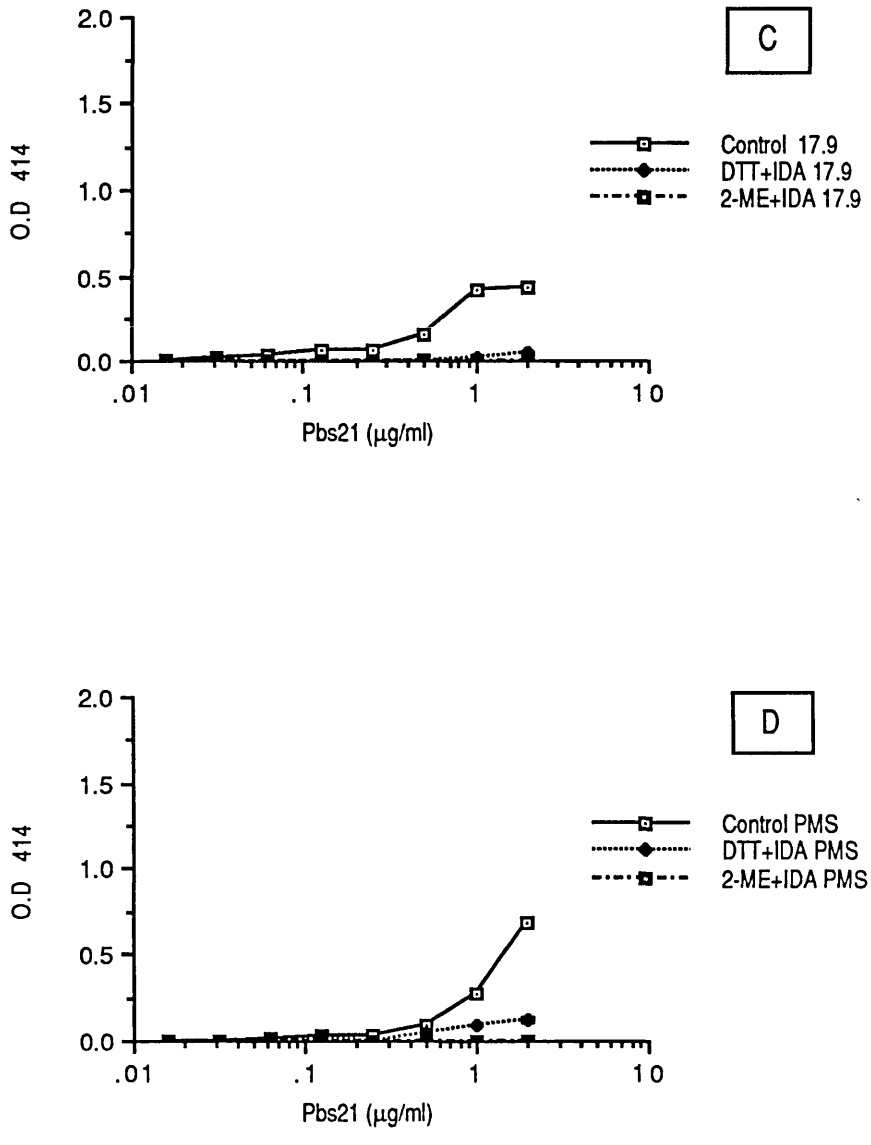


Figure 4.16 (continued)

3) Determination of the binding efficiency of the control and reduced-alkylated proteins to the microtitre plate using ^{125}I-labelling

Using ^{125}I-labelled double affinity-purified Pbs21 (described in section 3.2.2.3 a), the binding efficiency of the ^{125}I-labelled control (or non-reduced) and reduced-alkylated protein preparations was compared. Wells of the microtitre plates were coated with a doubling dilution series of the control and reduced (with either DTT or 2-ME) & alkylated (with iodoacetamide) proteins (1.150-0.018 µg/ml). Individual well was cut off and the radioactivity was determined as described in Materials and Methods. The results are shown in Figure 4.17.

Dose-response curve was observed in the binding of the protein to the microtitre plate in a similar manner to the antigen-antibody reactions in ELISA i.e. binding of the protein in control preparation > DTT-reduction > 2-ME reduction (Figure 4.16 A-D). The control antigen showed greater efficiency in binding to the plate than the reduced-alkylated proteins. Significant reduction of the 2-ME and DTT-reduced proteins in binding to the microtitre plate compared to the control antigen was varied between 59.27-100%. The 2-ME reduced protein (except at the initial concentration) showed less binding efficiency to the plate compared to DTT-reduced protein by 61.18-100%. Reduction of the binding efficiency of ^{125}I-labelled Pbs21 following DTT and 2-ME treatments to the microtitre plates also paralleled the degree of reduction in antibody reactivity in ELISA.

4.3.4) Study of the post-translational modification of the transmission-blocking epitopes in Pbs21

The role of carbohydrate for the immunological activity of Pbs21 epitopes was studied using mild periodate oxidation in combination with two-site ELISA for the study of 12.1 and 13.1 epitopes. Specific binding of 17.9-HRP to the antigen bound to 12.1 or 13.1 IgG pre-coated wells in two-site ELISA was not established (Figures 4.9 A and 4.10 A), such assay was not possible for the study of 17.9. Thus, direct one-site ELISA was modified for the study of 17.9 epitope. The results are shown in Figures 4.18 A, B and C for the study of 12.1, 13.1 and 17.9 epitopes respectively.

All 3 MAbs still reacted with the protein antigen after periodate treatment. Only a slight decrease in the optical density is observed when the controls (or native antigen ; solid lines) were compared with the periodate-treated samples (dotted lines). When the antigen was omitted in negative control wells, binding of the conjugates was negligible.

Therefore, it is suggested that the role of carbohydrate to the antigenicity of the epitopes recognised by 3 transmission-blocking MAbs might not be crucial.

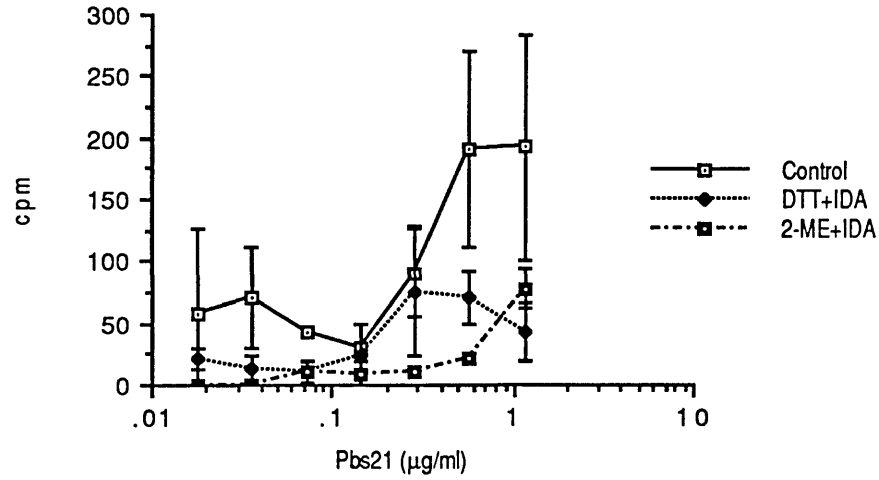
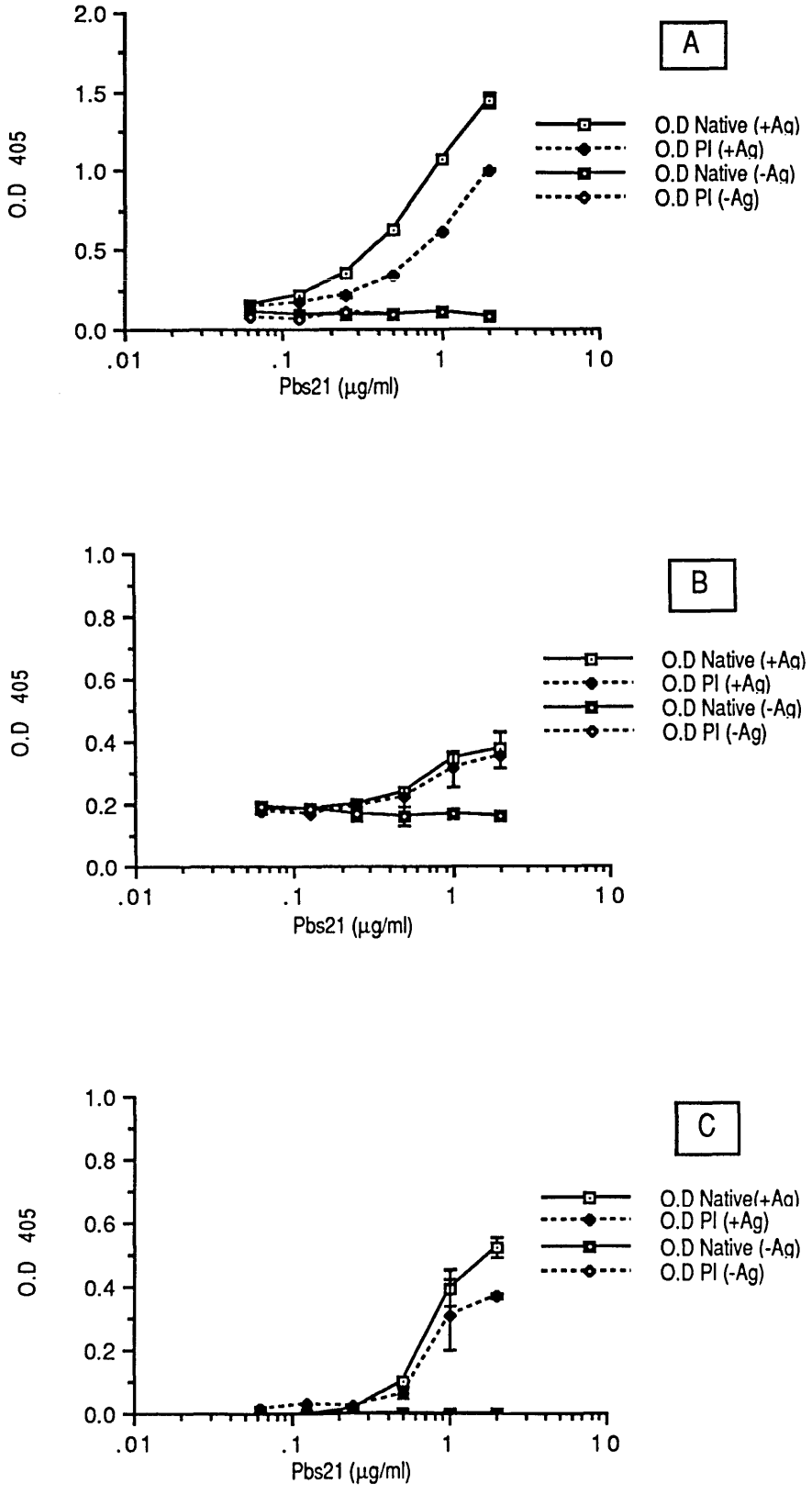


FIGURE 4.17 Binding efficiency of the ^{125}I-labelled the double affinity-purified Pbs21 under non-reducing (denoted as control in solid line), 0.01 M DTT-reducing (denoted as DTT+IDA in fine dotted line) and 5% 2-ME reducing (denoted as 2-ME+IDA in course dotted line) conditions & alkylated with 0.02 M iodoacetamide (IDA). Wells of the microtitre plate were coated with a doubling dilution series of 3 different preparations of the antigen (1.150-0.018 µg/ml) overnight. After the wells were washed to remove unbound materials, radioactivity was counted in a γ-counter. All values given are the mean of duplicate samples after subtraction from the background values ± S. D.

FIGURE 4.18 ELISA demonstrating the effect of mild periodate treatment to the immunological activity of the epitopes recognised by the MAbs 12.1, 13.1 and 17.9 in Pbs21. Two-site ELISA was used to study 12.1 and 13.1 epitopes and one-site ELISA was used to study 17.9 epitope :- (A) 12.1 epitope ; wells pre-coated with 20 µg of the ammonium sulphate-precipitated 13.1 IgG were incubated with a doubling dilution series of Pbs21 (2.000-0.063 µg/ml) either before or after periodate treatment (denoted as native ; solid line and PI ; dotted line respectively) and subsequently with 1:1000 dilution of 12.1-HRP. (B) 13.1 epitope ; wells pre-coated with 12.1 IgG were incubated with native (solid line) and periodate-treated (dotted line) antigens and subsequently with 1:100 dilution of 13.1-HRP. (C) 17.9 epitope ; wells pre-coated with native (solid line) and periodate-treated (dotted line) antigens were incubated with 1:1000 dilution of 17.9-HRP. All values given are the mean of duplicate samples ± S.D. The antigen is omitted for the negative controls of native (solid lines) and periodate-treated samples (dotted lines).


A
B
C
O.D 405
Pbs21 (µg/ml)
O.D Native (+Ag)
O.D PI (+Ag)
O.D Native (-Ag)
O.D PI (-Ag)
2.0
1.5
1.0
0.5
0.0
.01
.1
1
10
0.8
0.6
0.4
0.2

4.4) Discussion

4.4.1) Epitope mapping in Pbs21 using the monoclonal antibodies

The purpose of two-site ELISA was to study the number of independent antigenic sites of Pbs21 taken together with the use of competitive binding assay to determine the number of non-overlapping sites recognised by the MAbs. The results indicated that there are at least 3 B-cell epitopes (epitope I-III) recognised by the MAbs 12.1, 13.1 and 17.9 respectively in Pbs21.

The MAbs 12.1 and 13.1 recognised two distinct epitopes (epitope I and II respectively). A lack of concentration-dependent competition between these 2 MAbs in competitive ELISA (Figure 4.5 A) was consistent with the characteristic of a concentration-dependent curve in two-site ELISA (Figure 4.8). It is interesting to point out that the binding of 12.1 & 13.1-HRP to the antigen was enhanced in the presence of heterologous MAbs in competitive ELISA (Figure 4.5 A). The same phenomenon was reported by Carter *et al.*, (1985) which was correlated to the synergistic suppression of infectivity of gametocytes to mosquitoes. Our results from membrane feed and passive immunisation experiments indicated that individual MAb 12.1 and 13.1 showed suppression of mosquito infectivity by 93-100 and 92-94.88 % respectively (Winger *et al.*, 1988). Combination of these 2 MAbs in either membrane feeding or passive transfer experiments has not yet been performed.

Using 13.1-HRP as an enzyme-antibody conjugate and 12.1 as a capture antibody in two-site ELISA, the enzyme activity (maximum $O.D_{405}$ = 0.768 after 20 min of incubation at 37°C ; Figure 4.8 A) was higher than direct 1-site ELISA (maximum $O.D_{405}$ = 0.24 ; Figure 4.4 B). It could be reasoned that in direct 1-site ELISA, 13.1 epitope was sterically modified by surface-binding of the antigen to the plate and which reacted poorly with 13.1-HRP. When 12.1 was used as a capture antibody in two-site ELISA, 13.1 epitope was not modified and, therefore, exposed more effectively to further binding with 13.1-HRP.

In our study, sensitivity of two-site ELISA using 13.1 as a capture antibody and 12.1 as an enzyme-antibody conjugate was able to detect the affinity-purified Pbs21 antigen down to 0.016 µg/ml (Figure 4.8 B). Sensitivity of the assay was able to detect the recombinant circumsporozoite (CS) protein of *P. falciparum* and *P. vivax* down to ~ 0.1 µg/ml (T. Burkot pers. comm.). The idea of using two-site ELISA to detect the parasite antigen from different species of *Plasmodium* in mosquito vector has already been applied for the study of sporozoite infection rate in mosquitoes (Burkot *et al.*, 1984a, b ; Ferreira *et al.*, 1986 ; Beier *et al.*, 1987 ; Collins *et al.*, 1988). The method was proved to be successful in terms of both sensitivity and specificity. In this regard, it should be possible to extend the advantage of two-site ELISA using 13.1 as the capture antibody and 12.1 as the enzyme-antibody conjugate to study the persistence of Pbs21 in mosquito vector which could lead to further applications i.e. oocyst determination (instead of mosquito dissection which is time-consuming, cumbersome and made routine assays uneasy) in transmission-blocking studies in the future. However, using IFA on cryostat sections of mosquito, Pbs21 was still detectable in the oocysts 6 days after the bloodfeed (Sinden *et al.*, 1987).

Another epitope (epitope III) in Pbs21 was recognised by the third MAb 17.9. In competitive ELISA, a simple concentration-dependent competition occurred with the heterologous 12.1-17.9 and 13.1-17.9 assays was unexpected (Figures 4.5 B and C respectively). However, this phenomenon was already described in the epitope mapping of murine Leukemia virus proteins (Stone and Nowinski, 1980). It was reasoned that if two epitopes were closely located on a protein antigen, their corresponding MAbs would demonstrate (as a result of steric hindrance) competitive binding properties. On the other hand, if the 2 epitopes were at sufficiently distant sites on the protein (for instance, epitopes I and II in Pbs21), their corresponding antibodies would not bind competitively. Thus, epitope III is suggested to topologically overlap with epitopes I and II.

In two-site ELISA, the concentration-dependent curve was not well-defined in 12.1-17.9 and 13.1-17.9 assays (Figures 4.9 and 4.10 respectively). These results were consistent with competitve ELISA that 17.9 epitope was closely located to 12.1 and 13.1 epitopes. Unexpectedly, a non-specific binding of the enzyme-antibody conjugates to the antigen was observed at antigen concentrations above 0.5 µg/ml. This phenomenon was regarded as non-specific binding of the enzyme-antibody conjugates to the antigen, probably due to aggregation of the antigen at high concentration since the conjugates also showed non-specific binding characteristic in the wells pre-coated with Pb 3.28.1 (Figure 4.12 A-C).

A concentration-dependent curve was not well-defined in two-site ELISA using the MAbs and their homologues (Figure 4.11). Therefore, the results from two-site ELISA suggested that none of the 3 epitopes in Pbs21 were repeatedly expressed. However, it could not be considered conclusive as suggested by Carter *et al.*, (1985). For instance, a small number of repeated epitopes clustered in a single region of a native protein molecule might be rendered sterically unavailable to further binding when one of them reacts with a plate surface-bound antibody.

The absolute number of epitopes in Pbs21 is not known due to a limited number of anti-Pbs21 MAbs. Having established that the 3 epitopes (epitopes I-III) were recognised by the transmission-blocking MAbs, it is not yet clarified if any of the epitope is immunodominant. This aspect of study needs further investigation and it will be possible using the assay modified from Graves *et al.*, (1988b) and the immune sera from the mice vaccinated with Pbs21 (reported in Appendix 3 and the mice subgroups N 2 and O 2 that had been immunised with 5/20 and 20/20 µg of the protein respectively ; described in Chapter 3). Binding of 12.1-HRP, 13.1-HRP and 17.9-HRP to the antigen pre-coated wells of the microtitre plates in competition with the immune sera can then be examined. This will, therefore, allow further understanding of the host immune response to Pbs21.

4.4.2) Study of the conformational structure and post-translational modification of the epitopes I-III in Pbs21

It is generally accepted that reduction of a protein will cause a loss of the secondary and tertiary structure of the polypeptide, whereby the biological activity and the immunological reactivity (in the case of conformational epitopes) of the protein are also lost. Re-oxidation of the reduced protein under suitable conditions may restore the native conformation and consequently the biological and immunological activities. But when the protein is reduced and alkylated, the free SH-groups are blocked resulting in the inability to fold back into its native conformation and its activity is not restored (White, 1972).

From the preliminary study in Chapter 2 (Figure 2.4) and Chapter 3 (Figure 3.6), the MAbs 12.1, 13.1 and polyclonal monospecific sera (from the mice immunised twice with 5/20 μg Pbs21 ; subgroup N 2) have been shown to recognise Pbs21 on both non-reducing and 2-ME reducing SDS-PAGE. In the present study, observations from immunoblot analysis were also consistent with the previous study. The MAbs 12.1, 13.1 and polyclonal sera still recognised Pbs21 on both non-reducing and reducing (with DTT or 2-ME) SDS-PAGE (Figure 4.15, lanes 1-6 and 10-12). From visual judgement of Pbs21 protein band intensity in the autoradiograph (Figure 4.15), the hierarchy of reactivity of the MAbs 12.1, 13.1 and the polyclonal sera to Pbs21 was non-reducing ≥ 2-ME reducing > DTT-reducing conditions. Whether the relative reduction of the protein band intensity of DTT-reduced antigen compared to control (or non-reduced) antigen was due to (i) a genuine effect from the reagent or (ii) less binding efficiency of the DTT-reduced protein to the nitrocellulose membrane compared to 2-ME reduced and non-reduced proteins is not known.

Immunoblot analysis using the MAb 17.9 was not included in the present study due to an unfortunate shortage of the ascitic fluid at that specific moment. However, the MAb has already been shown to recognise only non-reduced Pbs21 in Chapter 2 (Figure 2.5).

ELISA was also developed to study the conformational structure of the epitopes recognised by the transmission-blocking MAbs. All antibodies 12.1, 13.1 and 17.9 recognised non-reduced Pbs21 protein (Figure 4.16, A, B and C respectively). The MAbs 12.1 and 13.1 but not 17.9 also combined with DTT-reduced protein with a reduced reactivity. Similar results were observed with the polyclonal sera (Figure 4.16 D). Hierarchy of the reactivity was, therefore, non-reducing > DTT-reducing > 2-ME reducing conditions. This was consistent with the observations that binding of ^{125}I-labelled Pbs21 to the microtitre plate under non-reducing > DTT-reducing > 2-ME reducing conditions (Figure 4.17).

Despite the similarity between immunoblot analysis and ELISA under non-reducing and DTT-reducing conditions, a major discrepancy between the 2 techniques also emerged. In immunoblot techniques, the MAbs 12.1, 13.1 and the polyclonal monospecific sera recognised 2-ME reduced Pbs21 (Figure 4.15, lanes 3, 6, 12 respectively) with no significant difference from non-reduced protein (Figure 4.15, lanes 1, 4, 10 respectively). In ELISA, reactivity of the

antibodies to 2-ME reduced protein was totally lost (Figure 4.16 A, B and D for the MAbs 12.1, 13.1 and polyclonal monospecific sera respectively). This was probably due to the lack of binding of 2-ME reduced protein to the microtitre plate as assessed using the ^{125}I-labelled protein (Figure 4.17).

The mechanism of protein interactions to nitrocellulose membrane and microtitre plate might not be the same. Binding of proteins to the microtitre plate was by hydrophobic interaction (J. E. Lillywhite, pers. comm.). Protein interactions with nitrocellulose were not clearly understood (Gershoni and Palade, 1983). At pH ~8, i.e. the pH at which protein electroelution was usually performed, nitrocellulose was negatively charged as were the proteins being adsorbed. Hydrophobic effects probably played a role in this interaction.

Therefore, it has to be borne in mind that although ELISA is more advantageous in terms of the simplicity and speed compared to immunoblot analysis, it could be less advantageous for the study of the conformational structure of a protein antigen, due to loss of the binding capacity of the protein upon reduction to the microtitre plate.

Having established that the MAbs 12.1 and 13.1 reacted with Pbs21 on both non-reducing and reducing SDS-PAGE, it is therefore suggested that the epitopes I and II were unlikely to be conformation-dependent. By contrast, the epitope recognised by the MAb 17.9 (epitope III) was conformation-dependent. The conformational structure of the epitopes I-III in Pbs21 can thus, be proposed in Figure 4.19.

Using ELISA, all MAbs recognised periodate-treated Pbs21 suggested that post-translational modifications by glycosylation might not be crucial in contribution to the immunogenicity of the epitopes I-III that involved in the transmission-blocking immunity. This was consistent with the previous findings that the protein antigen was not likely to be heavily glycosylated (A. L. Dearsly ; pers. comm.). It has been indicated that the immunological activity of the major epitopes in Pfs25 was not carbohydrate-dependent. In fact, post-translational modification with fatty acid (palmitic acid) was more important to the immunogenicity of the epitopes than glycosylation (Fries *et al.*, 1989). By contrast, the antigenicity of the asexual stages proteins of *P. faciparum* was determined by carbohydrate moieties (Ramasamy and Reese, 1985 and 1986).

In human malaria parasite *P. falciparum*, the number of epitopes on Pfs48 and 45 proteins was characterised using two-site immunoradiometric assay (IRMA) (Carter *et al.*, 1985 ; Vermeulen *et al.*, 1985b). There were at least 3 B-cell epitopes (epitopes I-III) in Pfs48/45 and none of the epitopes were repeatedly expressed (Carter *et al.*, 1988a). It was observed that the antibodies to epitope I blocked transmission when they were present singly. The 2 other epitopes (epitopes II and III) were recognised by the MAbs which must be present together to suppress infectivity. Epitope II was found to be variant in a minority of *P. falciparum* isolates (Graves *et al.*, 1985 ; Carter *et al.*, 1988a). The study was extended to identify the epitopes

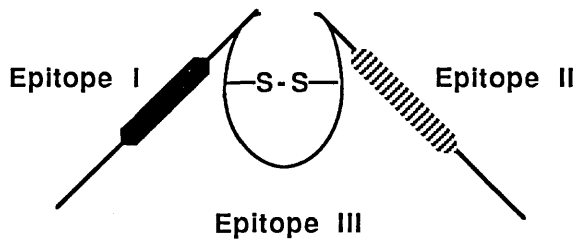


Figure 4.19 Proposed conformational structure of the epitopes I-III recognised by the transmission-blocking MAbs 12.1, 13.1 and 17.9 respectively in Pbs21 antigen :- Epitopes I and II are not likely to be conformation-dependent. However, the degree of linearity of these 2 epitopes is not known. By contrast, Epitope III is conformation-dependent and the epitope topologically overlapped between the first 2 epitopes. In this proposal, epitope III lies within an area of the loop. S-S represents a number of disulphide bonds in epitope III. Apparently, none of the epitope is repeatedly expressed.

recognised by naturally occurring antibodies using competitive ELISA (Graves *et al.*, 1988b) and epitope III was found to be a naturally immunogenic epitope. All of these 3 epitopes on Pfs48/45 proteins were conformation-dependent (Carter *et al.*, 1988a). Recently, 4 epitopes (namely A-D) recognised by the transmission-blocking MAbs in Pfs48/45 proteins were reported (Prof. G. A. T. Targett, pers. comm.). Epitope C was found to be a linear epitope and the other 3 epitopes were not different from epitopes I-III reported by Carter *et al.*, (1988a).

Not only Pfs48/45 but also Pfs230 protein was identified as the target of transmission-blocking MAbs on gametes of *P. falciparum* (Quakyi *et al.*, 1987). The presence of antibody against Pfs230 protein in immune human sera form Papua New Guinea correlated strongly with the suppression of gametocyte infectivity to mosquitoes (Graves *et al.*, 1988a). There were at least 2 conformation-dependent epitopes in the protein antigen (Carter *et al.*, 1988a). However, further details of the epitopes in Pfs230 are not known.

The number of epitopes on Pfs25 were also characterised using two-site IRMA (Vermeulen *et al.*, 1985b). Two major epitopes based on the recognition of transmission-blocking and non-blocking MAbs were non-repeatedly expressed (Vermeulen *et al.*, 1985b & 1986). Moreover, the epitopes were conformation-dependent (Fries *et al.*, 1989). The protein after reduction by DTT was not recognised by the MAbs. Recently, the gene encoding this protein antigen was cloned and 4 repeated EGF (Epidermal Growth factor)-like domains were discovered (Kaslow *et al.*, 1988). The relationship of the repeated sequence to the immunogenicity of the epitopes is not known. A notable feature was a high content of cysteine residues (11%) in the deduced amino acid sequence of Pfs25. This finding was consistent with the biochemical study of the protein that all the MAbs recognised only non-reduced Pfs25 in immunoblot analysis (Carter *et al.*, 1988a).

In *P. gallinaceum*, there was at least 1 epitope in Pgs31 (Carter *et al.*, 1988a). Details of the epitope study for this antigen are limited. Moreover, epitope study on the sexual stages antigens of other rodent malaria parasites is not well-characterised.

CHAPTER 5

GENERAL DISCUSSION

In this Chapter, the discussion seeks to present a summary of the major conclusions and to outline aspects of future work.

5.1) Transmission-blocking immunity in *P. berghei*

MAbs against the zygotes-ookinetes have been produced and the ascites shown to suppress gametocyte infectivity to mosquitoes by greater than 93% using membrane feed and passive transfer experiments (the results presented in Chapter 2 and Winger *et al.*, 1988). The MAbs also suppressed ookinete formation *in vitro* by 91.87-100% (Table 2.5). Using SDS-PAGE and immunoblot analysis, the MAbs recognised a protein determinant of M_r 21 kD (Pbs21) (Chapter 2). The protein was purified using affinity chromatography and the purified protein showed considerable homogeniety which was used in subsequent studies (Chapter 3). Full suppression of transmission was achieved when the mice were immunised twice with 20 µg of Pbs21 by the intramuscular route without adjuvant. Persistence of transmission-blocking immunity in the mice was at least 34 weeks after the first immunisation. Subsequent challenge did not boost the immunity.

5.2) B-cell epitopes in Pbs21

Epitopes in Pbs21 recognised by the transmission-blocking MAbs were analysed (Chapter 4). Apparently, there were at least 3 B-cell epitopes (I-III) recognised by the MAbs 12.1, 13.1 and 17.9 respectively. Epitopes I and II were unlikely to be conformation-dependent, as the ascitic fluids 12.1 and 13.1 still reacted with the protein on both non-reducing and reducing SDS-PAGE (the results in Chapters 2 and 4). However, the degree of the linearity of the epitopes I and II was not known. By contrast, epitope III was conformation-dependent as MAb 17.9 recognised the protein on non-reducing but not reducing SDS-PAGE. From the results of competitive and two-site ELISA (Chapter 4), epitopes I and II were at a distant sites on the protein molecule while epitope III topologically overlapped with both epitopes I and II. It is not yet known if any of the 3 epitopes is immunodominant.

Diversity of the epitopes I-III in Pbs21 among other strains of *P. berghei* is not yet resolved. Nor is it known whether the epitopes I and III cross-reacted with other species of murine malaria parasites. However, the MAb 13.1 showed cross-reactivity with *P. yoelii* (R. E. Sinden, unpublished data) suggested that epitope II might be less variant. The observations that synthesis of the protein was detected after gametogenesis of gametocytes in mosquito midgut (Sinden *et al.*, 1987 ; A. L. Dearsly, pers. comm.) imply that the host immune response to the protein would not be induced during 'natural' infection. Pbs21 might not, therefore, be under selective pressure to evolve immune evasion and hence the antigenic diversity.

5.3) Future aspects of the study

Whilst Pbs21 has already been characterised to some extent in this thesis, other aspects of the study still need further investigation.

1) Biochemical aspects of Pbs21

Properties of the target antigens of transmission-blocking MAbs on zygotes-ookinetes of *P. berghei* (Pbs21), *P. falciparum* (Pfs25) and *P. gallinaceum* (Pgs31) are summarised in Table 5.1. Although Pbs21 shares common features with Pfs25, considerable differences also emerge.

a) Pfs25 was poorly labelled with ^{125}I (Vermeulen *et al.*, 1985a & 1986) and ^{35}S-methionine (cited by Kaslow *et al.*, 1988). In contrast, Pbs21 was readily labelled with ^{125}I. The specific activity of the ^{125}I-labelled protein was between 1.13-2.59 x 10^7 cpm/µg protein (Chapter 3 ; Figure 3.1). The protein was also readily labelled with ^{35}S-methionine (A. L. Dearsly, pers. comm.).

The difficulty in labelling Pfs25 with ^{125}I was consistent with the observations of Good *et al.*, (1988) that the anti-Pfs25 antibody was more readily detected when immune sera were used to immunoprecipitate the protein from ^{35}S-cysteine metabolically labelled zygotes rather than ^{125}I surface-labelled zygotes. The contents of tyrosine residues in Pbs21 & Pfs25 are different and Pbs21, therefore, appears on this evidence to have a higher tyrosine content than Pfs25.

(b) The epitopes recognised by the transmission-blocking MAbs in Pfs25 were all conformation-dependent (Vermeulen *et al.*, 1985a, 1986 ; Carter *et al.*, 1988a ; Fries *et al.*, 1989). This is consistent with the content of cysteine residues from the deduced amino acid sequence which was as high as 11% (Kaslow *et al.*, 1988). In Pbs21, most of the epitopes recognised by the transmission-blocking MAbs were not likely to be conformation-dependent (the results presented in Chapter 2 and 4). It is, therefore, suggested that cysteine content in Pbs21 might not be as high as Pfs25. However, this remains to be further examined by the assessment of the incorporation of radioactive material into the protein from ^{35}S-cysteine metabolically labelled ookinetes or prediction from the sequence of amino acids or nucleic acids.

Other biochemical aspects of Pbs21 not yet resolved include the question as to whether the protein is acylated, and whether it is important to the immunogenicity of the protein. The study of acylation in Pbs21 would highlight this aspect of post-translational modification in another malarial antigen and hence have an impact on transmission-blocking vaccine development. Both Pgs31 and Pfs25 have been reported to be labelled with ^{3}H-palimitic acid and ^{3}H-myristic acid (Kumar and Carter, 1985a ; Fries *et al.*, 1989). Additionally, post-translational modification via acylation has a more important role than glycosylation for the immunogenicity of Pfs25 (Fries *et al.*, 1989).

TABLE 5.1 Summary of the characteristics of the target antigens on zygote-ookinetes of 3 species of *Plasmodium*.

Remarks	Pbs21*	Pfs25**	Pgs31***
Expression of the protein in gametocytes prior to gametogenesis	-	-	-
Cytoplasmic precursors detected	-	-	-
Post-translational modification of the protein via :-			
glycosylation ♠	+	+	+
Acylation ♣	not known	+	+
Approximate pI values	~7	5.6 ± 0.1	not known
Minimum number of epitopes in the proteins detected to date	3 (Epitope I-III)♦	2 (Epitope I-II)	1
Immunoreactivity of the epitopes on reducing SDS-PAGE & immunoblot	Epitope I + Epitope II + Epitope III -	Epitope I - Epitope II -	not known

*Taken from Sinden *et al.*, (1987) ; Winger *et al.*, (1988) and A. L. Dearsly, pers. comm.

**Taken from Vermeulen *et al.*, (1985a, b & 1986) ; Carter *et al.*, (1988a & 1989) ; Fries *et al.*, (1989).

***Taken from Kumar and Carter (1983 & 1985a, b) ; Carter and Kaushal (1984) ; Kumar (1985 & 1987) and R. Carter, pers. comm.

♠Glycosylation was determined from the observations that ^{3}H-mannose or ^{3}H-glucosamine was incorporated into the protein.

♣Acylation was determined from the observations that ^{3}H-palmitic acid or ^{3}H-myristic acid was incorporated into the protein.

♦Epitope I, II and III are recognised by the MAbs 12.1, 13.1 and 17.9 respectively.

Covalent binding of fatty acids (palmitic acid) in an ester (serine, -OH group) or thioester (cysteine, -SH group) linkage has been shown to be a post-translational modification of parasite proteins e.g. variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Ferguson and Cross, 1984), Pgs31 (Kumar and Carter, 1985a) and Pfs25 (Vermeulen *et al.*, 1985a & 1986 ; Fries *et al.*, 1989). The functional significance of protein acylation in the ookinete is not known. However, it is suggested that fatty acids covalently linked to the ookinete surface antigens might facilitate the interaction of ookinetes with mosquito tissue, during their passage through the mosquito gut wall (Kumar and Carter, 1985a).

However, it is not usual for myristic acid to be bound to membrane-bound proteins via ester or thioester linkages (Towler and Glaser, 1986), and it is possible that myristic acid (or palmitic acid) is a component of a diacylglyceride linked to the C-terminus of a protein via a glycosyl-phosphatidylinositol bond. This mechanism of membrane anchoring of proteins by a covalently attached glycosyl phosphatidylinositol moiety involving myristic acid has been reported in parasitic protozoa e.g. *T. brucei* (Ferguson and Cross, 1984) and *P. falciparum* (Haldar *et al.*, 1985).

Pbs21 can be isolated by temperature-dependent phase separation using the non-ionic detergent Triton X-114 (A. L. Dearsly, pers. comm.) which implies that it is an integral membrane protein. The mechanism of membrane anchoring of the protein is not known. However, if the protein could be labelled with ^{3}H-myristic acid and cleaved with phosphotidylinositol-specific phospholipase C (which remains to be investigated), it might be possible that the protein is anchored to the membrane by a covalently attached glycosyl phosphatidylinositol moiety.

2) Transmission-enhancing effect

Not only transmission-blocking but also transmission-enhancing has been reported in *P. vivax* (Mendis *et al.*, 1987b, Peiris *et al.*, 1988). Using a single anti-Pvs42/36 MAb at different concentrations, both suppression and enhancement of transmission was mediated. These observations indicated that the same target epitope must be involved in both effects of transmission. In our system, the enhancement of transmission was also observed using a single dilution of the ascites 1.2 (Chapter 2 ; Table 2.3) and in some immunisation trials using Pbs21 as an immunogen (Appendices 3 and 4). However, the mechanism of the enhancement of transmission by the MAb and the immune sera might be different. Using a single MAb, the same target epitope must be involved in both effects of transmission. Using immune sera, on the contrary, the effects could be mediated by antibodies against the same or different epitopes. It is, therefore, not conclusive whether a single epitope in Pbs21 would exhibit both blocking and enhancing effects of transmission. The detailed study of both effects of transmission is feasible by reconstituting different concentrations of purified IgG from the MAbs 12.1, 13.1, 17.9, or

polyclonal monospecific mouse sera (produced from mice that had been immunised with Pbs21) with *P. berghei* gametocyte-infected blood and then offered to mosquitoes in membrane feeding.

3) Transmission-blocking vaccine

As described earlier, Pbs21 served as a transmission-blocking vaccine candidate which prevent infected individuals from transmitting the parasite to the biting vector throughout the duration of infection in the host. In future studies, 3 kinds of immunogens could be tested for their efficacy as a vaccine candidate i.e. native molecule, recombinant and synthetic peptide proteins.

a) Native molecule

Although T cell-mediated mechanisms have not yet been examined in the transmission-blocking immunity of *P. berghei*, it is anticipated that antibody-mediated mechanisms might have important roles in such immunity and the T cell will play a helper role. In addition, Pbs21 is not naturally immunogenic. To improve the immunisation scheme, the use of adjuvants has to be sought in an attempt to produce a single vaccination that conveys long-lasting immunity in the host.

b) Recombinant protein molecule

It has been established that most of the epitopes in Pbs21 were not likely to be conformation-dependent and glycosylation might not have a crucial role in the immunogenicity of the epitopes (as the MAbs 12.1, 13.1 and 17.9 still recognised periodate-treated Pbs21 ; Chapter 4), it is, therefore, feasible to screen the DNA library of *P. berghei* using the MAbs 12.1 and 13.1. This is now underway (M. Paton, pers. comm.). It might be possible to screen for clones producing recombinant protein equivalent to the whole molecule (~ 570 bp) or a single epitope of the protein (e.g. clones that are positive to the MAb 13.1 but negative to 12.1) from the DNA library. Efficacy of a single epitope (compared to native molecule) as a vaccine candidate could then be assessed.

c) Synthetic peptide

Peptides could be constructed from the amino acid sequence of a single epitope (available from amino acid sequencing or prediction from the gene sequence) then assessed as a vaccine candidate.

The efficacy of a subunit vaccine or recombinant vaccine could be investigated. Optimal methods for immunisation against certain synthetic antigens may require the use of adjuvants e.g. aluminium hydroxide (Alum), liposomes or combinations of liposomes with lipid A. The recombinant protein $R32tet_{32}$ (which contained 32 tetrapeptide repeats from the CS protein of *P. falciparum* and 32 amino acids derived from the tetracycline resistant gene of the cloning vector) has been shown to be more immunogenic when the protein was encapsulated with the Alum-adsorbed liposomes containing Lipid A (Richards *et al.*, 1988). Alternatively, Pbs21

peptides could be coupled to a carrier molecule (proteosome-lipopeptide) as reported by Lowell *et al.*, (1988).

The use of the protein from peptide synthesis and recombinant DNA technology as a vaccine candidate would be more advantageous than the native molecule in terms of the unlimited production of the materials.

In case of the transmission-blocking vaccine candidate Pfs25, the protein was not shown to be naturally presented since the antigen was not recognised by the immune human sera from Papua New Guinea (Carter *et al.*, 1989). This may be because the expression of the protein was found only on the sexual stages of the parasite in the mosquito bloodmeal but not in the asexual stages circulating in the human host and thus, the protein may not have been under evolutionary selection pressure to be of restricted immunogenicity in human population (Carter *et al.*, 1989). This idea was supported by the observations in animals that there was a widespread of the antibody response to the protein in various H-2 congenic mice (Good *et al.*, 1988) and Kaslow *et al.*, (1989b) have failed to detect major variation in nucleotide sequence of Pfs25 in a number of *P. falciparum* isolates. Carter *et al.*, (1989) suggested that as a vaccine candidate, the protein could be widely recognised as an immunogen but it would suffer a drawback from the inability of the immunity to be boosted by natural infections. An ideal transmission-blocking vaccine remains, therefore, to be resolved.

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APPENDIX 1 SDS-PAGE and NEPHGE

Recipe of the reagents for SDS-PAGE and NEPHGE will be described here. All reagents were ultrapure grade obtained from BDH, Sigma or Pharmacia.

1.1) SDS-PAGE ; Electrophoresis was performed at 4 mA overnight with tap water cooling system.

1) 30% Acrylamide stock solution

	gm in 250 ml
Acrylamide	75.0 gm
Bis-acrylamide	2.0 gm
Amberlight ion exchange resin	2.0 gm

The solution was filterred through Whatman number 1 filter paper and store in the dark at 4°C.

2) 20% Acrylamide stock solution

	gm in 250 ml
Acrylamide	50.0 gm
Bis-acrylamide	1.25 gm
Amberlight ion exchange resin	2.0 gm

All steps were processed as described above.

3) Separating gel buffer (1.5 M Tris pH 8.8)

	gm in 250 ml
Tris	45.4 gm

pH to 8.8 with concentrated HCl.

4) Stacking gel buffer (0.5 M Tris pH 6.8)

	gm in 250 ml
Tris	15.2 gm
10% SDS	10.0 ml

pH to 6.8 with concentrated HCl.

5) Separating gel

a) 15% Homogeneous gel

	per 1 gel plate (size 16 cm x 18 cm x 0.3 cm)
30% Acrylamide stock	25.0 ml
Separating gel buffer	12.5 ml
Distilled water	12.5 ml

The solution was degassed before following reagents were added.

SDS	0.06 gm

10% ammonium persulphate (AMPS)	0.03	ml
TEMED	0.03	ml

b) 8-20% gradient SDS-PAGE

	per 1 gel plate			
	8%		**20%**	
30% Acrylamide stock	8.64	ml	21.44	ml
Separating gel buffer	8.0	ml	8.0	ml
Distilled w ater	15.36	ml	-	ml
Glycerol	-	ml	3.20	ml
The solutions were degassed before following reagents were added.				
10% SDS	0.32	ml	0.32	ml
10% AMPS	0.028	ml	0.028	ml
TEMED	0.028	ml	0.028	ml

Sixteen ml of each solution were mixed in gradient former.

6) Stacking gel (3%)

	per 1 gel plate	
20% Acrylamide stock	2.4	ml
Stacking gel buffer	4.0	ml
Distilled water	9.4	ml
The solution was degassed.		
10% AMPS	0.066	ml
TEMED	0.01	ml

1.2) Two-dimensional gel electrophoresis

First dimensional electrophoresis : Non-equilibrium pH gradient electrophoresis (NEPHGE), Electrophoresis was performed at 500 v for 4-5 h.

1) Acrylamide stock solution ; 28.38% Acrylamide and 1.62% bis-acrylamide.

2) NEPHGE Gel mixture

Urea	5.5	gm
Distilled water	2.0	ml
Acrylamide stock	1.33	ml
Ampholines pH 3.5-10	0.50	ml
After the gel was degassed, following reagents were added.		
10% NP-40	2.00	ml
TEMED	0.014	ml

10% AMPS	0.020	ml

The gel was poured into the glass rods and left for polymerisation.

3) Lysis buffer

Urea	2.75	gm
10% NP-40	1.0	ml
Ampholines pH 3.5-10	0.25	ml
DTT	0.04	gm
DNase stock	0.004	ml
RNase stock	0.004	ml

Added distilled water up to 5 ml, aliquoted and stored at -20°C.

For sample preparation, x µl of the sample was added with x µl of lysis buffer and x mg urea.

4) DNase stock (1 mg/ml DNase in 0.01 M Tris pH 7.4, 0.005 M $MgCl_2$).

RNase stock (50 mg/ml RNase in 0.01 M Tris pH 7.4, 0.005 M $MgCl_2$).

5) Overlay solution (1% ampholines in 8 M Urea).

The sample was loaded into the gel rod following with 20 µl of the overlay solution. The upper electrophoresis buffer was then used to overlay to the top of the rod.

6) NEPHGE electrophoresis buffer

Anodic buffer (Upper chamber) ; 0.01 M H_3PO_4

Cathodic buffer (Lower chamber) ; 0.02 M NaOH

The buffers were degassed before use.

7) Equilibration buffer

Glycerol	10	ml
10% SDS	50	ml
1 M Tris pH 6.8	6.25	ml
2% Bromophenol blue	1	ml
2-ME	5	ml
Distilled water	27.75	ml

pH to 6.8 with concentrated HCl

After the first dimensional electrophoresis was finished, the gel rod was equilibrated in the equilibration buffer for 15-60 min before it was loaded onto 8-20% gradient SDS-PAGE (described in section 1.1.5 b) for second dimensional electrophoresis .

1.3) Coomassie brilliant blue stain

1) Staining solution

Stock solution ; 2.5 gm of Coomassie brilliant blue R250 (Sigma) was dissolved in 454 ml methanol. The solution was then filterred through Whatman number 1 filter paper and kept at room temperature in the dark.

Working solution ;

Stock solution	138	ml
Glacial acetic acid	30	ml
Distilled water	138	ml

2) Destaining solution

For rapid destaining ; 40% methanol, 10% glacial acetic acid solution was used.

For destaining storage ; 10% methanol, 10% glacial acetic acid solution was used.

APPENDIX 2 Protease Inhibitors

Substance	MW	Final conc.(M)	Properties
EDTA (disodium salt)	364	0.001	Metal ion chelator
EGTA	380.4	0.001	Divalent metal ion chelator
N-ethylmaleimide	125.12	0.001	Protects sulfhydryl groups
p-hydroxymercuribenzoate	360	0.001	Protects sulfhydryl groups
1, 10-phenanthroline	180.2	0.001	Divalent cation chelator
PMSF	174.2	0.001	Serine & thiol protease inhibitors
TLCK	369.3	0.00135	Trypsin & thiol protease inhibitors
TPCK	351.9	0.0001	Chymotrypsin inhibitor

All reagents were obtained from Sigma.

APPENDIX 3 Immunisation summary

Immunisation trials (groups A-M) were performed. In each group, the mice were divided into 2 subgroups (1 and 2) (3 mice/subgroup). Subgroup1 was the control and subgroup2 was the immunised mice.

In the trials groups A, D, F, G, H, I, L and M, the mice subgroup2 were vaccinated with the affinity-purified Pbs21 once.

In the trials groups B, E, J and K, the mice were vaccinated and boosted once with appropriate concentration of the antigen (designated as x/y µg in the column under treatment). The intervening period between the 2 immunisations was ~ 1 month.

In the trial group C, the mice were boosted twice. Second boost was carried out 1/2 month later.

To assess the efficacy of the vaccines, the mice were infected with 10^7 *P. berghei* gametocyte-containing blood 2 weeks after the last boost. They were offered to mosquitoes 3 days later. The mosquitoes were kept at 19°C for 2 weeks before dissection. Number of oocysts was determined. %Transmission and % Blockade were calculated as described in section 3.2.3.5.

Mice group	Treatment	Mean no.oocysts /gut♦ ± S. D. (Range)	% Transmission	% Blockade	No. infected mosquito/ total
A 1	PBS	102.19 ± 48.16	100	0	*
2	0.5 µg Soluble Ag (Sol Ag)+CFA;SC	120.49 ± 105.54	117.91	-17.91	*
B 1	PBS	93.73 ± 66.27	100	0	*
2	0.5/1 µg Sol.Ag +CFA;S. C.	96.05 ± 62.77	102.48	-2.48	*
C 1	PBS	61.02 ± 52.08	100	0	*
2	0.5/1/5 µg +CFA S. C.	141.23 ± 91.63	235.5	-135.5	*
D 1	PBS	93.73 ± 66.27	100	0	*
2	1 µg Sol.Ag;I. V.	81.80 ± 71.59	87.27	12.72	*
E 1	PBS	61.02 ± 52.08	100	0	*
2	1/1 µg Sol.Ag;I.V.	161.24 ± 7.82	264.24	-164.24	*
F 1	PBS	202.32 ± 76.51 (0-525)	100	0	40/42
2	2 µg Sol.Ag;I. V.	193.09 ± 57.54 (0-591)	95.44	4.56	31/33
G 1	PBS	202.32 ± 76.51 (0-525)	100	0	40/42
2	20 µg Sol.Ag;I. V.	219.75 ± 54.34 (0-680)	108.62	-8.62	40/43
H 1	PBS	202.32 ± 76.51 (0-525)	100	0	40/42
2	2 µg Acetone precipitate Ag;I.V. (Ac.Ag)	338.34 ± 192.51 (1-1,282)	167.23	-67.23	25/25

Mice group	Treatment	Mean no.oocysts /gut♦ ± S. D. (Range)	%Transmission	%Blockade	No. infected mosquito/ total
I 1	PBS	202.32 ± 76.51 (0-755)	100	0	32/56
2	20 µg Ac.Ag;I. V.	120.67±18.45 (0-611)	59.64	40.36	48/53
J 1	PBS	106.9 ± 44.43 (0-755)	100	0	32/56
2	2/2 µg Ac.Ag;I.V.	98.2 ± 72.24 (0-413)	91.86	8.14	50/63
K 1	PBS	106.9 ± 44.43 (0-755)	100	0	32/56
2	20/20 µg Ac.Ag; I. V.	36.08 ± 7.85 (0-298)	33.75	66.25	37/65
L 1	DEA eluate**	76 ± 47.24 (0-942)	100	0	49/66
2	5 µg Ac.Ag;I. M.	110.11 ± 28.75 (0-657)	144.88	-44.88	87/102
3	5 µg 7.8 Ac.Ag*** ;I. M.	109.1 ± 28.44 (0-704)	143.55	-43.55	60/70
M 1	PBS	110.12	100	0	53/66
2	50 µg Ac.Ag;I. M.	100.01 ± 22.32 (0-391)	93.26	6.74	31/102

Subgroup 1 = Control mice

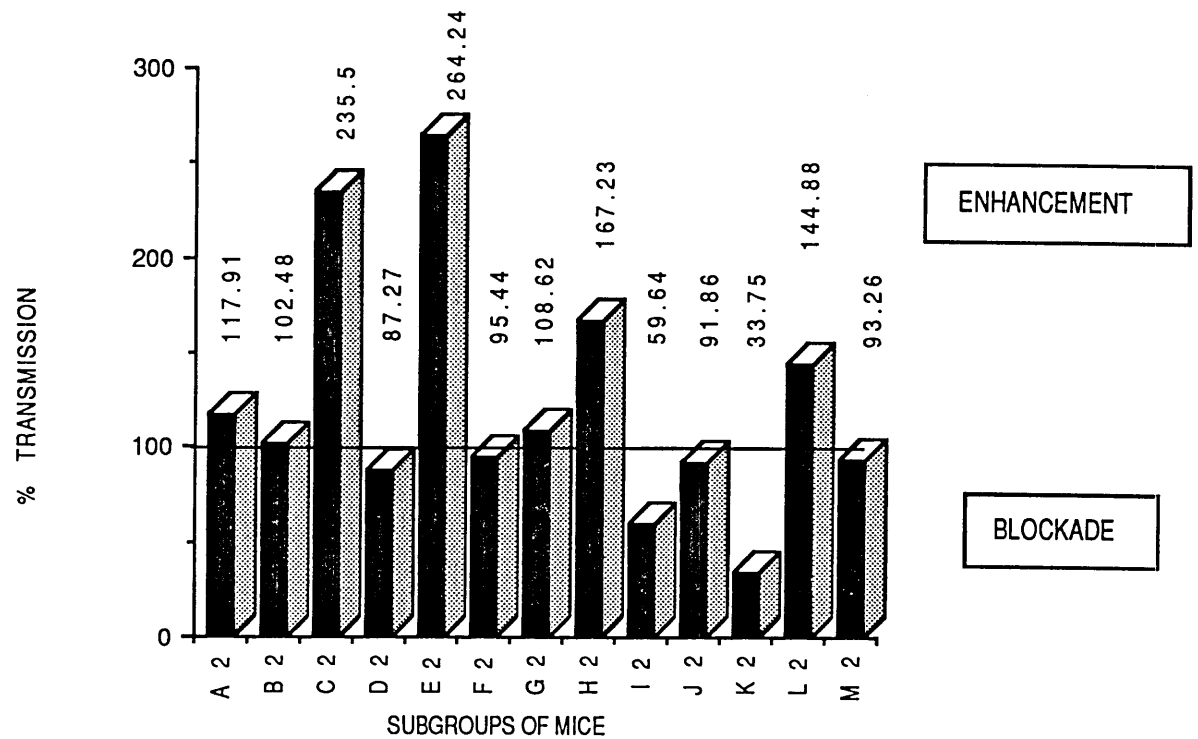
Subgroup 2 = Vaccinated mice

* = Dr. L. A. Winger did not have the detailed data.

** = Peak fractions eluted from the affinity column before the solubilised parasite proteins were loaded for the purification of Pbs21.

*** = Merozoite antigen of *B. divergens* ; used as a negative control.

♦ = The arithmetic mean number of oocysts/gut from total mosquitoes.



APPENDIX 4 Diagram summarised the immunisation trials from Appendix 3

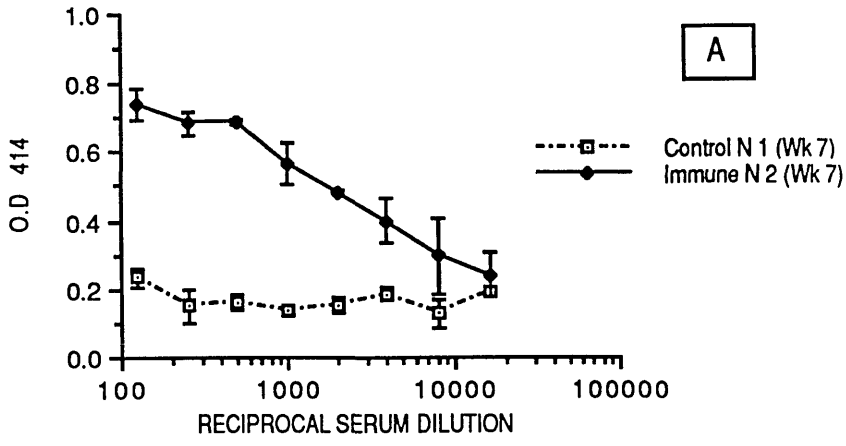
APPENDIX 5 ELISA demonstrating antibody titration of the control and immune sera from the mice groups N and O against Pbs21 antigen. Details of the immunisation trials were described in Chapter 3 (section 3.2.3.2).

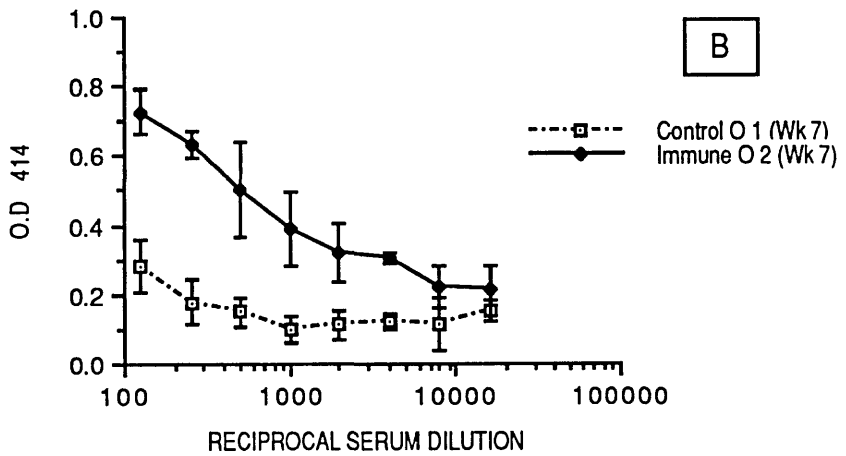
A :- Group N sera collected at week 7 post-immunisation. The mice were divided into 2 subgroups (3 mice/subgroup) i.e. N 1 (control) and N 2 (vaccinated mice that were immunised twice with 5/20 μg of Pbs21).

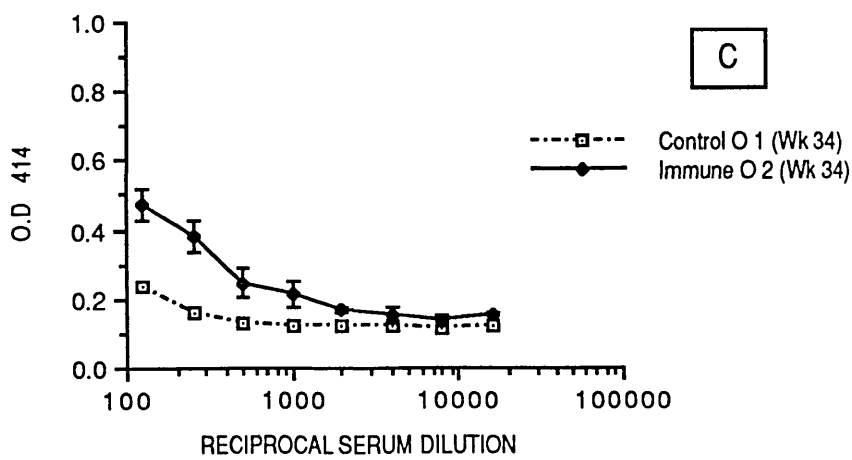
B :- Group O sera collected at the first blockade assessment (week 7 post-immunisation). The mice were divided into 2 subgroups (3 mice/subgroup) i.e. O 1 (control) and O 2 (vaccinated mice that were immunised twice with 20/20 μg of Pbs21).

C :- Group O sera collected at the second blockade assessment (week 34 post-immunisation). Details were as described in B.

At each point, all $O.D_{414}$ values are the arithmetic mean from 3 individual mouse serum in each subgroup (except group O at the second blockade assessment, the values were obtained from 2 mice/subgroup) ± S. D. The control sera (subgroup N 1 and O 1) are illustrated in dotted lines and the immune sera (subgroup N 2 and O 2) are illustrated in solid lines.


A
Control N 1 (Wk 7)
Immune N 2 (Wk 7)
O.D 414
1.0
0.8
0.6
0.4
0.2
0.0
100
1000
10000
100000
RECIPROCAL SERUM DILUTION


B
Control O 1 (Wk 7)
Immune O 2 (Wk 7)
O.D 414
1.0
0.8
0.6
0.4
0.2
0.0
100
1000
10000
100000
RECIPROCAL SERUM DILUTION


C
Control O 1 (Wk 34)
Immune O 2 (Wk 34)
O.D 414
1.0
0.8
0.6
0.4
0.2
0.0
100
1000
10000
100000
RECIPROCAL SERUM DILUTION

APPENDIX 6 Level of the 'anti-Pbs21' IgG in the immunised mice subgroups N 2 (that were immunised twice with 5/20 µg) and O 2 (that were immunised twice with 20/20 µg) calculated using ELISA as described in section 3.3.3. Sera from the mice subgroup N 2 (3mice) were collected by the time the mice were offered to mosquitoes (week 7 after immunisation). Sera from the mice subgroup O 2 (3 mice) were collected from week 7-39 after immunisation.

Subgroup of mice			
Week post-immunisation		µg IgG/ml serum	
	N 2.1	**N 2.2**	**N 2.3**
7	279.33	277.29	275.33
	O 2.1	**O 2.2**	**O 2.3**
7	95.50	278.00	176.50
9	38.42	dead	72.33
11	44.25		11.50
15	55.33		33.33
16	70.0		26.67
27	23.75		38.38
28	20.00		38.50
30	10.25		36.25
34	38.00		37.99
39	dead		27.12