BLOOD-STAGE MURINE MALARIA : IDENTIFICATION OF PROTECTIVE ANTIGENS FOR VACCINATION

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ABSTRACT

A Triton X-100 soluble lysate prepared from heavily parasitized erythrocytes of blood-stage *P.yoelii* malaria has been shown to be an effective vaccine against this fatal infection in mice. The large number of antigens present within this lysate were fractionated and tested for their ability to protect against live challenge and for their ability to stimulate parasite-specific T-helper cells and T cells responsible for delayed hypersensitivity (DTH). Enzymatic digestion of the protective fractions with either proteases or lipases suggested that the protective moiety of the antigens is mainly protein. Amino acid sequence analysis of the most protective antigens was attempted.

A two-step physical separation procedure was established for fractionation of the lysate. Isoelectric focussing (IEF) or Sephacryl S.200 gel filtration chromatography (S.200) were used as first step purification procedures. This was essential for the removal of impurities such as lipoprotein complexes and excess detergent from the lysate, thereby making it more suitable for final purification by High (HPLC) or Fast (FPLC) Performance Liquid Chromatography.

Several fractions with varying degrees of protection were isolated. The most consistently protective fractions appeared around the pH4, 6 and 8 regions on IEF. The pH4 fraction contained six protective FPLC purified components. The pH6 and 8 fractions contained one and six semi-protective HPLC purified components respectively. Other consistently protective fractions included Peaks I and II (S.200) and an HPLC purified fraction (fraction **h**) derived from Peak I. A partial amino acid sequence of fraction **h** has been obtained; weight for weight this is the most protective preparation available.

There was a significant correlation between protection and T- helper cell priming with the most protective antigens. However S.200 Peak I and its HPLC purified fraction **h** were relatively weaker stimulators of DTH. Thus although T-helper cell priming may be an essential requirement for the induction of protective immunity against malaria, strong priming for DTH

may not always be necessary. Selecting antigens that are strong stimulators of T-helper cell responses for antibody ("T_H2" type of T cell) and to a lesser extent for DTH ("T_H1" type), may therefore be important in terms of vaccine development.

To my wife Carol
and my children Neville and Naomi

"The hour of the greatest triumph must be the hour of the greatest humility".
Gandhi

Publications relevant to project

1) Playfair, J.H.L., de Souza, J.B., Freeman, R.R., and Holder, A.A. (1985). Vaccination with a purified blood-stage malaria antigen in mice: correlation of protection with T cell mediated immunity. *Clin. Exp. Immunol.*, **62**, 19-23.

2) Playfair, J.H.L., and de Souza, J.B. (1986). Vaccination of mice against malaria with soluble antigens. I. The effect of detergent, route of injection, and adjuvant. *Parasit. Immunol.*, **8**, 409-414.

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4) de Souza, J.B., and Playfair, J.H.L. (1988). Immunization of mice against blood-stage *Plasmodium yoelii* malaria with isoelectrically focused antigens and correlation of immunity with T cell priming *in vivo*. *Infect. Immun.*, **56**, 88-91.

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
A_{280}	Absorbance at 280nm
$Al(OH)_3$	Aluminium hydroxide
BCG	Bacille Calmette-Guerin
BMC	Bone marrow cell
CD4	T cell marker of MHC class II restriction
CD8	T cell marker of MHC class I restriction
cDNA	Complementary deoxyribose nucleic acid
CSP	Circumsporozoite protein
CFA	Complete Freunds Adjuvant
CFF	Crisis forming factor
$D.H_2O$	Distilled water
DNA	Deoxyribose nucleic acid
DTH	Delayed type hypersensitivity
EDTA	Ethylenediamine tetraacetate
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
FSV-1	Falciparum sporozoite vaccine - 1
FIRA	Falciparum interspersed repeat antigen
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosylphosphatidyl inositol
Hcl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Histidine rich protein
IEF	Isoelectric focussing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
i.v.	intravenous
kD	Kilo Dalton
KHARP	Knob-associated histidine-rich protein
LPS	Lipopolysaccharide
LSA	Liver stage antigen
MESA	Mature parasite-infected erythrocyte surface antigen
MHC	Major histocompatibility complex
MSA	Merozoite surface antigen
M_r	Relative molecular mass
MRBC	Normal mouse red blood cells
NANP	Asparagine-alanine-asparagine-proline tetrapeptide (from CSP repeat)
NO	Nitrous oxide
NK	Natural killer cell
OH	Hydroxyl radical

PBS	Phosphate buffered saline
Pb XAT	Irradiation attenuated *Plasmodium berghei*
Pf EMP 2	Mature parasite-infected erythrocyte surface antigen
PMN	Polymorphonuclear leucocyte
PMSF	Phenyl methyl sulphonyl fluoride
PMMSA	Precursor to the major merozoite surface protein
PVDF	Poly(vinylidene flouride)
RBC	Red blood cell
RESA	Ring infected erythrocyte surface antigen
RNase	Ribonuclease
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RPMI 1640	Cell culture medium (Rosewood Park Memorial Institute)
SALSA	Sporozoite and liver stage antigen
S-antigen	Heat-stable, soluble serum antigen
SE	Standard error
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRBC	Sheep red blood cell
S.200	Sephacryl S.200 gel chromatography
S.300	Sephacryl S.300 gel chromatography
T_H	T helper cell subset
TX-100	Triton X-100
TNF	Tumour necrosis factor
TNS	Tumour necrosis serum
TNP	Trinitrophenyl
Tris	Tris (hydroxymethyl) aminomethane
UV	ultra violet
µg	microgramme
V_e	Exclusion volume
V_o	Void volume
WHO	World Health Organization

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1.0 INTRODUCTION

Malaria, probably one of the most important tropical diseases in the world, is caused by infection with sporozoa of the genus *Plasmodium*, transmitted by species of *Anopheles* mosquitoes. The life cycle of the parasite is complex involving the vector (mosquito) cycle and a host (human or animal) cycle, the latter being characterized by an exoerythrocytic or liver stage and an asexual blood stage which is responsible for the clinical signs and symptoms of the disease.

1.1 Historical Background

The name malaria is derived from the Italian *mal'aria* or bad air, as it was then thought that the cause of the disease was related to the foul air common to marshy areas. Paludism, another word used to describe malaria, is derived from the Latin *palus* or marsh, which also associates the disease with a damp environment.

The disease has been a scourge of mankind for centuries and its existence in Britain can be traced back to the 16th century (Dobson, 1989), although it possibly originated in Africa. Its spread to other parts of the world probably arose as a result of human migrations and colonization, and until the early part of this century, nearly two-thirds of the world's population was affected. The first breakthrough in terms of treatment came about in the early 17th century with the discovery of "Peruvian bark" (obtained from the Cinchona tree), from which an extract called "Jesuit's powder" was used to treat the fevers. This was followed much later by the discovery of malaria parasites in the red blood cells of man in 1880 through the work of Laveran. In 1878, Manson had put forward a

theory that malaria may be transmitted from man to man by mosquitoes, however the actual mode of transmission was elucidated by Ronald Ross in 1897, when developing forms of the malaria parasite were found in the body of a female *Anopheles* mosquito that had previously fed on the blood of a malarious patient. Further field studies by Bignami, Bastianelli and Grassi in 1889-99 and Manson in 1900 confirmed Manson's earlier theories that malaria was transmitted to man through the bite of an infected female Anopheles mosquito.

Control measures adopted during the 20th century - larvicides, insecticides, and antimalarial drugs - were very promising and in 1955 the World Health Organization (WHO) launched a global malaria eradication campaign. Its success was particularly impressive in Europe, North America, some parts of Asia, the USSR and Australia; however, success rates were somewhat lower in tropical countries, largely due to socio-economic factors. Success rates were also hampered by vector resistance to insecticides and resistance of the parasites to drugs. This led to a search for new antimalarial drugs and research into the development of new insecticides.

By the late 1960s the disease had been eradicated from the whole of Europe, most of North America including the whole of the USA, most of the Caribbean, large parts of northern and southern South America, Australia, Singapore, Japan, Korea and Taiwan (Bruce-Chwatt , 1985).

Despite these measures, there has been a resurgence of malaria in several tropical areas where initial eradication strategies were successful. Areas most affected by this resurgence were southern and south-east Asia, Central America and parts of South America but the situation in tropical

Africa to date remains unchanged. Imported malaria poses yet another problem, especially in areas where the disease had been eradicated. In Britain alone there were, on average, 1843 cases reported annually in the last decade, many of them acquired during short stays in the tropics (Bradley, 1989).

1. 2 MALARIA PARASITES

The microorganisms causing malaria, or the malaria parasites as they are commonly known, belong to the family Plasmodiidae and to the genus *Plasmodium.* Parasites from the family Plasmodiidae undergo two types of multiplication; asexual division (*schizogony*) in the vertebrate host and a single sexual multiplication (*sporogony*) in the mosquito host. Species of Plasmodia occur in reptiles, birds, and mammals and are transmitted by mosquitoes; mammalian Plasmodia are transmitted by anophelines and those of birds by culicines.

Over 120 species of Plasmodia have been described and of these about 55 occur in reptiles, 25 in birds, 20 in primates excluding man, 4 in man and 20-25 in rodents, bats or other mammals.

Plasmodium species of mammals have been the most intensively studied. Four species occur in man, *P. vivax, P. ovale, P. falciparum,* and *P. malariae.* Of these, only *P. malariae* occurs in African great apes, which do not, however, act as reservoirs of human infection. Several primate species have been maintained in laboratories and these include the most commonly used *P. knowlesi,* which has no counterpart in man. *P. cynomolgi, P. fieldi, P. fragile,* and *P. brasilianum* resemble *P. vivax, P. ovale, P. falciparum* and *P. malariae* respectively. Other species include *P.*

inui and *P. simium.* Primate malarias are very similar to those of man and are also transmitted by anopheline mosquitoes. They can infect humans and whilst accidental transmission is not uncommon, natural transmission is extremely rare.

Another major group of Plasmodia, now classified under the subgenus *Vinckeia,* occur in various murine and non-murine rodents, lemurs, bats, and other mammals. Plasmodia of African rodents are the most intensively studied of all the malaria parasites. There are four murine species, *P. berghei, P. yoelii, P. vinckei,* and *P. chabaudi.* Within these there are a number of subspecies, most of which can be identified only by using biochemical techniques. *P. berghei* was the first of these to be discovered in 1948 by Vincke and Lips, and was soon adapted to laboratory mice and rats, in which it produces full-blown infections. It is the most widely used species of rodent malaria parasite, particularly for chemotherapeutic trials. *P. yoelii* is less virulent than *P. berghei* and is therefore widely used by immunologists. While both these parasites invade reticulocytes, *P. vinckei* and *P. chabaudi* prefer mature red cells; the former being virulent and the latter less virulent. Virulent strains of *P. yoelii* and *P. chabaudi* also exist, thus providing malariologists with a wide range of model systems for various studies.

1. 2. 1 Life cycle of malaria parasites

The life cycle involves a phase of development in an invertebrate host (mosquito) followed by a phase of development in a vertebrate host. In mammals, the infection is caused by inoculation of infective forms (sporozoites) into the host by a female anopheline mosquito. The sporozoites circulate in the bloodstream and eventually enter the

parenchymal cells of the liver where they undergo a process of development and multiplication. This process, known as exoerythrocytic schizogony, results in the production of merozoites which enter the circulation, where they initiate an asexual blood-stage infection resulting in the release of merozoites which reinvade other red blood cells and after several asexual cycles they differentiate into gametocytes which are infective for mosquitoes. Merozoites released during the pre-erythrocytic phase were once thought to invade other hepatocytes and repeat the exoerythrocytic cycle, but this does not take place, and in many species once the liver stage is complete, the rest of the infection is confined to the blood. In some species, sporozoites in the liver remain dormant and mature to invade the blood months later (Garnham, Bray and 1982). Such forms, known as hypnozoites, are responsible for relapses. Relapses, which differ from recrudescences that result from persisting but low levels of parasites in the blood, occur in *P. vivax* and *P. ovale* infections of man. Recrudescences are common in *P. falciparum* infections.

1.3 HUMAN MALARIA

Human malaria is caused by four species of *Plasmodium* transmitted by about 60 species of *Anopheles* mosquito. These four species are, *P. malariae* (Laveran, 1880), *P. vivax* (Grassi and Felatti, 1890), *P. falciparum* (Welch, 1897) and *P. ovale* (Stephens, 1922). The disease at the present time is largely confined to the tropics and subtropics and the majority of people infected live in Africa where *P. falciparum* is the dominant parasite; in hyperendemic areas 90-95% of the population may become infected, although there is considerable variation from place to place. The number of clinical cases worldwide is estimated at 100 million per year and in 1986 alone, 489 million cases (of infection with all species) were

recorded (Sturchler, 1989) and 234 million of these were due to *P. falciparum*, of which 2.3 million were fatal. In Africa, high mortality rates due to *P. falciparum* are principally amongst children under 5 years old. This species of malaria parasite can also be fatal in non-immune adults such as visitors. In other parts of the world, considerable progress has been made in the control of malaria but large numbers of people, probably in the order of millions in India and Pakistan, for example, are still infected, but the death rate outside Africa is much lower.

1. 3. 1 Distribution of species

P. falciparum and *P. vivax* are the two most common species of human malaria parasite and are found in most malarious areas. *P. falciparum* is the predominant species in Africa, Haiti, Dominican Republic, French Guinea, Surinam, parts of Asia and Papua New Guinea. *P. vivax* predominates in Latin America, Turkey, the Indian subcontinent and China. *P. malariae* is widely distributed but is not as common as either *P. falciparum* or *P. vivax*. *P. ovale* occurs mainly in Africa and very rarely in other continents. *P. vivax* is not found in West Africa (Spencer, 1986).

1. 3. 2 Stable and Unstable Malaria

Unstable malaria occurs in countries such as India, Sri Lanka, Brazil and Ethiopia where the frequency of transmission is low during the dry season, but increases to epidemic proportions during the wet season. Consequently little immunity is developed, except during periods immediately following widespread epidemics, and as a result children and adults alike remain susceptible to infection. Stable malaria which is found throughout the countries of tropical Africa, where transmission is

frequent and where inhabitants receive multiple infections throughout the course of the year, by contrast leads to the development of effective immunity in all individuals except children under 5 years old.

1. 3. 3 Clinical Course

Clinical signs and symptoms of malaria appear during the erythrocytic phase of the life cycle. The hepatic (exoerythrocytic) phase of the cycle following sporozoite invasion is asymptomatic and represents the incubation period of the infection. The interval between the infecting mosquito bite and elevation of temperature above 37.8^{o} C is 11 to 14 days in *P. falciparum*, 13 to 17 days in *P. vivax* and *P. ovale*, and 28 to 30 days in *P. malariae* infections. These incubation times vary with the intensity of the infection, immune status of the patient, type of exposure to malaria (mosquito bite, transfusion or congenital), and with the dose and frequency of any chemoprophylactic agents taken by the patient.

All forms of human malaria are characterized by periodic fevers which coincide with schizont rupture and which occur every 48 hours for *P. vivax*, *P. ovale* and *P. falciparum* and 72 hours in the case of *P. malariae*; these infections are designated benign tertian, ovale tertian, malignant tertian, and quartan malaria respectively. *P. falciparum*, referred to as malignant tertian malaria because of its potential lethality, invades both young and mature erythrocytes and its cycle of multiplication is asynchronous. *P. vivax* and *P. ovale* prefer young erythrocytes, parasitaemias therefore being limited by the degree of reticulocytosis, and their cycle of multiplication is synchronous. In *P. malariae* infections, only senescent erythrocytes are invaded and parasitaemias are therefore

low; the cycle of multiplication is synchronous.

Following the incubation period, the clinical course of malaria proceeds via three successive stages. The first one, lasting 15 minutes to one hour, is the cold stage which starts with shivering (rigors), accompanied by a feeling of intense cold, a rapid but weak pulse, and cyanosis of the fingers and lips. The second, or the hot stage is marked by a distressing rise in body temperature to 40^{o}C (102^{o}F) or more. Other common symptoms of this phase are a dry and burning skin, intense headache, nausea and vomiting . This stage lasts from two to six hours. In the third stage (sweating stage), which lasts from two to four hours, there is profuse sweating initially, followed by a rapid drop in body temperature. Fatigue and weakness are also common. A typical attack, often beginning in the early afternoon, may last from eight to twelve hours.

1. 3. 4 Treatment

Clinical symptoms of malaria are treated with drugs called *schizonticides.* These compounds are, the 4-amino-quinolines, quinine, mepacrine, proguanil, pyrimethamine and a Chinese compound, qinghaosu (artemisinine). Antibiotics such as tetracyclines and sulphonamides are also used. Whilst these drugs inhibit the development of the erythrocytic forms of the parasite, the exoerythrocytic (liver) stages and gametocytes are inhibited by the 8-aminoquinolines, such as primaquine.

1.4 RODENT MALARIA

The discovery of rodent Plasmodia by Vincke and Lips in 1948, and later by Meir Yoeli, revolutionized malaria research and made the rodent model an important research tool. Of particular importance were early chemotherapeutic studies which led to the discovery of the mode of action of a number antimalarials, and immunological studies, which are to date attempting to unravel some of the complexities of the host-parasite association.

The natural hosts of rodent Plasmodia are the African thicket rats, from *Thamnomys* and *Grammomys* species, however these Plasmodia have been successfully adapted to infect the laboratory mouse, rat and hamster. Whilst infections in the natural hosts are characterized by parasitaemia of long duration (Landau & Boulard, 1978), the same parasites in laboratory mice give rise to short-term infections which are either lethal or self-resolving (described in section **1.2**); some of the characteristics of rodent malarias are summarized in Table 1. Based on serological cross-reactivity, Cox and Turner (1970) have shown that the four malaria parasites of mice can be classified into two antigenically distinct groups: (a) *P. chabaudi* and *P. vinckei,* and (b) *P. berghei* and *P. yoelii.*

Rodent malarias do not exactly resemble the human infections but, they do serve as useful model systems for studying various aspects of the immune response to malaria which cannot be studied in humans; immunosuppression, the role of cell-mediated immunity and antibody, immunopathology, and genetics of the immune response to malaria being the most important.

Table 1

The origin and some characteristics of rodent malarias

Species	Subspecies	Origin	HOST		Characteristics of infection
			Natural	Laboratory	
	P. berghei. (Vincke & Lips)	Katanga (Zaire)	Thicket rats *Grammomys surdaster, Praomys jacksoni, Leggada bella*	mice rats hamster musk rats	Non-fatal infection in adult rats (natural or laboratory). Lethal infection in mice
P. berghei. Group.	*P. yoelii.* Landau & Killick Kendrick (1966)	Central African Republic (CAR)	Thicket rat *Thamnomys rutilans*	mice, rats hamsters	Non-fatal infection in all types of host
	P. killicki. Landau, Michel & Adam (1968)	Brazzaville (Zaire)	*T.rutilans*	"	"
	P. nigeriensis. Killick-Kendrick (1973)	Nigeria	*T.rutilans*	"	Lethal in 50% of lab. mice
	P. vinckei. Rodhani (1952)	Katanga (Zaire)	*T. rutilans*	mice	Lethal infection in laboratory mice
	P. v. petteri. Carter & Walliker (1975)	CAR	*T. rutilans*	mice	"
P. vinckei. Group.	*P. v. lentum.* Landau, Michel, Adam & Boulard (1975)	Brazzaville (Zaire)	*T. rutilans*	mice,wild rat *Hybomys univittatus*	"
	P. v. brucechwatti. Killick-Kendrick (1975)	Nigeria	*T. rutilans*	mice, multimammate rats	"
P. chabaudi. Group.	*P.c.chabaudi.* Landau (1965)	CAR	*T. rutilans*	mice, multimammate rats	Non-lethal infection in all types of host
	P.c.adami. Carter & Walliker (1977)	Brazzaville	*T. rutilans*	"	

1. 5 IMMUNOLOGY OF MALARIA

Immunity to malaria under natural conditions is achieved only after repeated exposure to infection, may take years to develop and is not complete, as parasitaemias may persist in the absence of clinical illness. It has been suggested that maintenance of effective immunity depends on regular antigenic stimulation (Targett, 1984), and this is supported by evidence that immune individuals who return to areas of high transmission after spending periods of less than a year away from the endemic area become susceptible to infection (Cohen and Lambert, 1982). However, acquired immunity is not necessarily dependent on coexisting parasitaemia.

Immunity to blood-stage malaria operates via: a) natural or innate resistance mechanisms which determine whether an animal is susceptible to infection or not, and b) protective immunological mechanisms which control the parasitaemia and maintain a state of immunity in a susceptible host.

1. 5. 1 Innate immunity

Innate factors operating at the level of the red cell include: a) the absence of appropriate receptors for the parasite which are necessary for red cell invasion; this phenomenon is observed in West Africans whose erythrocytes lack the Duffy blood group antigens Fy^a and Fy^b, who are consequently immune to *P. vivax* infections (Miller *et al,* 1976). b) red cell haemoglobinopathies, which interfere with the intracellular environment of the parasite; these include G6PD deficiency, β–thalassaemia and Sickle cell anaemia, the latter being found in

approximately 20% of the population in some endemic areas (Marsh, Otoo, and Greenwood, 1987).

1. 5. 2 Role of antibody

There is no doubt that antibody plays a major role in malarial immunity. Epidemiological studies provide evidence that levels of specific antibody correlate with the degree of immunity to infection. Antibody titres to the asexual blood stage parasites are high in infants born to immune mothers, but wane within weeks after birth (McGregor *et al.*, 1965). Maternal anti-malarial antibodies provide protection against infection in the newborn infant, however during the first year of life, when these antibody titres have decayed, infants become susceptible to recurrent and severe attacks of the disease. A progressive rise in antibody levels throughout childhood is associated with clinical immunity.

The most direct evidence for the role of antibody in protection against infection comes from passive transfer studies, first carried out in humans by Cohen *et al.* (1961). Purified IgG from immune adults was effective in controlling the parasitaemia of *P. falciparum* and *P. vivax* infections of infants with acute malaria.

This protective effect has since been confirmed in rodents, although results varied according to the host-parasite combination in question. Young rats were protected against a lethal *P. berghei* infection by passively administered hyperimmune serum (Diggs and Osler, 1969). Similar treatment of mice only delayed the parasitaemia and the animals usually succumbed to the disease (Golenser *et al.*, 1975; Wells & Diggs, 1976); bolus quantities of hyperimmune serum were required for complete protection

(Wells & Diggs, 1976). T-cell depleted rats were protected against *P. berghei* by passively administered hyperimmune sera (Lourie & Dunn, 1972), suggesting that antibody alone is sufficient for protection. However, animals protected by passive antibody are not resistant to rechallenge (Zuckerman & Golenser, 1970).

The non-fatal *P. yoelii* infection in mice can also be controlled by antibody. In general, low doses of hyperimmune serum given at the time of challenge significantly delayed the onset of parasitaemia, whereas higher doses prevented infections (Freeman & Parish, 1981). Similar doses of protective serum did not, however, protect T cell-deprived mice from infection (Jayawardena *et al.*, 1978), suggesting that a host T cell response was required to prevent infection. Protection by vaccination in the lethal *P. yoelii* system correlates with antibody which can be passively transferred to give protection (Playfair & de Souza, 1979).

In *P. chabaudi* infected mice, protection does not always correlate with antibody activity. Hyperimmune sera and sera collected after a single infection cause a delay in patency and reduce peak parasitaemias in *P. chabaudi* infected mice (McDonald & Sherman, 1980), but large doses of high titre antibody given at the time of challenge exacerbate the infection (Wallace, 1989). Furthermore, cross-protection between different species of malaria found in recovered or drug-cured mice did not correlate with antibody cross-reaction (Cox,1970; Cox and Turner, 1970). Other studies using purified antigens also suggest that protection does not always correlate with antibody activity. For example, antibodies raised against a 230kD merozoite surface protein which acts as a potent vaccine against lethal *P. yoelii* in mice are not protective when passively transferred

(Freeman & Holder, 1983). This antigen does however induce a potent DTH-type T cell response (Playfair *et al.*, 1985).

Antibody also plays an important role in sporozoite immunity. Mice vaccinated with irradiated sporozoite developed high prechallenge titres of anti-sporozoite antibody which appeared to correlate with protection (Hansen, de Silva & Strickland, 1979). Although passive transfer of large quantities of anti-sporozoite antibodies do not totally protect mice from infection (Spitalny & Nussenzweig, 1973), low doses of monoclonal antibodies (10-100μg) raised against the CS protein protected mice from challenge with 1000 viable sporozoites (Potocnjak *et al.*, 1980). The most recent vaccination studies in humans (discussed in **1.6.1** & **1.7.1**) using an immunogenic portion of the CS protein as a vaccine suggest that only a few individuals develop antibodies to the peptide and there is no booster effect (discussed later).

1. 5. 3 The role of B cells

As antibody seems to play a major role in defence against infection, depletion of B cells should result in exacerbation of the disease. This is not always true and tends to vary with the host-parasite system being studied. Balb/c mice rendered B cell deficient by treatment with anti-μ sera from birth died when infected with the non-lethal 17X *P. yoelii* (Weinbaum *et al.*, 1976b; Roberts & Weidanz, 1979), suggesting that mice cannot control this non-fatal infection in the absence of antibody. On the other hand, when B cell deficient Balb/c mice infected with the same parasite were drug-rescued by treatment with clindamycin (Roberts & Weidanz, 1979), the majority of animals developed long-term chronic

infections in the apparent absence of antibody; thus these mice are able to develop an alternative antibody-independent mechanism to survive a *P. yoelii* infection. By contrast, μ-suppressed mice are able to control primary *P. chabaudi adami* infections in the total absence of antibody (Grun & Weidanz, 1981), but require antibody to eliminate the parasitaemia ultimately.

Naive mice or rats can be partially protected against *P. berghei, P. yoelii* and *P. chabaudi* via immune B cells (Phillips, 1970; Gravely, 1976; Jayawardena 1978; McDonald & Phillips, 1978). The subset of B cells involved in protection against *P. yoelii* was shown to be of the $CD5^+$ phenotype (Hunter *et al.*, 1979; Jayawardena *et al.*, 1979). B cells of both $lyb5^+$ and $lyb5^-$ phenotypes are involved in inducing immunity; the IgM response is primarily under T cell regulation, and both IgM and IgG antimalarial antibodies are required for maximal immunity.

1. 5. 4 Role of T cells

The direct involvement of T cells in protection against malaria is still not clear. They do, however, play an important role, as suggested by at least three lines of evidence: a) the development of immunity is associated with T cell activation, both *in vivo* and *in vitro*, (b) T cell-deficient hosts are more susceptible to infection, and c) T cells from immune donors or antigen-specific T cell lines or clones, can transfer protection to non-immune recipients.

Jayawardena *et al.* (1975), showed that T cell proliferation was high in mice infected with the non-lethal 17X *P. yoelii*, while infection with the

lethal *P. berghei* failed to give comparable levels of proliferative activity. This finding was confirmed *in vitro* by Weinbaum *et al.* (1976a), who showed that protection correlated with the ability of splenic T cells to proliferate in response to malarial antigens. In other studies, T cell activation, as measured by the expression of delayed-type hypersensitivity (DTH) responses in immunized mice after challenge with malarial antigen, was shown to correlate with protection (Cottrell, Playfair & de Souza, 1978; Playfair *et al.*, 1985).

T cell-deprived rats (Brown *et al.*, 1968) or mice (Jayawardena *et al.*, 1977), cannot control normally self-resolving infections. Similar findings were reported for nude mice (Clark & Allison, 1974), who died after infection with non-lethal *P. yoelii* or *B. microti* . By contrast, B cell-deficient mice can overcome an acute blood stage infection caused by at least four murine haemoprotozoan parasites including, *P. chabaudi adami,* (Grun & Weidanz, 1981), *P. vinckei petteri, P. chabaudi chabaudi,* and *B. microti* (Cavacini *et al.*, 1990). Mice that are drug rescued during the course of a *P. chabaudi adami* infection can resist reinfection (Grun & Weidanz, 1983). Similar treatment of nude mice led to their death (Roberts *et al.*, 1977), suggesting that immunity to reinfection is T cell dependent. Hence, T cell-dependent cellular mechanisms can function to prevent reinfection and to suppress the acute disease.

Spleen cells from immune animals can transfer immunity to non-immune animals (Phillips, 1970; Jayawardena *et al.*, 1978). T and B cell enriched fractions from rats immune to *P. berghei* and from mice immune to *P. yoelii* are capable of transferring immunity (Gravely & Krier, 1976; Brown *et al.*, 1976).

T cells of the $CD4^+$ helper phenotype protect mice against a non-lethal *P. yoelii* infection (Jayawardena *et al.*, 1982). Similar results were reported for *P. chabaudi* infections (Langhorne, 1989). $CD4^+$ T cells are thought to be important for immunity against both hepatic and blood stages of the infection. In the liver stage, $CD4^+$ T cells may act as helpers for antibody production, but of greater importance is their ability to help $CD8^+$ cytotoxic T cells to kill the parasite within the hepatocyte, via a γ-interferon (IFN-γ)-mediated pathway or by TNF (Schofield *et al.*, 1987; Weiss, 1988). Control of blood stage infections is dependent mainly on $CD4^+$ T cells, as mice depleted of this population of cells cannot control their infection (Suss, 1988) and immunity can be adoptively transferred to naive recipients via immune $CD4^+$ T cells (Cavacini, 1986). $CD4^+$ T cells of mice have been classified into two subsets, T_H1 and T_H2 cells. When activated by antigen (*P. chabaudi* peptides presented on MHC class II molecules of macrophages or B cells), the former subset produces the cytokines interleukin-2 (IL-2) and IFNγ, and provides help for DTH and cytotoxicity responses, while the latter produces IL-4, IL-5, IL-6 and IL-10, and provides help for B cell synthesis of antibody. During a *P. chabaudi* infection there is an early T_H1 response between the 7th and 14th day of infection and a later T_H2 response occurring between the 18th and 60th day of infection (Langhorne, 1989). Control of a *P. chabaudi* parasitaemia is thus ultimately associated with antibody, and this observation agrees with the finding that μ-suppressed mice required antibody to clear this parasite from their blood (Grun & Weidanz, 1981).

Perhaps the most important contribution of T cells in malarial immunity, and indeed immunity to parasites in general, is in the production of the various cytokines which act as regulators of humoral and DTH-type T cell responses. Cytokines secreted by the two subsets of T cells are mutually antagonistic; for example IFN-γ can inhibit the effects of IL-4, and IL-10 can inhibit the effects of IFN-γ (Mossman & Moore, 1991). Whilst both subsets of T cells are involved in protective immunity to *P. chabaudi* malaria, in *Leishmania,* the outcome of infection is dependent on which type of cytokine response is stimulated. Protective epitopes stimulate effective T_H1 responses (IFN-γ and IL-2) which induce macrophage activation and consequent resolution of the disease. Suppressor epitopes, by contrast, stimulate T_H2 (IL-4) responses which have a negative effect on macrophage activation, leading to disease progression (Locksley & Scott, 1991).

1. 5. 5 Role of non-specific immunity

A wide range of agents such as BCG (Clark *et al.*, 1976), *C.parvum* (Cottrell *et al.*, 1977) and LPS (Martin *et al.*, 1987), have been shown to induce protection against certain species of *Plasmodia* and *Babesia* non-specifically. The protection achieved in these studies ranged from a brief delay in patent parasitaemia to complete protection against the disease. The degree of protection depended on the stage of infection being tested and was expressed selectively against different species of *Plasmodia* (Clark, Cox and Allison, 1977). Immunity achieved by these agents was short lived, lasting for a few days to months, lacked a memory response (Allison *et al.*, 1979), and was not attributable to the presence of protective antibody (Cox, 1970). The mechanism by which these substances act is

largely unknown; however, in the case of BCG, it has been suggested that it activates a potent form of non-specific immunity mediated by natural killer cells (NK cells), monocytes and macrophages (Allison & Clark, 1977).

1. 5. 6 Cellular mediators of non-specific immunity

Natural Killer Cells

NK cells, thought to play a significant role in resistance to tumours (Haller *et al.*, 1977), have also been implicated in resistance to malaria. Strains of mice, such as the A/J mouse, which have low levels of NK activity are susceptible to *P. chabaudi* infections, whereas those with high NK activity (CBA mice) are the most resistant (Eugui & Allison, 1980). However, the role of NK cells in malaria remains controversial. While the above studies suggest their apparent importance, other studies clearly oppose this theory of NK cell mediated killing of malaria parasites. Plasmodial infections were not exacerbated in mice who were depleted of their NK cells or, in beige mice who are NK cell deficient (Skamene *et al.*, 1983; Wood & Clark, 1982). Furthermore, nude mice develop fatal infections with various *Plasmodial* species (Clark & Allison, 1974; Cavacini *et al.*, 1986) despite their high NK cell activity (Kindred, 1979).

1. 5. 7 Polymorphonuclear Leucocytes (PMN)

These cells may inhibit parasite growth either by phagocytosis or by secretion of soluble mediators. Phagocytosis has been reported for *P. falciparum* merozoites (Kharazmi & Jepsen, 1984), parasitized red cells (Tosta & Wedderburn, 1980), schizonts (Brown & Smalley, 1981), and gametocytes (Sinden & Smalley, 1976), and this may contribute to protection. The multiplication of asexual blood stage of *P. falciparum* can be inhibited *in vitro* by neutrophils (Kharazmi & Jepsen, 1984; Nnalue &

Friedman, 1988), via a combination of phagocytosis and extracellular killing mechanisms, involving singlet oxygen (Nnalue & Friedman, 1988). Eosinophils may exert an antiparasitic effect extracellularly via secreted granule proteins, and other degranulation products such as arylsulphatase B, phospholipase D, lysophospholipase may also be involved (Waters *et al.*, 1987).

1. 5. 8 Macrophages and monocytes

The role of macrophages and monocytes in malarial immunity was made clear in 1930 through the work of Taliaferro and Mulligan, who observed the presence of parasites at various stages of phagocytosis. More recent studies suggest that phagocytosis may contribute to the clearance of parasitized erythrocytes by the spleen and liver during infection (Quinn& Wyler, 1979; Dockrell, de Souza & Playfair, 1980). Phagocytosis may be enhanced by the presence of antibodies, which direct parasitized erythrocytes or free parasites on to effector macrophages which express surface Fc and C3b receptors. However, recovery from infection does not necessarily depend upon phagocytosis as parasites may be killed within red cells, giving rise to degenerate parasites termed crisis forms; these have been seen in blood films of mice recovering from murine malaria infections (Clark, Allison and Cox, 1976). Macrophages may also exert their protective effect by releasing soluble factors that are toxic to blood stage parasites (Allison & Eugui, 1982; Clark & Hunt, 1983). During infection, these cells increase in number (Lee *et al.*, 1986), and following activation by cytokines, for example IFN-γ, they release products such as reactive oxygen intermediates (ROI) that are toxic to parasites (Ockenhouse, Schulman & Shear, 1984; Dockrell, Alavi & Playfair, 1986).

1. 5. 9 Soluble mediators of malarial immunity

Reactive oxygen intermediates

ROIs released from activated macrophages during the so called oxidative burst, include superoxide anion, hydrogen peroxide (H_2O_2), singlet oxygen, and hydroxyl radical, all of which are toxic to the parasite. Hydrogen peroxide is toxic to blood-stage *P. falciparum* (Wozencraft *et al.*, 1984), and to *P. yoelii* and *P. berghei* (Dockrell & Playfair, 1984) *in vitro* at physiological concentrations. Some rodent parasites can also be killed *in vivo*, by H_2O_2 (Dockrell & Playfair, 1983), or by various agents known to generate free radicals, for example, alloxan (Clark & Hunt, 1983), t-butyl hydroperoxide (Clark *et al.*, 1984a), and divicine (Clark *et al.*, 1984b). When these substances are injected into infected animals, they cause a rapid drop in parasitaemia, and crisis forms within erythrocytes are seen on blood films. The effect of these agents was prevented by injection of desferrioxamine (Clark & Chaudhri, 1989), a substance that inhibits the iron-catalysed formation of OH radicals. Finally, singlet oxygen, released by human neutrophils, inhibits the *in vitro* growth of *P. falciparum* (Nnalue & Friedman, 1988); this reaction can be partially abrogated by histidine and tryptophan, agents which quench singlet oxygen.

Reactive nitrogen intermediates (RNI)

Monocytes from patients with chronic granulomatous disease, are unable to mount a respiratory burst but can kill blood-stage *P. falciparum* *in vitro* (Kharazmi *et al.*, 1984, Ockenhouse & Shear, 1984). Furthermore, mice deficient in activated macrophages are still capable of controlling their parasitaemia (Cavacini *et al.*, 1989). These observations suggest that other factors may be involved in parasite destruction and the most recent evidence focuses on reactive nitrogen intermediates (RNIs,). These

molecules which include nitric oxide (NO) and its oxidized forms, nitrite (NO_2^-) and nitrate (NO_3^-) inhibit the *in vitro* growth of bacteria (Granger *et al.*, 1988), *Leishmania major* (Green *et al.*, 1990), *Plasmodium* liver stages (Nussler *et al.*, 1991) and indeed blood-stage *P. falciparum in vitro* (Rockett *et al.*, 1991). NO is also effective *in vivo*; when the lesions of *L. major* infected mice were injected with L-*N*MMA, an inhibitor of NO synthase (enzyme that generates NO from L-arginine), the infection was exacerbated (Liew *et al.*, 1990). Although the precise mechanism of RNI action remains unknown, Rockett *et al* (1991) propose that RNIs may diffuse into red blood cells to form nitrosothiol groups which react in various ways to reduce the parasite oxidant defense capacity.

1. 5. 10 Cytokine - induced killing mechanisms

Besides RNIs, other non-oxidative mechanisms appear to be toxic to malaria parasites. For example tumour necrosis serum (TNS) obtained from animals treated with substances such as BCG, *P. acnes*, or indeed malaria parasites, to activate macrophages and injected with lipopolysaccharide (LPS), to trigger the release of several cytokines including, tumour necrosis factor (TNF), IL-1 and various lysosomal enzymes into the serum, is toxic to human (Wozencraft *et al.*,1984) and rodent (Taverne, Dockrell & Playfair, 1981) malaria parasites *in vitro.* TNS is also toxic to some rodent parasites *in vivo* (Taverne, Depledge & Playfair, 1982), but its toxic effects are probably not attributable to the presence of TNF, as recombinant TNF does not interfere with the growth of *P. falciparum in vitro* (Hviid *et al.*, 1988). Whilst the parasitotoxic component of TNS is yet to be discovered, certain macrophage enzymes should be considered as likely candidates. For example, polyamine

oxidase, in the presence of a substrate such as spermine or spermidine, generates H_2O_2 and aldehydes which inhibit the growth of *P. falciparum in vitro* (Egan *et al.*, 1986). Since TNF itself does not kill parasites directly (Taverne *et al.*, 1987; Jensen *et al.*, 1987), even in the presence of IFNγ, the antimalarial effect *in vivo* is probably mediated by an unknown TNF responsive cell.

It was reported that another substance, termed crisis form factor (CFF), is present in the serum of residents living in endemic areas of Sudan (Jensen *et al.*, 1982). Sera from these individuals inhibited the growth of *P. falciparum in vitro*, giving rise to crisis forms (Jensen *et al.*, 1987) and although the nature of CFF is not yet known, it appears to be different from both immunoglobulin and TNF (Jensen *et al.*, 1982; Jensen *et al.*, 1983).

Other cytokines have also been implicated in malarial immunity, where they act via the host cell, affecting the intracellular environment and parasite function indirectly. Although cytokines such as IFN-α and IFN-γ (Jensen *et al.*, 1983), do not have a direct effect on *P. falciparum* blood stage parasites, liver stage parasites are inhibited *in vitro* by IL-1α (Mellouk *et al.*, 1987), IFN-β and IL-2 (Mazier *et al.*, 1988) and TNF (Schofield *et al.*, 1988).

1. 5. 11 Pathology of malaria

Individuals infected with malaria for the first time usually develop severe symptoms, although their blood parasitaemias may be as low as 1%. Blood films can be misleading, as mature forms of *P. falciparum* can

adhere to the vascular endothelium of blood vessels, causing their sequestration from the general circulation. It has been proposed that this cytoadherence in small capillaries, may be associated with the pathology of the disease, in particular with cerebral malaria, which can be fatal. Other pathological changes include, hypoglycaemia, pulmonary oedema, circulatory collapse, severe anaemia, thrombocytopenia, acute renal tubular necrosis and foetal death (White, 1986).

Possible causes of these pathological changes may be:

i) The release of malarial toxins during schizogony, either as directly acting toxins, although a directly toxic product has not yet been found, and

ii) An inflammatory reaction in response to the parasite. The latter explanation, which is immunopathological, has been the topic of intensive research in recent years, and may involve TNF and other inflammatory mediators.

Clark *et al.* (1981) suggested that at schizogony an endotoxin-like molecule induces the release of TNF and other monokines (prostaglandins, leucotrienes, IL-1, proteolytic enzymes) from activated macrophages, and that these inflammatory mediators not only lead to the development of crisis forms within circulating erythrocytes but also contribute to the pathology of malaria. By injecting recombinant TNF into mice, it is possible to reproduce some of the pathological changes associated with the disease - erythrophagocytosis, dyserythropoesis, and foetal death, all of which are seen in human (Warrell, 1987) and mouse malaria (Clark & Chaudhri, 1988a, and 1988b). Furthermore, antibody to mouse TNF protected against cerebral malaria occurring during a *P. berghei* infection, although the animals remained parasitaemic (Grau *et al.*, 1987). Clark *et al* (1991) have recently suggested that TNF and other

cytokines can induce the release of NO (from endothelial cells, vascular smooth muscle, neutrophils or macrophages), the local production of which could lead to the clinical signs seen in cerebral malaria.

Taken together, these findings suggest that, besides acting as antimalarial agents, TNF and indeed NO may also contribute to the pathology of malaria. As most deaths in malaria are due to toxic or pathological changes in the host, it seems logical that treatment regimes might include the use of antibody to TNF.

1. 5. 12 Summary of malarial immunity

The relative importance of the various mechanisms involved in protective immunity to malaria remain largely unresolved. It is nevertheless clear, that in self-resolving infections or in systems using effective vaccination against lethal parasites, there is a rapid recruitment of a number of protective responses, involving T cells, antibody, monocytes and macrophages and various cytokines which act in combination to inhibit parasite growth. In lethal infections such as *P. berghei* in mice, activation of these responses is limited, and instead suppressor responses are probably activated with fatal consequences.

The study of cytokines in malaria has further strengthened our understanding of the critical interactions between the parasite and the immune system. T cell activation by parasite antigens, can lead to production of various cytokines which regulate cytotoxic mechanisms in both liver stage and blood stage immunity. Cytokine activation of macrophages, however, can result in beneficial as well as harmful responses.

1. 6 VACCINATION AGAINST MALARIA

The extent of malaria worldwide is often described in terms of infant mortality rates and the number of cases of clinical malaria per year. The disease can also be very debilitating, lasting for up to five years in some cases of *P. falciparum* infection (Guerrero *et al.*, 1983), and this aspect of malaria is said to be linked with the slow economic development of third world countries.

Both the limited success from the 1950s to the 1970s, of malaria control programmes based on destruction of the mosquito vector and the resistance of the parasite to chemotherapy have created an urgent need for vaccination against the disease. Adults from endemic areas probably never achieve sterile immunity, however they do develop clinically effective immunity with transient parasitaemia, suggesting that a human vaccine should be possible. Futhermore, successful vaccination in animal models can be achieved, using either whole parasites or purified antigens (discussed later).

The complexity of the parasite's life cycle, with different stages possessing their own repertoire of antigens, rules out the possibility of a vaccine based on a single antigen. In addition, immunity to malaria is stage specific, and therefore an ideal vaccine, besides being cheap, safe and totally effective, should contain elements directed against the key stages of the parasite life cycle. Vaccine targets presently under consideration include; i) sporozoites, ii) asexual erythrocytic stages, and iii) gametes and other forms developing in the mosquito mid-gut.

1. 6. 1 Sporozoite vaccines

Sporozoites initiate malaria infections and would therefore seem to be obvious targets. An effective vaccine would prevent entry of the infective stage into the liver and/or block subsequent development.

Irradiated sporozoites induce protection in a broad range of hosts, including mice, monkeys, and humans (Nussenzweig *et al.*, 1967; Gwadz *et al.*, 1979; Clyde *et al.*, 1975) however, large numbers of irradiated sporozoites were required and they had to be administered intravenously. Although this approach is not clinically feasible for obvious reasons, these studies led to the important finding that vaccination with irradiated sporozoites induces antibodies directed against a repetitive epitope on the circumsporozoite (CS) protein (Zavala *et al.*, 1983). This repetitive CS epitope appears to be immunodominant since a number of murine anti-sporozoite monoclonal antibodies recognize it. The genes coding for the CS protein of *P. falciparum* have been cloned (Dame *et al.*, 1984), the amino acid sequences determined, and it has been shown that the immunogenic part of the molecule, resides in a short repeated sequence, asparagine-alanine-asparagine-proline (NANP). This segment of the CS protein has already been used in two vaccine trials in human volunteers. In one trial, a synthetic vaccine, $(NANP)_3$ conjugated to tetanus toxoid, was injected intramuscularly into three volunteers. All subjects developed antibodies, and following challenge by infected mosquito-bite, one was totally protected while the two others experienced slightly delayed infections (Herrington *et al.*, 1987). The other trial, which used a recombinant vaccine designated falciparum sporozoite vaccine 1 (FSV-1), alum-adsorbed, produced similar results (Ballou *et al.*, 1987). Although these results are not very encouraging, they at least confirm the feasibility

of an antisporozoite vaccine. Vast improvements in the immunogenicity of such vaccines are essential; perhaps epitopes that stimulate both T and B cells will have to be incorporated so that a potent response is generated, capable of destroying the hundreds to a few thousand sporozoites inoculated by an infected mosquito during a blood meal. A sporozoite vaccine has to be 100% efficient, as a single surviving sporozoite can initiate infection.

1. 6. 2 Asexual blood stage vaccines

As the erythrocytic stage of the infection is responsible for the clinical signs and symptoms of the disease it would appear to be a strategic target for the development of malaria vaccines. In this case although sporozoites would be able to enter the liver and develop normally an effective vaccine would prevent the subsequent development of asexual blood stages, thereby limiting clinical illness.

In the 1970s, effective blood stage vaccines consisting of merozoites, schizonts, or both were developed in a variety of host-parasite combinations (Targett & Fulton, 1965; Mitchell *et al.*, 1977; Cohen *et al.*, 1977; Playfair, de Souza & Cottrell, 1977). Immunity induced was species-specific and did not act on the pre-erythrocytic stage. Development of the *in vitro* culture system for malaria parasites (Trager & Jensen, 1976) greatly facilitated the bulk production of merozoites which were used in various vaccination studies, mainly in simians. However, the feasibility of such preparations as human vaccines was ruled out, as they required Complete Freunds Adjuvant for efficiency, and the crude nature of the preparation, containing erythrocyte membranes, was likely to induce autoimmune reactions; they may also have contained antigens

responsible for immunosuppression and pathology. In the 1980s, parasite surface antigens (reviewed in **1.7**), were identified in an effort to develop subunit vaccines. Protective antigens have been identified and used as successful vaccines in mice (Holder & Freeman, 1981) and in monkeys (Dubois *et al.*, 1984, Perrin *et al.*, 1985). The amino acid sequences of these protective antigens have been determined, and in one case the peptides were biochemically synthesized and a mixture of three, of relative molecular mass 83kD, 55kD and 35kD, induced protection against a *P. falciparum* challenge in *Aotus* monkeys (Patarroyo *et al.*, 1987a & 1987b). Two polymeric synthetic hybrid proteins based on these peptides were alum-adsorbed and tested in humans (Patarroyo *et al.*, 1988). When challenged with ring-infected *P. falciparum* erythrocytes, one out of nine individuals was totally protected; four others experienced low level parasitaemia (less than 0.5%), which were cleared by day 21 after challenge. The remaining four volunteers required chemotherapy as their parasitaemias reached the agreed danger level of 0.5%. The same vaccine, subsequently tested by Patarroyo and colleagues in a large-scale clinical trial in South America (unpublished data), was said to be very encouraging. It is however, difficult to draw any scientific conclusions about this trial as the data have not yet been published.

1. 6. 3 Transmission blocking vaccines

This type of vaccine is targeted at the sexual stage of the parasite life cycle, which develops from the differentiation of erythrocytic parasites into male and female gametocytes. Gametocytes ingested by a mosquito during a blood-meal, undergo gametocytogenesis in its mid-gut, and within 10-20 minutes, gametes released from the red cells begin fertilization, thereby infecting the mosquito. However, the presence of an

antigametocyte antibody in the ingested blood-meal, would block fertilization of female by male gametes in the mosquito gut or inactivate the fertilized zygote and prevent the development of sporozoite-containing oocysts. This strategy of transmission-blocking immunity does not confer protection on the vaccinated individual, but would be expected to reduce the overall rate of malaria transmission in endemic areas and thus play a vital role in control.

Transmission-blocking vaccines consisting of male gametocytes have been shown to inhibit sexual reproduction and consequently transmission of avian, simian (Gwadz, 1976; Gwadz & Green, 1978), and rodent (Mendis & Targett, 1979), *Plasmodia*. Specific transmission-blocking antibodies can also be found under natural conditions in humans, in endemic areas (Mendis *et al.*, 1987); antibody titers against extracellular female gametes, are high in the sera of patients who have experienced repeated malarial attacks. One of the problems of this type of vaccine is that low-levels of antigametocyte antibody can enhance the production of infective sporozoites in the mosquito.

1. 6. 4 Anti - disease vaccines

This recently proposed vaccine by Playfair *et al.* (1990) would immunize against the toxic effects of the disease rather than against the parasite itself. The vaccine would be analogous to the currently used tetanus or diphtheria toxoid vaccines, which act by inducing neutralizing antibodies against the toxins whilst having no effect on the organisms themselves. Toxins may be released during schizogony, but none have yet been identified. Indirect toxicity may result via the production of cytokines and indeed, there is evidence that antigens inducing the release of cytokines

such as TNF are produced during infection (Bate, Taverne & Playfair, 1989), and may be involved in the pathology of malaria. Antigens responsible for the induction of TNF would serve as ideal anti-disease vaccines.

1. 6. 5 Summary of vaccination

In summary, the asexual blood-stage vaccine would seem the most likely to induce immunity similar to that acquired by individuals living in endemic regions, where natural infection does not appear to give rise to an effective anti-sporozoite immunity or to the development of effective transmission-blocking immunity. An asexual blood-stage vaccine on its own may be most appropriately used in such areas for children under five years of age, who suffer severe malaria infections which are often fatal. This vaccine would induce an immune response in the young child which would reduce the morbidity and mortality rate and which would be boosted on reinfection.

Sporozoite and transmission- blocking vaccines would need to be given to entire populations in endemic areas to be effective. Repeated boosting at appropriate intervals would be necessary to maintain effective immunity until transmission is totally interrupted. Both these vaccines may be of immense value in limiting the epidemic spread of malaria. Sporozoite vaccines would seem useful in areas where drug-resistant parasites are prevalent, particularly for protecting non-immune subjects such as visitors, immigrant workers and the military.

Ideally, a cocktail containing all three vaccine types would be the most effective in terms of disease control. However, economic and logistical

factors may necessitate the use of single rather than multiple vaccines. Besides the specific requirements of safety and efficacy assessment, all vaccine types will have to be tested and evaluated independently in field trials before their use in combination is contemplated.

1.7 MALARIA STAGE-SPECIFIC ANTIGENS AS TARGETS FOR VACCINE DEVELOPMENT

Each stage of the complex life cycle of the malaria parasite, independently expresses antigens that are stage-specific. However, cross-reactions, based on antibody cross-reactivity, do occur between stages for example: (a) between Pf155/RESA (discussed in para. **1.7.3**) and some blood-stage antigens (Wahlin *et al.*, 1992) and (b) epitopes of the major merozoite surface protein MSA-1 (discussed in para. **1.7.3**) are also found in the hepatic stages of *P. berghei* (Suhrbier *et al.*, 1989). Nevertheless, protective antigens from one stage do not confer protection against another. For example, animals vaccinated with sporozoite antigens, remain protected against a virulent inoculation of sporozoites, but are fully susceptible to infection induced by asexual blood-forms. Furthermore, the asexual blood-stage parasites themselves display remarkable antigenic diversity, both through the developmental cycle within the red cell, and also between genetically different isolates or strains of each species of *Plasmodium*. Not surprisingly therefore, malaria parasites contain a broad range of diverse and unusual antigens which have been studied in much detail.

1. 7. 1 Sporozoite antigens

The circumsporozoite protein (CSP), which is the major antigen located on the outer membrane of the sporozoite (Nussenzweig & Nussenzweig,

1985a), is the only antigen that has been studied in any detail. It has a molecular weight of 30-60kD in *P.falciparum* (Nussenzweig & Nussenzweig, 1985b), and the gene encoding it has been well characterized in a number of *Plasmodium* species (Nussenzweig & Nussenzweig, 1986; Lockyer, 1989). As mentioned earlier (**1.6.1**), the immunogenic portion of the protein residing in the NANP repeat region was used in two clinical trials, with limited success. Of 40 individuals tested, only nine developed significantly high antibody titres, which were considered high enough to permit live challenge, and only one person from each trial was successfully immunized. Antibody responses in both trials were poor and there was no evidence of boosting, suggesting that the CS protein itself is poorly immunogenic and therefore unable to generate an effective cell-mediated immune response (Herrington *et al.*, 1987; Ballou *et al.*, 1987). This seems unusual as the CS antigen is known to contain an immunodominant B cell epitope (Ballou *et al.*, 1987; Zavala *et al.*, 1983), as well as a T cell epitope (Good *et al.*, 1988a). The limited success of the vaccine may be due to a lack of epitopes which induce T cell effector as well as T cell helper activity. Epitopes that induce T helper activity (Good *et al.*, 1987), and cytotoxic T cells, specific for the CS protein (Kumar *et al.*, 1988), have been identified and their use in human clinical trials will be of great interest.

1. 7. 2 Liver stage antigens

Antigens of the hepatic, pre-erythrocytic stage, once thought to be of lesser importance in immunity to malaria, are now being considered as attractive targets for immunological attack. Their importance dates back to the earlier observation, that irradiated (14K.Rads) sporozoites are living organisms capable of invading hepatocytes and transforming into young

trophozoites but, they are unable to develop further and consequently remain for unknown periods of time as uninucleate intrahepatic bodies (Druilhe & Marchand, 1989), probably acting as an immunogenic depot. Sporozoites irradiated with higher doses (23K.Rad. - lethal dose), and killed sporozoites do not enter the hepatocyte, and this is thought to be the reason for their poor immunogenicity. Hence the production of young liver forms appears to be crucial for the induction of immunity.

The importance of liver stage antigens was demonstrated by Schofield *et al.* (1987), who also pointed out the involvement of antibody independent mechanisms of immunity to malaria. Immunity to sporozoites is mediated by cytotoxic $CD8^+$ T cells and IFN-γ (Schofield *et al.*, 1987). Hence the targets for these responses cannot be circulating sporozoites as they do not express MHC class I antigens. The most likely targets for cell-mediated responses are the infected hepatocytes, as they express MHC class I antigens and receptors for IFN-γ.

Liver stage antigens are now being investigated in more detail and to date at least two have been identified. One of these designated LSA (liver stage antigen), encodes an epitope present in *P. falciparum* liver stages, and is made up of 17 amino acid repeats organized in an alpha helix (Marchand-Guerin, Druilhe & Galey, 1987). The other, termed SALSA (sporozoite and liver stage specific antigen), has its reactive epitopes incorporated within an 87 amino acid polypeptide which is analogous to a 70kD protein in *P. falciparum* sporozoites. An important finding to emerge from studies on these antigens was that in some African

populations, the prevalence of antibodies to LSA and SALSA was much higher than those for the CS antigen.

Unlike the CS antigen which induces mainly antibody - mediated immunity, the liver stage antigens are most likely to stimulate both antibody and a cell-mediated response, making them attractive antigens for vaccination.

1. 7. 3 Asexual blood stage antigens

The asexual blood stage of the malaria parasite's life cycle occupies a central position in the disease, where it is responsible for clinical malaria and, not surprisingly, antigens from this stage have to date received the most attention. Early vaccination studies using merozoites, schizonts or a mixture of erythrocytic forms yielded significant levels of protection in a variety of host-parasite systems. The finding that merozoite reinvasion of red cells at schizogony could be inhibited by immune sera (Butcher & Cohen, 1972) led to extensive studies with merozoite vaccines, again in a variety of host-parasite combinations, including monkeys immunized with *P. falciparum* (Siddiqui, 1977), or *P. knowlesi* (Mitchell, Butcher & Cohen, 1975) merozoites. The results were promising, especially in mice (Playfair & de Souza, 1978), where 100% protection was achieved by a single intravenous dose of formalin-fixed parasites plus *B.pertussis* as adjuvant. However, there are several drawbacks in every system studied. Intravenous inoculation is not practical for humans, and even the successful intramuscular vaccination route used in monkeys had an absolute requirement for Freund's Complete Adjuvant, which is unethical in humans as it results in severe chronic granulomatous ulceration.

Mass production of merozoites for large scale vaccination trials is not feasible and finally, host red cell membranes present within these crude preparations are likely to induce autoimmune phenomena.

Priority was accordingly directed towards defining antigens on the surface of merozoites, because of their potential as components of a malaria vaccine, with the eventual aim of developing subunit vaccines. This task was greatly facilitated by the introduction of a continuous *in vitro* culture system for *P. falciparum* (Trager & Jensen, 1976), whereby large quantities of merozoites, schizonts or gametes could be cultured for analysis of membrane proteins and production of antigens.

Several antigens have been identified by means of a number of techniques, including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional SDS-PAGE, surface labelling, Western blotting, the use of monoclonal antibodies of defined specificity, and perhaps the most advanced of these being molecular biology, where libraries have been constructed of antigen-expressing cDNA clones containing several distinct asexual blood-stage antigens, some of which are likely to be candidate vaccine molecules. Only a few of these antigens have been characterized in any detail however, but a number have been identified as potential asexual blood-stage vaccine candidates.

Merozoite surface antigens

An antigen identified on the merozoite membrane by surface labelling techniques, is derived from the proteolytic processing of a high molecular weight precursor polypeptide, known as the Precursor to the Major Merozoite Surface Antigen (PMMSA). This antigen, which is now referred

to as MSA-1 (Merozoite Surface Antigen), is the major merozoite surface antigen which is shed from the merozoite when it invades host erythrocytes. The molecular weight of MSA-1 in *P. yoelii* is 230kD (Holder & Freeman, 1984), 250kD in *P. chabaudi* (Boyle, Newbold, Smith & Brown, 1982), and 195kD in *P. falciparum* (Holder, Freeman & Newbold, 1983; Holder & Freeman, 1982). These high molecular weight proteins are synthesized at schizogony and undergo proteolytic prossessing into a number of smaller fragments, which are exposed on the surface of the merozoite. The 230kD *P. yoelii* protein, an analogue of the 195kD protein of *P. falciparum* (Holder 1988), is highly immunogenic in mice, protecting the animals from challenge with the lethal infection (Holder & Freeman, 1981). The 195kD *P. falciparum* analogue is processed into fragments of 83kD, 28kD, 38kD, and 42kD (Holder & Freeman, 1982). The 83kD polypeptide, which is the main merozoite surface component, has been found in culture supernatants, suggesting that it is shed from the merozoite before erythrocyte invasion (Holder, 1988). Studies using a series of monoclonal antibodies suggest that the molecule is antigenically diverse (McBride, Newbold & Anand, 1985). The gene encoding this molecule has been cloned and sequenced from several *P. falciparum* isolates and can be divided into several units ranging in homology from 10-80% at the amino acid level (MacKay *et al.*, 1985). Vaccination trials with MSA-1 given with Freund's Complete Adjuvant induced partial (Perrin *et al.*, 1984) to complete (Siddiqui *et al.*, 1987) protection in experimental monkeys. Vaccination with synthetic peptides corresponding to the conserved -NH_2 part of the molecule induced protection in monkeys (Patarroyo *et al.*, 1987).

Another merozoite surface antigen termed MSA-2, not related to

MSA-1, has been identified on *P. falciparum* merozoites (Eping *et al.*, 1988). MSA-2 is a smaller antigenic polypeptide of relative molecular weight 45kD, which has been cloned and sequenced (Smythe *et al.*, 1988). Both MSA-1 and MSA-2 share at least two common features: a) they are both post-translationally modified by the attachment at the C-terminus of a glycosyl phosphatidylinositol moeity (GPI) which anchors the mature polypeptides into the surface membrane of the merozoite (Smythe *et al.*, 1988); however, the functional significance of the GPI anchor is not known, and b), both molecules exist in antigenically diverse forms. DNA sequencing of MSA-1 (Holder, 1988) and MSA-2 (Smythe *et al.*, 1990) from different strains of *P. falciparum* shows regions of conserved, semi-conserved and variable sequence within the genome. Monoclonal antibodies against MSA-2 are inhibitory to parasites *in vitro* (Clark *et al.*, 1989). Recently, Saul *et al.* (1992) showed that the MSA-2 protein also exists in *P. chabaudi* and that the invariant peptides of the *P. falciparum* MSA-2 antigen could induce protective immunity in mice challenged with a lethal strain of *P. chabaudi.*

Antigens associated with the erythrocyte membrane

The surface of the infected erythrocyte becomes associated with a number of different parasite proteins during the various stages of the asexual life cycle. Some of these antigens have been characterized and include a ring infected erythrocyte surface antigen (RESA) and two proteins associated with structures known as knobs - electron dense projections of the plasma membrane of infected erythrocytes. These antigens include, KHARP or HRP 1 (knob-associated histidine-rich protein), and MESA or Pf EMP 2 (mature parasite-infected erythrocyte surface antigen).

RESA, of molecular mass 155kD, is synthesized in trophozoites, accumulates in micronemes (apical structures of merozoites), and becomes associated with the membrane of the ring-infected erythrocyte following invasion (Perlmann *et al.*, 1984; Brown *et al.*, 1985). Antibodies against RESA inhibit erythrocyte invasion *in vitro*, and it is therefore thought to be involved in erythrocyte invasion (Brown *et al.*,1985). The gene coding for RESA has been cloned and sequenced, and it has been established that the molecule contains two blocks of repetitive sequences which are the immunodominant epitopes for antibody responses (Favaloro *et al.*, 1986). RESA molecules from different isolates of *P. falciparum* analysed to date appear to be conserved. This lack of diversity is unusual as the antigen can induce protective immune responses in monkeys (Perrin *et al.*, 1985; Collins et al 1986). In vaccination trials in monkeys, partial protection was achieved using recombinant peptides corresponding to various parts of the molecule, and antibody levels correlated with protection (Collins *et al.*, 1986); however, synthetic peptide vaccines based on the 8 amino acid 3′ repeat were less effective (Patarroyo *et al.*, 1987). A copy of the 5′ RESA repeat, was included in the synthetic peptide vaccine that was used in the human clinical trial (Patarroyo *et al.*, 1988) discussed earlier.

Erythrocytic schizonts and trophozoites of *P. falciparum* adhere to the endothelial linings of venules in deep tissues, via their so-called knobs. This cytoadherence, which is a recognized evasion tactic of the parasites, prevents their passage through the liver and spleen where they are thought to be destroyed by cell-mediated immune reactions (Playfair *et al.*, 1979). KHARP and another histidine-rich protein HRP 2 (Ellis *et al.*, 1987), and MESA (Coppel *et al.*, 1986), have been cloned and characterized. Both proteins vary in size , contain repeated amino acid sequences and differ

antigenically among the various isolates of *P. falciparum.* The apparent molecular mass of KHARP is in the range 85kD-105kD, and 240kD-300kD for MESA.

All these antigens, except HRP 2 (secreted from the infected cell), appear to be associated with the cytoplasmic face of the red cell membrane and are unlikely to be important in protective responses. Antigens located on the external surface of infected erythrocytes are of greater importance, particularly as targets of protective responses. A number of techniques have been used to identify these antigens and the most sensitive of these, radio-iodination and immunoprecipitation, have detected a large molecular mass polypeptide called Pf EMP-1, which is polymorphic in size and in common with other surface proteins it displays considerable antigenic diversity (Howard *et al.*, 1988).

Rhoptry proteins

Rhoptries are apical organelles connected by pore-like structures to the micronemes on the surface of the merozoite. Both organelles are involved in penetration of the erythrocyte. During invasion the contents of the rhoptries are released into the erythrocyte. Several antigens from this organelle have been identified in *P. falciparum* and *P. yoelii* merozoites. A purified 235kD rhoptry antigen from *P.yoelii* YM strain induces a relatively weaker protection in vaccinated mice compared with the 230kD merozoite surface antigen, which induces a potent protective response (Holder & Freeman, 1981). However, monoclonal antibodies recognizing the 235kD antigen were protective on passive transfer, while monoclonals to the 230kD antigen were not (Freeman *et al.*, 1980). The authors concluded that humoral immunity to *P. yoelii* may be mediated by

antibody to the 235kD antigen, while the 230kD antigen was probably involved in protective cell-mediated responses. This antigen was later confirmed to induce a potent delayed-type hypersensitivity (DTH), T cell response in vaccinated mice. (Playfair, de Souza, Freeman & Holder, 1985).

Several rhoptry antigens have been identified in *P. falciparum.* Monoclonal antibodies against a 41kD-82kD rhoptry component inhibit the growth of asexual blood-stages *in vitro* (Perrin & Dyal, 1982). The 82kD protein is processed into fragments of 76kD and 65kD which are not related to the 41kD fragment (Braun-Bretton *et al.*, 1986). In addition, the fragments differ functionally; the 82kD and 76kD fragments are proteases, while the 41kD fragment displays aldolase activity (Certa *et al.*, 1988). All these fragments induce protective immunity in vaccinated monkeys (Dubois *et al.*, 1984; Perrin *et al.*, 1985). Rhoptry antigens of similar size (80kD, 66kD & 42kD) have also been described by other laboratories (Schofield *et al.*, 1986; Bushell *et al.*, 1988), and monoclonal antibodies to this complex inhibited *P. falciparum* merozoite invasion of erythrocytes *in vitro.*

An antigen located in the peduncle of the rhoptries has been described for *P. falciparum.* It is synthesized at the trophozoite stage in the form of a 240kD protein which is processed into a 225kD polypeptide during schizogony (Roger *et al.*, 1988). Various other rhoptry antigens identified in *P. falciparum,* once again consisting of sets of proteins, include a complex containing a major component of 110-100kD and minor components of 155kD, 140kD and 130kD, identified by a monoclonal antibody (Sam-Yellowe *et al.*, 1988). There is also a set composed of three proteins of molecular mass 105, 130 and 140kD which are co-precipitated.

Although several rhoptry proteins have been identified, only a few have been tested as potential vaccines.

Other candidate antigens

Molecules located on the surface of merozoites which are involved in penetration of host erythrocytes are obvious targets, and indeed a lectin-like polypeptide has been shown to bind to the erythrocyte membrane or to glycophorin A (Jungery *et al.*, 1983). The malaria transferrin receptor, identified at the surface of erythrocytes, is yet another target. The parasite is dependent on exogenous iron which is transported as bound ferrotransferrin to the intracellular parasite via the transferrin receptor however, the use of this protein as a vaccine will depend on its divergence from the human receptor (Roderigues & Jungery, 1986). A parasitophorous vacuole antigen of *P. falciparum* , molecular mass 126-140kD, is cleaved on schizont rupture into three fragments of 50,47 & 18kD. The gene encoding this protein has been cloned and shown to code for amino acid repeats. This antigen protects monkeys from a *P. falciparum* challenge infection (Perrin *et al.*, 1984).

A large molecular mass antigen (M_r >300,000) isolated from an antigen-expressing λgt 11 clone (Stahl *et al.*, 1985) is expressed in all isolates of *P. falciparum*. This antigen, termed FIRA (Falciparum Interspersed Repeat Antigen), induces appreciable antibody levels in the majority of inhabitants of endemic areas, and is therefore unlikely to be a target of protective antibody responses. The antigen is nevertheless of interest because it contains repeat regions (as in RESA and the S antigens - discussed below) which encode naturally immunogenic epitopes. Indirect

immunofluorescence studies with anti-FIRA antibodies indicate that FIRA is external to the parasite within the infected erythrocyte and can therefore be recovered in the supernatant following saponin lysis of infected erythrocytes.

Whilst RESA and the merozoite rhoptry proteins do not exhibit any diversity among different isolates of *P. falciparum*, the S antigens (heat-stable, soluble serum antigen - highly strain specific), by contrast, show a range of repeat structures which are totally unrelated to one another. For example, an isolate from Papua New Guinea has an S antigen with an 11 amino acid sequence repeated 100 times, while a Ghanian isolate has a repetitive 8 amino acid sequence alternating at one position throughout the molecule (Cowman *et al.*, 1985). Such "repetopes" are probably evasion tactics of the parasite, designed to confuse the immune system and may well be responsible for the incomplete, strain-specific immune response to malaria. The diversity of the S antigens has probably evolved as a result of selection pressure exerted by the host immune response. However, there is no evidence to suggest that protective, strain-specific responses are directed against S antigens. Their use as vaccine components is limited by the extreme diversity of the repeat regions.

The presence of repeat structures is a remarkable feature of all *P. falciparum* antigens so far sequenced. Interestingly, the same or related repeat sequence can occur in antigens from different stages of the life cycle. For example there is an immunological cross-reaction between repeat regions from CSP, RESA, and S antigens (Anders *et al.*, 1985). The significance of these cross-reactions is not clear, but they may enhance the ability of these immunodominant structures to mask the host's immune

response to protective epitopes. Repeats may also be involved in polyclonal B and T cell activation and play a role in immunosuppression.

1. 7. 4 Sexual blood - stage antigens

A number of gametocyte surface proteins have been identified as targets of transmission-blocking immunity, using monoclonal antibodies of defined specificity. At least two gametocyte-surface proteins have been identified in *P. falciparum*; these include a 230kD protein and a 48-45kD doublet. Gametocytes treated with antibodies against these antigens, are unable to transmit infection to mosquitoes (Vermeulen *et al.*, 1985). These antigens are present in blood stream gametocytes, and antibodies can be found in some naturally infected humans. The stages arising from gamete fusion, zygote and ookinete, express a protein of 25kD. Antibodies to this antigen do not prevent fertilization, but inhibit subsequent development of the parasite. This antigen has now been sequenced (Kaslow *et al* ., 1988), but has not yet been tested as a potential transmission blocking vaccine. By contrast to the 48/45kD doublet, sera from humans exposed to infection do not respond to the 25kD antigen. This is not surprising as the antigen appears only in the mosquito.

1. 7. 5 Enzyme Targets

Other attractive targets that might be considered for immunological attack are enzymes. Parasitic protozoan enzymes, particularly the cysteine proteases, have generated much interest during the last five years, and it is generally accepted that they are involved especially in many aspects of host-parasite interactions (North, Mottram & Coombs, 1990). Inhibitors showing a high degree of specificity for particular proteinases have been developed for their use as possible antiparasitic agents. Some have

antiparasitic activity *in vitro* (Robertson *et al* ., 1990; Coombs, Hart & Capaldo, 1982). Another exciting trend in this field is the development of synthetic peptides based on the structure of proteinase inhibitors, which were shown to be effective against bacteria (Bjorck *et al* ., 1989), and indeed against *P. falciparum* (Rockett *et al.*, 1990) *in vitro* .

1. 7. 6. Problems of malaria vaccine development

Unlike the simple surfaces of bacteria or viruses, parasite surfaces consist of a large repertoire of proteins, glycoproteins and glycolipids. In addition, the life cycle of malaria parasites is complex, involving developmental stages in both a human host and a mosquito vector. Immunity to malaria accordingly is a lot more complicated than bacterial or viral immunity, and a number of factors are likely to interfere with vaccine development; these include:

a) Stage specificity of antigens: Immunity to malaria is stage specific and there is very limited cross-reaction between protective antigens, thus antigens from one stage do not induce immunity to another. Cross-reactivity only exists between the sporozoite and liver stages.

b) Strain specificity: The occurrence of inter-strain polymorphism in malaria antigens is a serious problem in terms of vaccine design. Candidate vaccine antigens, including MSA-1 and the CS protein, contain regions in their amino acid sequences which differ between isolates. In the CS protein, the immunodominant B cell epitope (NANP repeat) is naturally invariant; however, diversity occurs in the T cell recognition sites.

c) Antigenic variation: The ability of the parasite to switch its antigenic

expression in response to immune pressure is yet another problem. It is generally associated with the erythrocytic antigens, and in one study antibody-induced variation has been reported (David, *et al.*, 1985). When a rhesus monkey was immunized with a 140kD merozoite surface antigen (not associated with MSA-1), which is naturally invariant but immunodominant, a limited protective response was achieved. Interestingly, when the resulting parasites were analysed, they lacked the 140kD antigen but showed the presence of a new variant molecule.

d) Immunogenicity: A better understanding of the mechanisms of acquired immunity to malaria would be of immense value in vaccine development. Epitopes that stimulate both antibody and cell-mediated immunity must be included as essential components of a potential vaccine. Specific T cell epitopes may be difficult to identify in the light of antigenic diversity of these epitopes; however, it might be possible to identify regions for universal MHC class II binding. An epitope of this sort has been identified by Sinagaglia *et al.* (1988). In mice the response to the CS antigen (Good *et al.*, 1988a) and RESA (Lew *et al.*, 1989) is MHC class II restricted, but the use of human recombinant IL-2 as an adjuvant has been reported to overcome genetic non-responsiveness to malaria sporozoite peptides (Good *et al.*, 1988b).

The fact that immunity to malaria is slow to develop and is usually incomplete, lasting only for months or years, shows how well the parasite has adapted itself to co-exist with its host. An equilibrium is therefore established between host immunity and parasite evasion; this may be facilitated by parasite induced immunosuppression or immunopathology.

1. 8. AIM OF THE PROJECT

Recombinant DNA technology and affinity purification using monoclonal antibodies are two widely used techniques for the identification of blood-stage malaria antigens from various species of *Plasmodia.* Electroelution of protein bands from SDS gels has also been used for immunization of monkeys. However, all these techniques rely on the reactivity of antigens with immune serum or protective monoclonal antibodies, which assumes in advance that the induction of antibody is the main objective of a vaccine. Other equally important factors that should be considered are the possible role of T cell recognition of antigen and macrophage activation. Furthermore, most of the currently identified protective blood-stage malarial antigens face the problem of antigenic polymorphism which rules out their use as potential vaccines. Nevertheless, there are a number of antigens on the surface of merozoites and schizonts, which remain unidentified. Two-dimensional SDS-PAGE shows the presence of several hundred *P.yoelii* blood-stage antigens (Fenton, Walker and Walliker, 1985), some of which have now been identified by affinity purification using monoclonal antibodies. Whilst antibodies raised against some of these antigens are protective in passive immunization studies, others are not (Freeman & Holder, 1983).

Hence the aims of this research project were as follows: 1) to isolate a panel of asexual blood-stage antigens using biochemical techniques, without reference to their reactivity with antibodies, 2) assay the protective effect of all antigens *in vivo;* the mouse model of malaria is ideally suited for this purpose where large-scale protection experiments can be carried out, 3) determine the mechanisms involved in protection, 4) attempt to obtain amino acid sequences of protective antigens and compare these

with a database of known antigens, 5) if novel antigens were detected, identification of the relevant genes from an existing DNA library would be attempted with the eventual aim of obtaining the human (*P. falciparum*) counterpart.

2.0 MATERIALS AND METHODS

2.1 Mice

(Balb/c X C57BL) F1 hybrid mice of both sexes were used at 10 to 15 weeks of age. These mice were bred in our laboratory from parent strains supplied by the National Institute for Medical research, Mill Hill, London, U.K.

2.2 Parasites

The lethal *P.yoelii* YM strain of parasite was kindly provided by Professor D. Walliker (University of Edinburgh, Edinburgh, Scotland). This parasite gives rise to a rapid parasitaemia in our mice, resulting in death by day 9. Parasites were cloned once a year, stored in liquid nitrogen and maintained by weekly blood passage. A fresh supply of cloned parasites were passaged from frozen stocks every three months. Infections were initiated by i.v. injection of 10^4 parasitized erythrocytes and parasitaemias were counted on Giemsa-stained tail blood films.

2.3 VACCINE PREPARATION

a. Extraction buffer for parasite membrane antigens

A Triton X-100 (TX-100) extraction buffer, described by Deans *et al.* (1982), was prepared as follows:

Values in brackets represent final concentrations.

12.5ml 0.1M Tris-Hcl pH8 (50mM)

2.5ml 5% (w/v) TX-100 in D.H_2O (0.5%)

0.625ml 0.2M EDTA (5mM)

0.25ml 0.5M PMSF (dissolved in isopropanol) (5mM)

The above solutions were mixed and heated to 60°C to ensure that the PMSF was completely dissolved. After cooling to room temperature the following were added:

92mg Iodoacetamide (20mM)

0.125ml Trasylol (Aprotinin) (5µg/ml)

0.0125ml Pepstatin A (dissolved in acetone/ethanol 1:3) (1µg/ml)

50µl Leupeptin (20µg/ml)

Finally the volume of the solution was made up to 25ml with D.H_2O.
All reagents were obtained from Sigma except Leupeptin which was obtained from Calbiochem.

b. Preparation of TX-100 lysates

Donor mice were infected i.v. with 10^7-10^8 parasitized erythrocytes and bled 4 days later into heparinized PBS when their parasitaemias approached 100%; 90% of the parasites were schizonts. The blood was washed three times in PBS, the buffy coat was carefully removed, and the pellet was lysed in 0.01-0.02% saponin in PBS at 37°C for 20-30 min, and washed three times in PBS until the supernatent was free of visible haemoglobin. The pellet was resuspended in TX-100 extraction buffer at a ratio of 1 volume pellet to 5 volumes buffer. Solubilization was carried out at 4°C for 3hr with vortexing at intervals. Cell debris and insoluble material were removed by microcentrifugation at 8000*g* for 10 min. The clear supernatent was dialysed against distilled water for IEF or against PBS for Sephacryl S.200 fractionation and for vaccination. The protein content of the lysate was determined by the Bio-Rad protein assay. The yield per

donor was approximately 400μg which was sufficient to protect about 20 recipients.

2.4 VACCINATION AND PREPARATION OF ANTISERA

a. Vaccination and challenge

Mice were given two i.p. doses of 10μg protein (lysate or fractionated antigens) plus 25μg of saponin HP3 (kindly provided by Dr. R. Bomford, Wellcome Research Laboratories, Beckenham, Kent.) two weeks apart, followed three weeks later by challenge with 10^4 viable parasites for protection studies. A similar vaccination regime was used for DTH studies but the challenge was different (described later). For T-helper cell studies mice were given a single dose of 1μg protein (of the appropriate fractionated antigens or the lysate) plus 25μg saponin; the latter two regimes had been shown previously to be optimal.

b. Preparation of anti-*P.yoelii* antisera

Antibody to *P.yoelii* was obtained from mice vaccinated with the TX-100 lysate, and challenged as described above. Animals were bled out ten days following challenge, which was usually three days after clearing their parasitaemia. This antiserum had an indirect immunofluorescence titre of 1:65000 against schizonts of *P.yoelii*.

c. Preparation of antiserum against HPLC fraction (h)

Mice were immunized i.p. with two doses of 5μg HPLC fraction **h** plus 25μg saponin as adjuvant, two weeks apart. Two weeks later they were

boosted i.v. with 5μg of the fraction in PBS and bled out two weeks later. This antiserum had an indirect immunofluorescence titre of 1:32000 against *P.yoelii* schizonts.

2.5 PURIFICATION PROCEDURES

a. Isoelectric focussing

Lysates prepared in a sucrose density gradient (5%-50%) containing 4.5% Ampholine were fractionated in a 110ml vertical LKB IEF column, according to the instructions in the LKB manual (LKB 8100 Ampholine Electrofocussing Column). Usually 5ml of lysate, containing 20mg protein was mixed with 5ml of Ampholine and 5ml of the mixture was added to a dense (50%) or a light (5%) sucrose solution. Each of these solutions was finally made up to 55ml with D.H_2O. A 5-to-50% gradient solution was introduced into the focussing column via a gradient former. The Ampholine mixtures used were pH3 to 10 (broad range). Narrow range Ampholines used in the initial studies included pH3.5 to 5, 5 to 8, 7 to 9 and 9 to 11. Electrofocussing was carried out for 48hrs using a power of 5 Watts, a current of 10mA and a variable voltage. The apparatus was cooled with a supply of coolant pumped through the cooling jackets. On completion, 3ml fractions (25 - 30) were collected and their pH was measured. Fractions were then dialysed against PBS overnight at $4^{o}C$ to remove the sucrose and Ampholines. Protein concentrations of the fractions were determined by the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Hertfordshire WD1 8RP, U.K.) Based on this assay the recovery of protein from the IEF column was 75 to 80%. The average yield of protein from 10^{10}

normal mouse erythrocytes was 2.1mg, and 17.5mg for schizont parasitized erythrocytes. Thus about 88% of the protein in the lysate appeared to be of parasite origin.

Diagrams of the IEF apparatus used in this project have not been shown as this particular model is now obsolete. Modern focussing columns are available from Bio-Rad Laboratories. With this new apparatus, known as a Rotofor preparative IEF cell, a complete focussing run takes only 5hrs.

b. Sephacryl S.200HR gel filtration chromatography

A Sephacryl S.200 or S.300 High Resolution gel (Pharmacia), equilibrated in PBS was used in a column of dimensions 1.6 X 90cms. The S.200 gel matrix separates molecules on the basis of size, between 10kD and 250kD, while the S.300 gel separates molecules of size between 1000kD and 20kD. The column was packed and standardized according to the manufactures instructions before use. 1ml - 1.5ml of millipore filtered (0.45µ pore size) lysate was loaded and usually 3.5ml fractions were collected at a flow rate of 20ml/hr at room temperature. A chromatograph representing the A_{280} of fractions from a typical experiment is shown in figure 2.5a. Four clear peaks are shown; the large fourth peak eluted at the end of the run represents the excess TX-100. The protein content of these fractions is shown in figure 2.5b, which shows that there are only three protein peaks.

Sephacryl S.200 chromatograph of *P.yoelii* TX-100 lysate

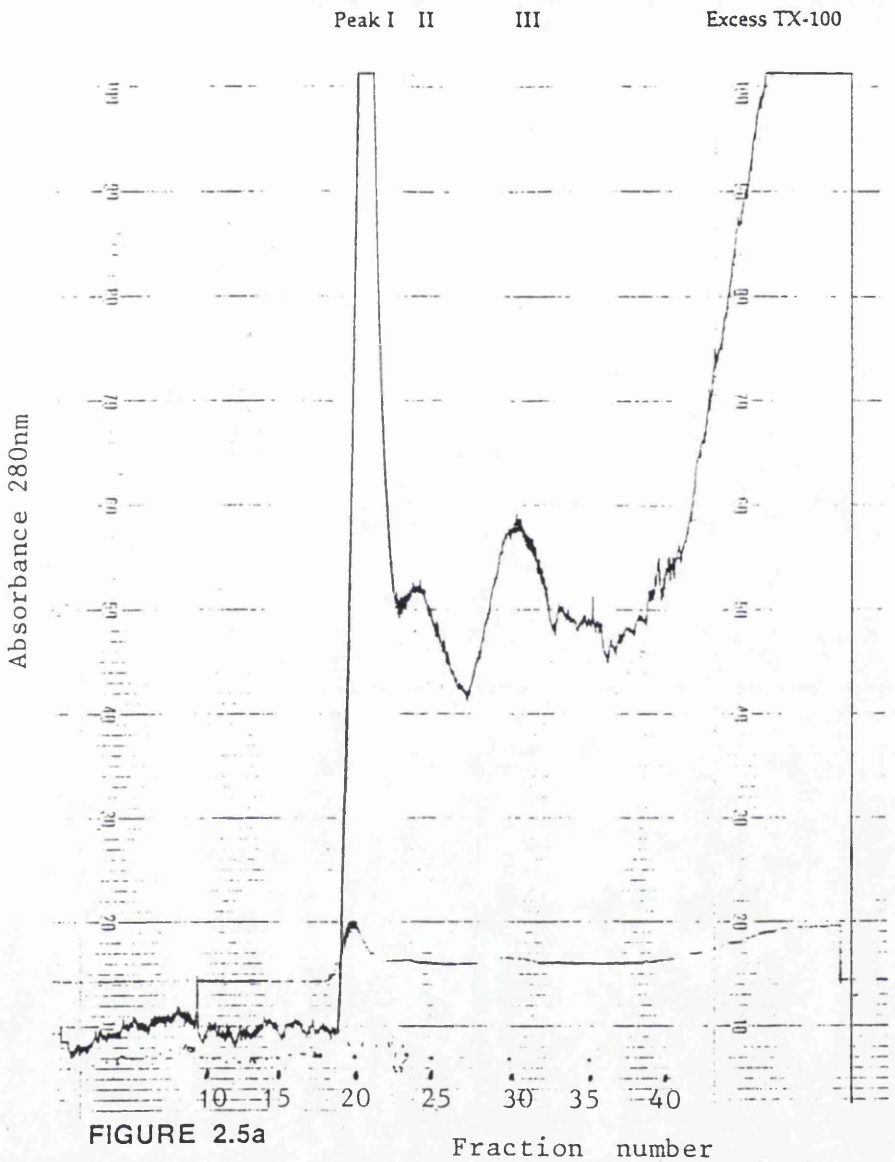


FIGURE 2.5a

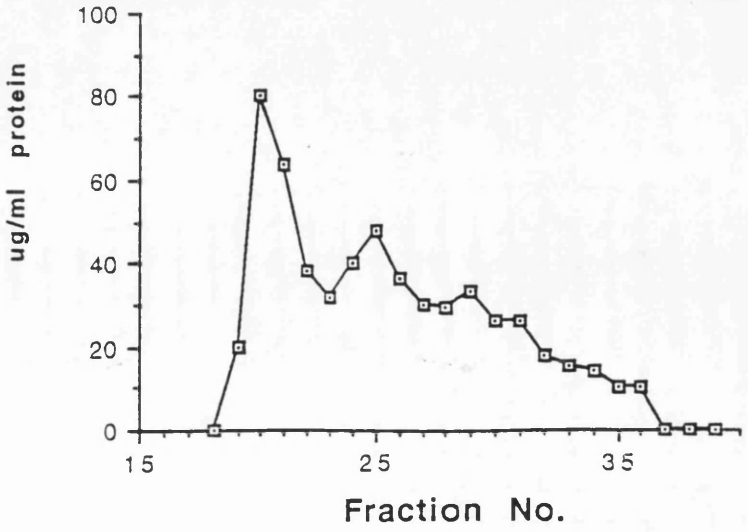


Figure 2.5b Bio-Rad protein concentration of fractions from above chromatograph

c. Reverse phase HPLC

This purification procedure was kindly carried out by Dr. B. Coles from the Molecular Toxicology Department, UCMSM.

A Brownlee Aquapore (300 A^{O}) (Gilson), C-8 HPLC purification column of dimensions, 30 X 4.6mm was used. This column separates molecules on the basis of their hydrophobicity. The hydrophilic molecules are eluted first while the hydrophobic ones are eluted towards the end.

Usually 200µl-400µl (40-80µg protein) of sample were loaded, and 1ml fractions were collected at a flow rate of 0.4ml/min using a solvent gradient ranging from 25% to 60% acetonitrile in D.H_2O; both gradient solutions were acidified with 0.06% trifluoroacetic acid in D.H_2O. As several different samples were purified by this procedure the elution chromatographs are shown together with the respective protection data in section 3.0.

d. Gel filtration FPLC

This procedure was carried out jointly with Dr. N. Sumar from our Department. It was used for purification of the IEF pH4 fraction. A Superose 12 HR 10/30 FPLC column (Pharmacia) equilibrated in PBS was used for separating molecules of size between 300kD and 10kD. The column was standardized using the Bio-Rad FPLC standardization kit (Cat. No. 151-1901). The IEF pH4 precipitate was dissolved in 0.05% SDS and millipore filtered (0.45µ pore size) before purification. Usually 0.2ml (200-300µg/ml protein) of sample were loaded and 1ml fractions (20 fractions) were collected. Protein concentrations of fractions were determined by the

Bio-Rad assay before they were used for vaccination.

e. Calculation of molecular weight of fractions from gel filtration columns

The Sephacryl S.200 and S.300 columns were standardized with molecular weight standard kits MW-GF-200 and MW-GF-1000 respectively, supplied by Sigma. Bio-Rad FPLC standards were used for the Superose-12 column as mentioned before. The void volume (V_o) of the columns was determined with Blue Dextran and the exclusion volume (V_e) of each standard was calculated. Molecular weight was plotted versus the V_e/V_o for each respective protein standard on semi-log paper. This standard curve was used to calculate the molecular weights of the fractionated antigens. The V_e/V_o of the fractions were determined and their molecular weights read off from the standard curve.

2.6 ANALYSIS OF FRACTIONATED ANTIGENS

a. Determination of protein concentrations

The Bio-Rad protein assay kit No. 500-0002 (Bio-Rad Laboratories, Watford, U.K.) was used. The microassay procedure was carried out as described in the instruction manual (Bio-Rad 1989).

b. SDS-PAGE and Western Blot analysis

An LKB/Pharmacia mini-gel system which is a slight modification of the Laemmli procedure (Laemmli, 1970) was used for molecular weight determination of fractionated antigens. This concentration of acrylamide is ideal for resolving proteins of M_r ranging from 200kD to 29kD. All samples were concentrated with polyethylene glycol 20kD and 10-50µg of protein were loaded, in volumes of 65µl, on to 7.5% acrylamide gels.

Electrophoresis was carried out at 20mA per gel.

Gels were stained with Coomassie brilliant blue R (Sigma) or with Bio-Rad Silver stain (Bio-Rad, U.K.).

For Western blot analysis, samples were electrophoresed as above and transferred to nitrocellulose sheets (Schleicher & Schuell BA 85), according to the method of Towbin *et al.* (1979). Transfer was carried out at $0.8mA/cm^2$ in a Hoefer TE 70 Semi-dry transfer unit (Hoefer Scientific) according to the instruction manual. The procedure was complete in about 1.5hr with mini-gels. This was determined by ensuring the complete transfer of the coloured bands of the molecular weight Rainbow markers (Amersham, U.K.). Unoccupied protein-binding sites were blocked by incubating in 50ml PBS containing 3% BSA, overnight at 4^oC. The blot was washed three times (10min per wash) in PBS containing 0.05% Tween, and then incubated in 50ml of a 1:50 dilution of antibody at room temperature. After 2hr the blot was washed three times as before and incubated in 50ml of a 1:1000 dilution of a rabbit anti-mouse peroxidase conjugate (Dako, U.K.). Two hours later the blot was washed again and protein bands were developed with 4-chloro-1-naphthol developer (Sigma) as described in Hudson and Hay (3rd Edition 1989).

c. Amino acid sequencing (Dr. B. Coles, Molecular Toxicology, UCMSM)

For amino acid sequencing samples were electrophoresed and transferred to a ProBlott™ protein sequencer membrane (Applied Biosystems, U.K.) as described above. The membrane was rinsed with $D.H_2O$, saturated with 100% methanol for a few seconds and stained with

0.1% Coomassie brilliant blue R in 40% methanol/1% acetic acid for one minute. The membrane was destained with 50% methanol until the background stain was removed and clear bands were visible. After rinsing with D.H_2O the bands of interest were excised and sequenced in an Applied Biosystems protein sequencer (Applied Biosystems, U.K.).

d. Spectrometry

The absorbance spectrum of the fractions of interest were obtained from a scan through the UV detector (Gilson 614 UV monitor) of the reverse phase HPLC apparatus; 100µl-200µl of sample were scanned between wavelengths - 200nm-300nm.

2.7 IMMUNOLOGICAL ASSAYS

a. T-helper cell assay

TNP coating of parasites

Parasitized erythrocytes were labelled with TNP, by the method of Ritteberg and Pratt (1969), as described previously (Playfair, de Souza and Cottrell, 1977a). Briefly, blood with at least 50% parasitaemia was washed three times in PBS and labelled with 2,4,6-trinitrobenzenesulphonic acid (TNBS) (Sigma) at a concentration of 5mg/ml in 0.28M cacodylate buffer pH6.9. Washed packed cells (1ml) were added to 7ml of TNBS solution with mixing and the reaction was allowed to continue for 30min at room temperature. The reaction was stopped by the addition of cold PBS and the TNP-labelled cells were washed with PBS until no free (yellow) TNP was visible in the supernatent. TNP-labelled parasitized erythrocytes (TNP-PY), or TNP-labelled normal mouse RBC, were injected i.v. immediately after preparation.

Plaque-forming cell assay

Four days after i.v. injection of 10^5 TNP-PY the mice were culled and their spleens were assayed for anti-TNP immunoglobulin M plaque-forming cells, using the method of Cunningham and Szenberg (1968). TNP-labelled sheep erythrocytes (prepared as described above for TNP-PY) were used as detector cells. This assay has been shown to reflect parasite-specific helper T cell activity (Playfair, de Souza & Cottrell, 1977a).

b. Delayed hypersensitivity (DTH) assay to parasite antigens

DTH to malarial antigens was assessed two weeks after vaccination by challenging mice in the right pinna with a subcutaneous injection of 10µl of the eliciting antigen. This test has shown that the strong protective immunity induced by a formalin-fixed *P.yoelii* vaccine correlates with the induction of specific DTH (Cottrell, Playfair and de Souza, 1977). After 24hrs the mice were injected i.v. with 1 X 10^7, ^{51}Cr-labelled bone marrow cells (BMC). The latter were obtained from femur washings of syngeneic mice, washed with RPMI and incubated with 100µci of ^{51}Cr (Sodium chromate, Amersham, U.K.) per 10^8 cells for 30 min at 37^{o}C. Labelled BMC were washed three times in RPMI and resuspended to a concentration of 5 X 10^7 cells per ml ready for i.v. injection. Twenty-four hours later mice were killed by cervical dislocation and their pinnae cut off at the hairline for radioactive counts in a Crystal Multi Detector Gamma Counter (United Technologies Packard, U.K.). The BMC-homing to the experimental pinnae was calculated as the percentage of the total injected cells specifically reaching the challenged pinnae. Thus, percentage homing (PH) was

calculated as follows:-

$$\text{PH (\%) value} = \frac{\text{(number of counts in right pinna) - (number of counts in left pinna) X 100}}{\text{(number of counts in total injected BMC)}}$$

This test has been shown to reflect parasite-specific T cells responsible for DTH activity (Cottrell, Playfair and de Souza, 1977).

c. Determination of antibody titres

Total anti-parasite antibody was measured by the slide fluorescence method of Voller and O'Neill (1971) using parasitized blood from an early infection as the antigen. Two-fold dilutions of serum were applied to the antigen-coated slides at room temperature for 30 min. Slides were washed with PBS and treated with fluorescein conjugated (FITC) polyvalent rabbit anti-mouse immunoglobulin for a further 30 min. After a final wash in PBS, slides were mounted with 10% glycerol pH8.6 (mounting medium) and the fluorescence was read in an u.v. incident light microscope. The end point was taken as the last dilution showing clear fluorescence.

2.8 ENZYMATIC DIGESTION OF ANTIGENS

a. Treatment with mixed proteases

Fractionated antigens were diluted 1:2 in PBS and digested with a mixture of proteinase K (Sigma, 10µg/ml) and pronase E (Sigma, 10µg/ml) for 24hrs at 37^{o}C on a roller. The reaction was terminated by boiling the enzymes for 15 min followed by extensive dialysis. Treated fractions were microfuged to remove aggregrates before use in vaccination experiments. Protein concentrations were checked before and after enzyme treatment.

b. Treatment with mixed lipases

Mixed lipases immobilized on to agarose beads (Sigma) were used at 5 units per ml of fractionated antigen. The reaction was allowed to proceed for 24hrs at 37^{o}C on a roller. Enzymatic digestion was terminated by the addition of an equal volume of cold PBS and the enzyme-coated beads were removed by microcentrifugation and the treated samples were dialysed against PBS before use.

c. Treatment with unrelated enzymes

Ribonuclease A (Sigma), essentially protease-free, or deoxyribonuclease I were used at 5 units per mg/ml of protein solution and used as described above for protease digestion.

2.9 STATISTICS

Significance levels were determined by the Student's *t*-test and values of $p>0.02$ were considered to be not significant.

The relationship between protection and T-helper priming was tested in two ways, by analysis of variance and by Spearman's rank correlation test. Conventional regression analysis is not appropriate because of the nonlinearity of the data for protection and mortality.

3.0 Isolation of protective *P.yoelii* (YM) antigens

Introduction

A saponin-lysed formalin-fixed preparation of parasitized red blood cells is an effective vaccine against lethal blood-stage murine malaria (Playfair & de Souza, 1977b). A single intravenous dose of between 10^6- 10^8 fixed parasites plus 10^7 *B.pertussis* as adjuvant, protected mice from a lethal *P.yoelii* YM infection but was less effective against *P.berghei* (Anka) where only 70% of the vaccinated mice were protected (de Souza, 1983).

This type of vaccine preparation is not practical for human use for three important reasons. First, mass production of merozoites is too costly. Second, as it is difficult to obtain merozoites that are entirely free from host red cell material, autoimmune phenomena may arise. Finally, it is impractical to conduct a large-scale field trial with a vaccine that has to be given intravenously. Identifying soluble parasite components for use as potential vaccines was considered as the most logical approach. Vaccination studies of soluble antigens prepared from heavily parasitized erythrocytes using various detergents showed that lysates prepared from Triton X-100 (TX-100) provided the best protection (Playfair & de Souza, 1986). Two intraperitoneal doses of 10µg protein plus 25µg saponin as adjuvant given two weeks apart, immunized mice effectively against a challenge infection. This lysate was used as the starting material for the isolation of various antigens for vaccination studies in mice.

Isoelectric focussing (IEF) or Sephacryl (S.200/S.300) gel filtration

chromatography were used as first step purification procedures for the lysate (fig. 3.0) and the major protective fractions from these procedures were further purified by HPLC or FPLC. All fractions were analysed by SDS-PAGE and Western Blotting and amino acid sequencing of single protective antigens was attempted. The type of immune response induced by the protective antigens was studied and an attempt was also made at characterizing these antigens. The basic experimental strategy is outlined in Figure 3.0.

Procedures for purification of TX-100 lysates of *P.yoelii* (YM) membrane proteins

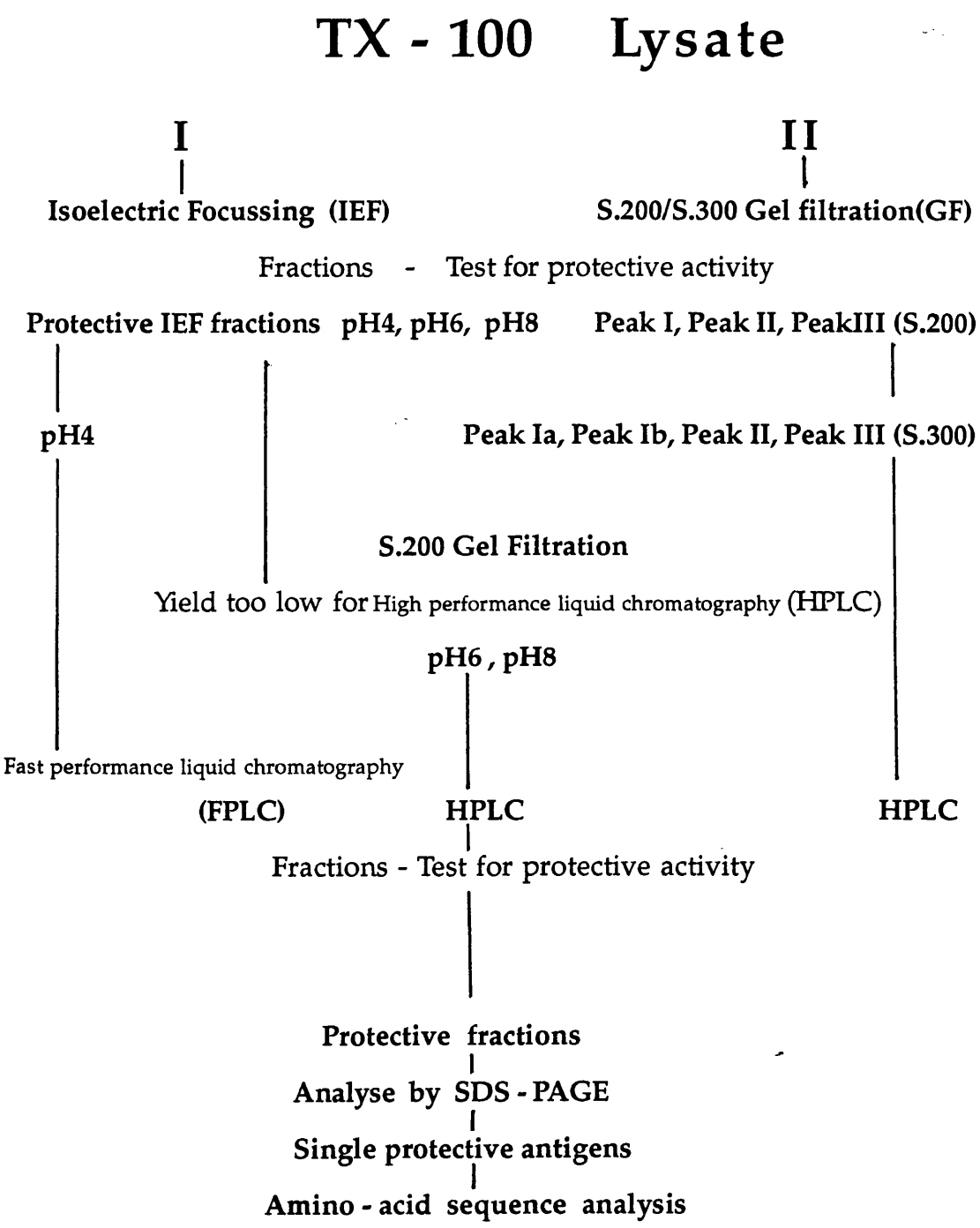


Figure 3. 0

RESULTS

3.1 Purification procedure I for *P.yoelii* (YM) TX-100 lysate

Protective effect of IEF fractions

Recovery in mice protected by vaccination with *P.yoelii* lysates or purified antigens was characterized as either **early recovery** - where parasitaemias were cleared by day 8 to day 12 after challenge, or **late recovery** - where parasitaemias were cleared around day 17 to 25. These two types of recovery are distinct. The distribution of recovery time is bimodal in mice vaccinated with a variety of fractionated *P.yoelii* membrane antigens; 66% of mice cleared their parasitaemia by day 12 and 24% after day 20 (figure 3.1a). The data in figure 3.1a was obtained from a total of 1000 vaccinated mice. Recovered animals remained totally resistant to rechallenge.

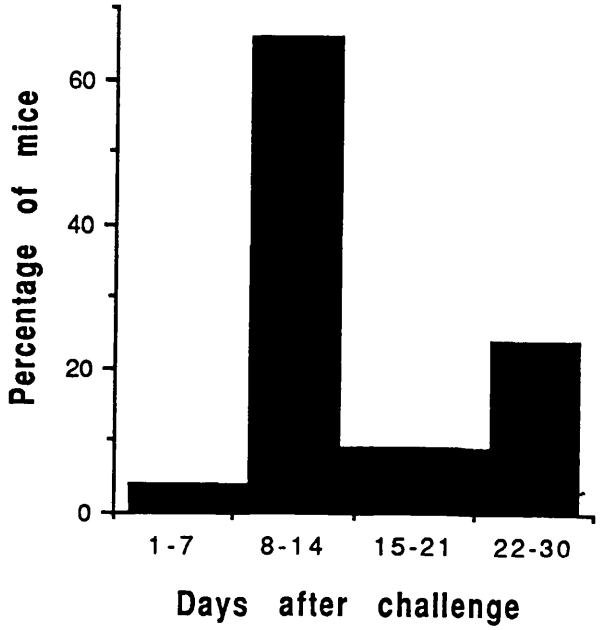


Figure 3.1a. The bimodal distribution of recovery time of mice vaccinated with P.yoelii membrane antigens

A broad range Ampholine of pH 3.5 to 10 was initially used to locate the areas where protective antigens were present. Preliminary experiments revealed protective activity in three regions around pH 4.1 to 4.6 (which contained the majority of the protein) and in two other regions at pH 6.5 to 7.2 and pH 7.8 to 8.3. The presence of these three protective regions was then confirmed in a study using narrow-range Ampholines in the range pH 3.5 to 5, pH 5 to 8, pH 7 to 9 and pH 9 to 11 (de Souza and Playfair, 1988).

Experiments were carried out with focussed fractions from four different lysates using the pH 3.5 to 10 Ampholine to assess reliability. The protein content of a typical experiment is illustrated in figure 3.1b where the majority of the protein focussed in the pH 4.1 to 4.6 region. This figure does not show a pH curve but instead the pH values of the collected fractions are plotted along the horizontal axis so that they can be directly compared with the pH values of the protection data shown in the figure below (figure 3.1c). The entire pH gradient is shown on the horizontal axis of figure 3.1b but the linear portion only includes fractions between pH3.8 and pH9.

Only antigens that were focussed along the straight portion of the pH curve (pH3.8 to 9) were injected into groups of 2 to 3 mice and fractions beyond the focussing range were excluded. The data in figure 3.1c includes values from the initial study with the pH3.5 to 10 Ampholine. Each bar corresponds to a range of 0.2 pH unit, represents a total pool of 17 to 21 mice and shows the percent of mice with early (solid) or late (shaded) protection. Vaccination with fractions from the first peak of pH 4.2 to 4.4

always gave 100% protection mainly of the early type. Fractions of pH6.0 to 6.8 and pH7.6 to 8.2 consistently gave over 75% protection. The pH5.0 to 5.8, pH6 to 6.2 and the pH7.2 to 7.4 fractions predominantly gave late type protection. Fractions of pH6.4 to 6.8 and pH7.8 to 8.2 gave mainly early protection.

Lysates prepared from normal uninfected mouse erythrocytes yielded only about a tenth of the protein as the same number of infected erythrocytes and the majority of this protein peaked at around pH 4.6 (figure 3.1b). This lysate and its IEF fractions did not have any protective activity in mice that were vaccinated with doses of up to 25µg of protein (Table 3.1.1b). These mice and the unvaccinated controls always died between days 8 and 9 after challenge.

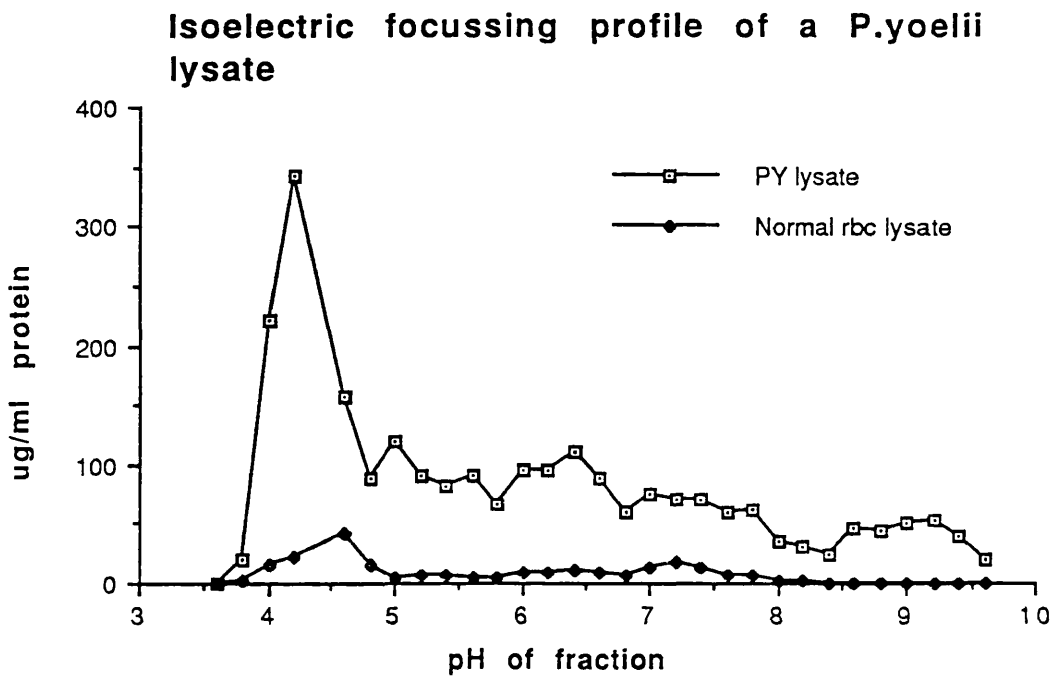


Figure 3.1b. Isoelectric focussing profile of a P.yoelii lysate from a single representative experiment

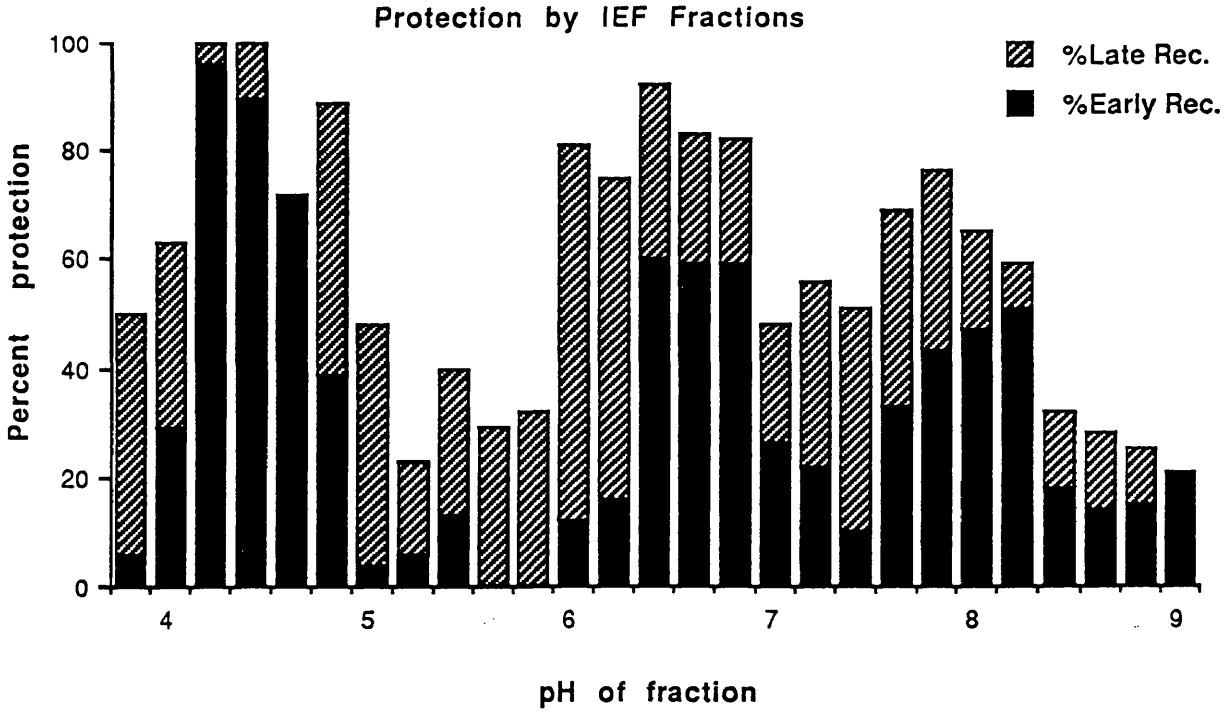


Figure 3.1c. Protective effect of fractions from 7 separate experiments using the Ampholine of range pH3.5 to 10. Each bar represents a total of 17 to 21 mice.

3. 1. 1 Protection by pooled IEF fractions

Fractions within each IEF region were pooled and concentrated in order to increase the protein yield for further purification by S.200 gel filtration or HPLC. IEF fractions within each region were pooled as shown in table 3.1.1a.

Table 3.1.1a Preparation of pooled IEF fractions

pH pool	Fractions pooled	Protein µg/ml
pH 3	pH 3.6 to 3.8	200
pH 4	pH 4.0 to 4.6	500 - 1mg/ml
pH 5	pH 5.0 to 5.6	200 - 300
pH 6	pH 6.0 to 6.8	300
pH 7	pH 7.0 to 7.6	300
pH 8	pH 7.8 to 8.4	300
pH 9	pH 9.0 to 9.2	200

IEF fractions selected on the basis of their protective activity were pooled as shown above. Each IEF pool was the product of a single focussing run.

Five different lysates were focussed and the resulting pooled IEF fractions were concentrated and their protein content determined. Ten µg of each IEF pool were tested in a group of 3 mice in all experiments. Five experiments were set up; thus each bar in figure 3.1.1a corresponding to one pH unit, represents a total of 15 mice and shows the percent of mice with early or late protection. Clearly the pH4 pool induced the strongest protective response which was similar to that of the lysate; recovery was total and almost always of the early type. The IEF pH6 and pH8 pools were less effective. Some preparations were potent enough to give early

recovery but the majority induced recovery of the late type. Parasitaemias of mice vaccinated with these pooled IEF fractions are shown in figure 3.1.1b. The parasitaemias of mice vaccinated with IEF pH3, 5, 7, 9 and the unvaccinated control group terminate on day 7 as deaths occurred on the following day. Similarly parasitaemias of groups vaccinated with IEF pH6 and 8 terminate on day 9. Mice vaccinated with the pH4 pool cleared their infections by day 7 or day 8 after challenge, their parasitaemias never exceeded 10% and they were significantly lower than the parasitaemias of animals vaccinated with the intact lysate (table 3.1.1b). Crisis forms were a marked feature of their blood films particularly on the day preceding recovery. Animals vaccinated with the pH6 and pH8 fractions could be classed into three groups: I) About 50% recovered early - by day 8 to 12 - and their parasitaemias were comparable with mice vaccinated with the lysate. II) 15%-20% of those recovering late developed higher parasitaemias which peaked above 50% on day 10 but decreased gradually by day 21-25 when the animals recovered. III) Finally, 20%-30% of the animals endured high parasitaemias which were fatal. Most of these deaths occurred at around day 10 to 20 which was significantly later than unvaccinated control mice.

In these experiments the pH7 pool gave encouraging results with more than 60% of the animals recovering. But as the majority were of the late type and a number of deaths occurred on day 8, this IEF fraction was excluded from further purification studies. The pH3, 5 and 9 antigens were always less effective as vaccines; recovery rates of vaccinated animals were usually less than 50% and deaths occurred by day 8 to 10.

The results obtained with the pooled IEF fractions confirmed the presence of the three protective regions at pH4, 6 and 8. These were further purified by Sephacryl S.200 gel filtration or HPLC and they were also analysed by SDS-PAGE and Western blotting.

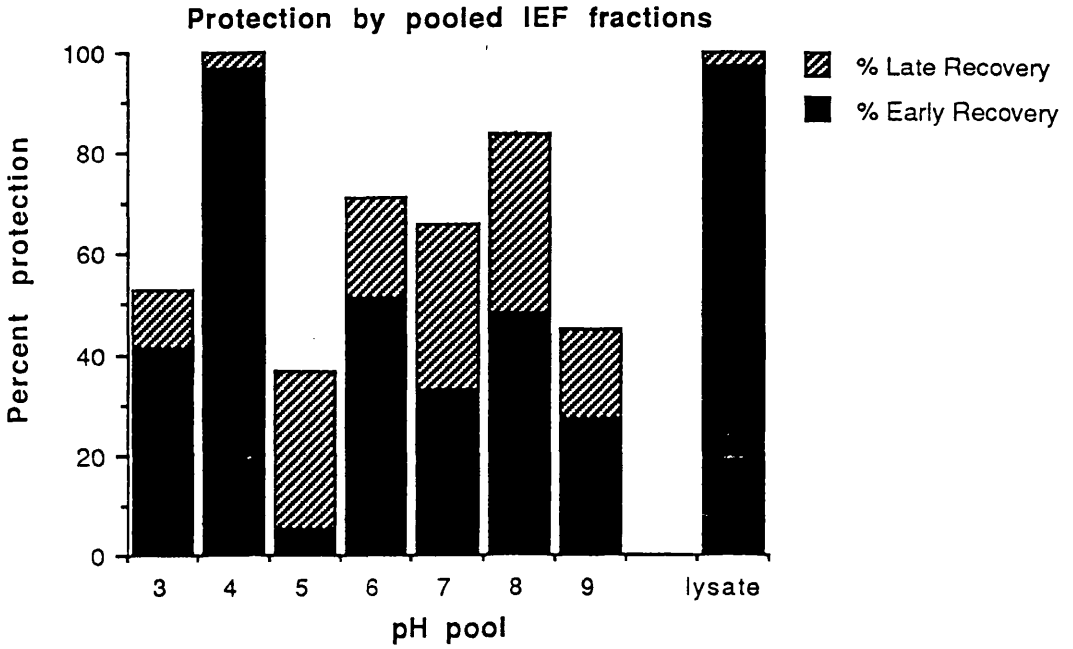


Figure 3.1.1a. Protective effect of pooled IEF fractions from 5 different experiments. Each bar represents 15 mice.

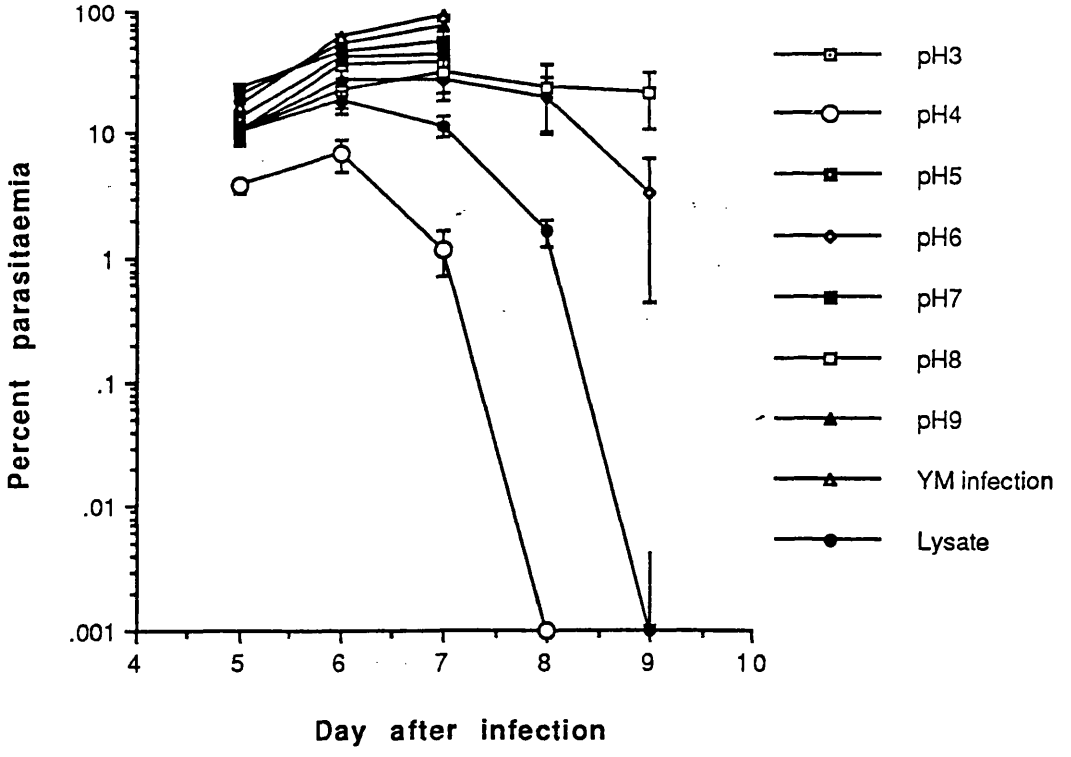


Figure 3.1.1b. Course of parasitaemia in mice vaccinated with pooled IEF fractions as described above

Table 3.1.1b

Parasitaemias of mice vaccinated with the *P. yoelii* lysate, MRBC control lysate or fractions from the three protective IEF regions. Pooled data from four experiments.

Vaccine	Percent parasitaemia ± SE						Day died
	Day after infection						
	5	6	7	8	9	10	
YM Lysate	10.2 ±1.5*	17.8 ±3.9@	11.3 ±2.1$	1.6 ±0.4	0.0	0.0	-
IEF pH4	3.6 ±0.4*	6.7 ±1.8@	1.2 ±0.4$	0.0	0.0	0.0	-
IEF pH6	10.2 ±1.4	26.1 ±6.3	27.1 ±8.1	18.5 ±9.2	3.3 ±12.8	NS	10-20
IEF pH8	10.2 ±1.9	21.6 ±5.9	31.3 ±10.6	22.5 ±12.5	20.6 ±10.3	NS	10-20
Unvaccinated control	16.1 ±2.0	59.8 ±4.5	90 ±2.1	D	-	-	8
MRBC control lysate	12.1 ±2.6	42.6 ±3.3	89.5 ±5.2	D	-	-	8

NS - data not shown as some mice died on this day.
D - day animals died

*$p < 0.001$

@$p < 0.019$

$$p < 0.0001$

3. 1. 2 SDS - PAGE and Western Blot analysis of pooled IEF fractions

All the IEF pooled fractions that were used in vaccination experiments were analysed by SDS-PAGE but only the most protective ones, pH4, 6 and 8 were analysed by Western Blotting so that correlations could be made between protective activity and the number of immunoreactive bands.

SDS-PAGE analysis of IEF pooled fractions

Each IEF pool was usually concentrated about five-fold for analysis and 20-30μg of protein were loaded on to gels. Usually consistent Coomassie Blue stain patterns were noted in experiments carried out with IEF fractions prepared from different batches. Variations were noted with some pH6 and 7 fractions which were occasionally contaminated with small amounts of haemoglobin.

The results of a typical experiment are shown in plate 3.1.2a. Each fraction had a characteristic staining pattern. The number of bands within each fraction are summarized as follows:

pH of frac.	Number of bands	Apparent Mol.wt.(kD)
3	none detected	-
4	7 clear; several diffuse	range 205-29
5	8	range 116-30
6	8	range 110-35
7	4 faint	range 60-40
8	2 clear	40, 35
9	1-2 clear	45, 40

SDS - PAGE analysis of IEF pooled fractions

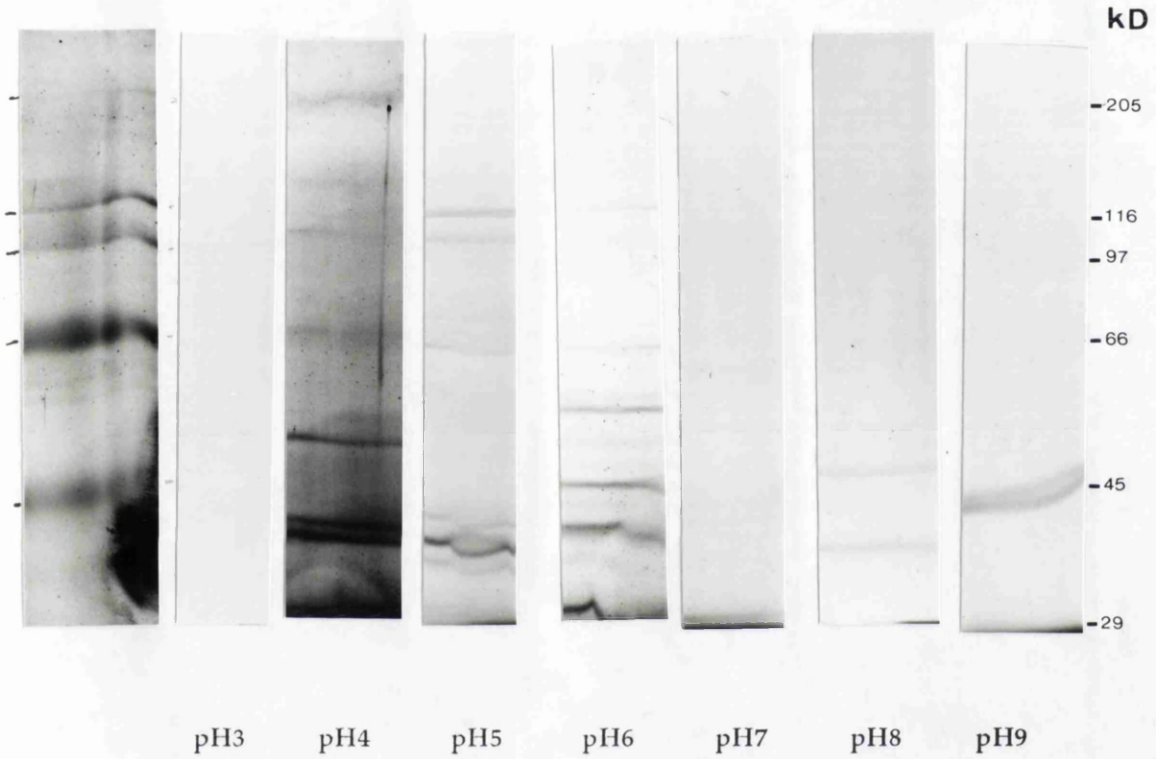


Plate 3.1.2a Coomassie Blue stained gel of 20-30ug protein of IEF pooled fractions.

SDS-PAGE and Western Blot analysis of IEF pH4, 6 and 8 fractions

Plate 3.1.2b (top photo) represents a Coomassie Blue stained SDS-PAGE gel of pooled IEF pH4, 6 and 8 fractions and the TX-100 lysate. The latter contained a number of bands of molecular weight ranging from 240kD to 20kD. Two different pooled pH4 samples have been shown as one of these (the second track) was frozen and thawed several times and shows diffuse bands. This fraction contained 12 visible bands and their molecular weights ranged from M_r 150kD to 20kD. The pH6 pool contained 6 bands of M_r 130kD, 84kD, 56kD, 36kD, 30kD and 22kD. The pH8 pool containd only 2 bands of M_r 40kD and 36kD.

Plate 3.1.2b (bottom photo) shows an immunoblot of the above fractions. In this experiment only 10μg of the lysate was loaded in order to obtain clear staining on the blot. Ten of the bands reacted with antibody, their molecular weights ranged from 140kD to 30kD. However, there are probably several other antibody-reactive bands present particularly between M_r 69kD and 30kD. These were seen when 25μg lysate were used as shown in plate 3.5.3a. In the pH4 pool 7 bands reacted with antibody; two of these, of M_r 45kD and 30kD, reacted particularly strongly. Other antibody-reactive bands with diffuse staining were seen at M_r 140kD, 90kD, 70kD, 50kD and 38kD. The pH6 pool contained a strongly stained band at 56kD and relatively weaker bands at 69kD - 60kD (four bands), 40kD, 38kD and 30kD. The pH8 pool also contained a strongly stained band at 56kD but four other strongly stained bands were seen at 60kD, 58kD, 40kD, 35kD and a weaker band at 38kD.

Whilst the Western blot staining patterns were very reproducible with

the pH4 fractions, variations were seen with different batches of the pH6 and pH8 fractions. The only variations seen with some freshly prepared pH4 fractions were stronger stained bands in the higher molecular weight region between 140kD and 70kD. All the pH6 batches tested contained antibody-reactive bands at 56kD, 38kD and 30kD; other antibody-reactive bands such as those shown in plate 3.1.2b were variant. The majority of the pH8 batches tested contained bands at 56kD, 40kD and 35kD which consistently reacted with antibody from various batches of hyperimmune serum.

SDS-PAGE and Western blot analysis of normal mouse red cell lysates (MRBC)

The SDS-PAGE gels shown in plate 3.1.2c were stained with Bio-Rad Silver stain in order to enhance the staining pattern of the MRBC lysate.Very weak bands were seen with Coomassie Blue stain. Bands representing high molecular weight membrane proteins such as glycophorin and spectrin were not seen, but strongly stained bands representing haemoglobin and presumably red cell membrane proteins were seen at M_r 65kD, 50kD, 45kD and 30kD. An equivalent quantity of a *P. yoelii* lysate was grossly over stained. The Western blot shows that some of the red cell membrane proteins reacted with anti-malaria antibody. These include bands at 90kD, 70kD, 50kD, 46kD, 40kD, 38kD and 30kD. However, the over-stained appearance of the *P. yoelii* lysate clearly indicates the presence of several parasite-specific proteins.

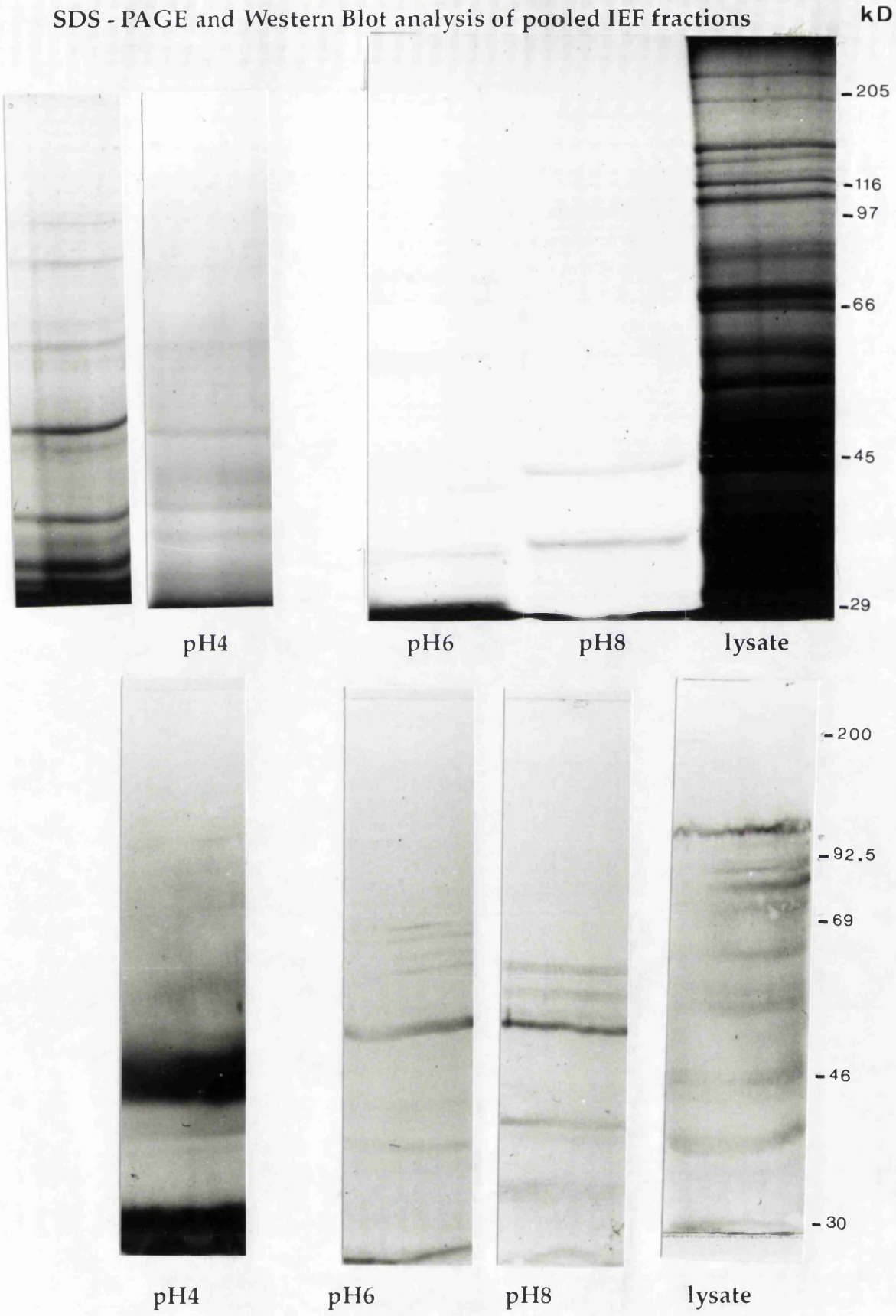


Plate 3.1.2b Top photo - SDS-PAGE analysis of IEF pH4 , 6, 8 and the lysate
Bottom photo - Western Blot analysis of above proteins

SDS-PAGE and Western Blot analysis of normal red blood cell membrane (MRBC) lysates

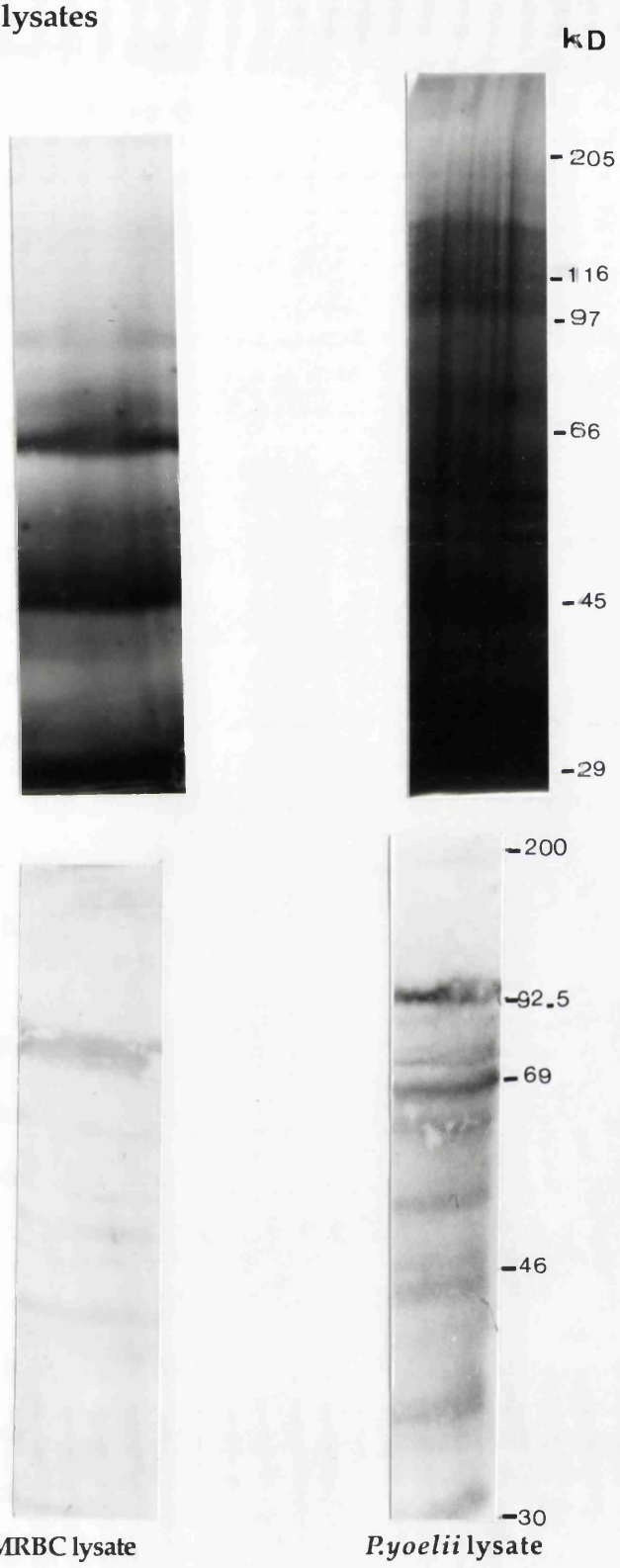


Plate 3.1.2c Top photo - Silver stained gel of 50µg MRBC lysate and 10µg *P.yoelii* lysate
Bottom photo - Western blot of above samples

3. 2 Purification procedure I, step 2

Sephacryl S.200 gel filtration of IEF pH4, 6 and 8 pooled fractions

As the three most protective IEF fractions contained a mixture of proteins as judged by SDS-PAGE it was necessary to determine which band (antigen or antigens) contributed towards the protective activity. Each IEF pool was accordingly further fractionated by S.200 gel filtration. In these experiments the pH4, 6, and 8 proteins were prepared in batches to increase their protein yield for further purification. Each batch consisted of pooled fractions (as shown in Table 3.1.1a.) from 3 to 4 focussing runs. In all experiments described below at least 3 mice were injected with each of the eluted S.200 fractions, giving a total of 18 to 25 mice tested per fraction. The protection data is shown in figures 3.2.1b, 3.2.2b and 3.2.3b where each bar represents values over a short range of molecular weights. For example the 300- bar represents values within the range 300-250kD and the 250- bar would represent 250-200kD. Single figures followed by a hyphen have been shown for reasons of convenience.

3. 2. 1 Protection by S.200 purified IEF pH4 pooled fraction

The pH4 fraction appeared as a precipitate and was therefore re-dissolved in 0.02% TX-100 before separation on the S.200 column.

Six different IEF pH4 batches were purified on the S.200 column and the eluted fractions were tested. The elution profile of a typical experiment, illustrated in figure 3.2.1a, shows that the majority of the protein appeared in the high molecular weight region. The best protection appeared in a fraction of relative molecular mass (M_r) 250kD where 100% protection of the early type was achieved - figure 3.2.1b. Fractions of M_r

ranging from 200 to 100kD also gave 100% protection but 15-20% of this was of the late type. Fractions of M_r 80 to 60kD protected less than 80% of the vaccinated mice and the protective effect of fractions decreased as their molecular weights dropped down to <10kD.

The most protective fractions were in the molecular weight range 300kD-100kD. By limiting the dose of each fraction it would have been possible to determine which was the strongest, however this was not done for three reasons: I) This technique was only used as an intermediate purification step to identify the main protective regions which could then be further purified by FPLC.

II) As each fraction consisted of antigens over a range of molecular weights they were not likely to be single antigens.

III) It seemed wasteful to use large numbers of mice to test fractions that contained mixtures of antigens.

The whole pH4 fraction was used for further purification by FPLC as the best protective activity was in the main protein peak (fig.3.2.1a) which accounted for over 90% of the fraction; also the yield of the separated S.200 fractions was too low for this purpose.

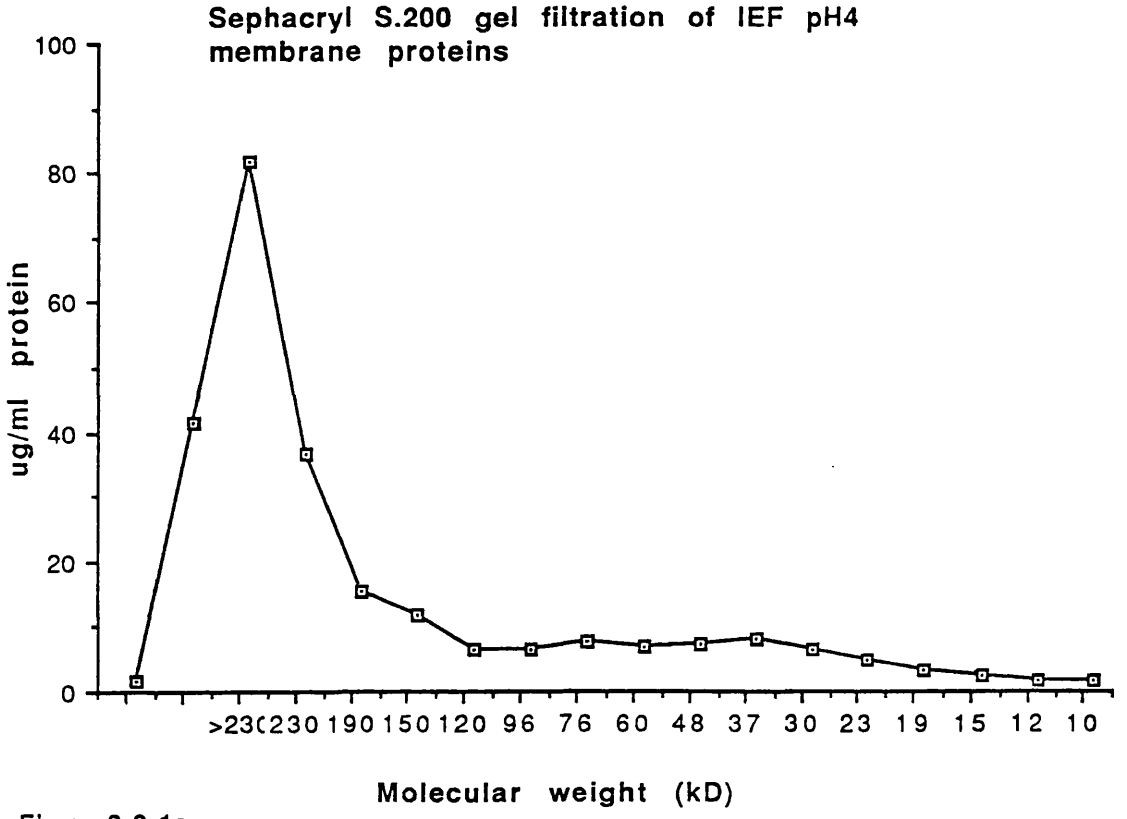


Figure3.2.1a.

S.200 profile of an IEF pH4 batch from a single representative experiment

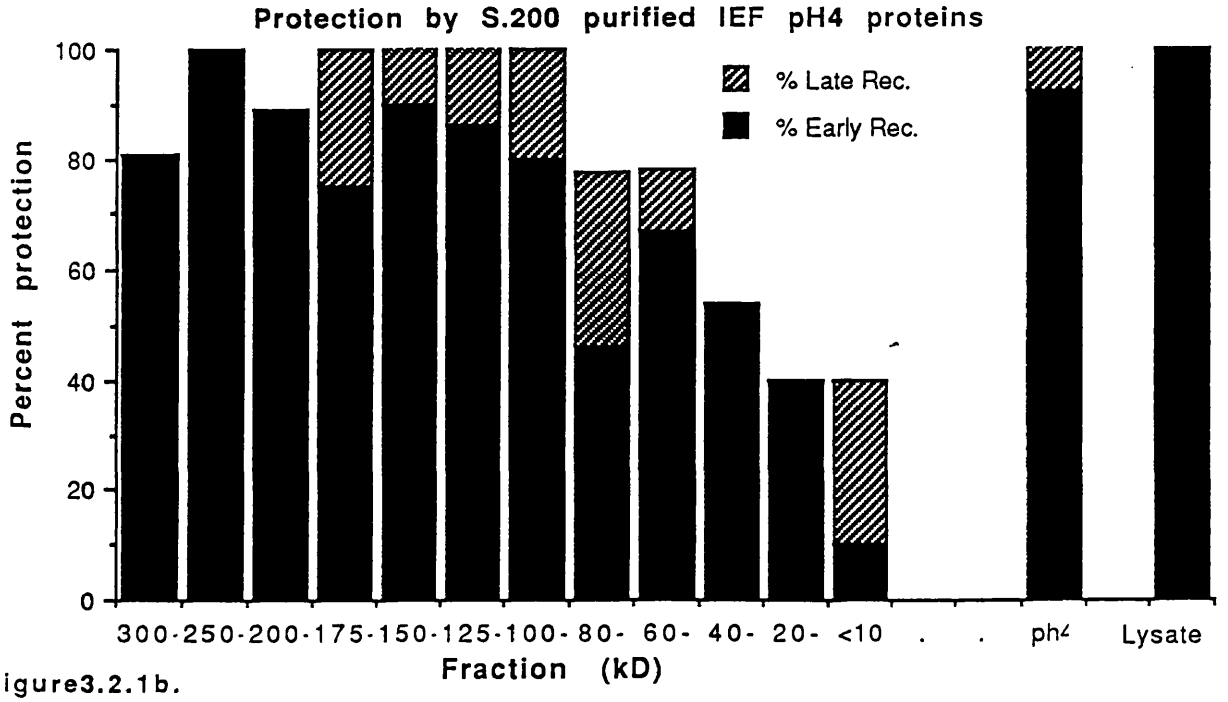


Figure3.2.1b.

Protective effect of S.200 separated IEF pH4 proteins; pooled data from 6 experiments

3. 2. 2 Protection by S.200 purified IEF pH6 pooled fraction

Six different IEF pH6 batches were separated on the S.200 column and the eluted fractions were tested. A typical experiment is illustrated in figure 3.2.2a which shows that the majority of the protein appeared around the 40kD to 60kD region with a smaller peak at the 23kD region. The best protective activity (over 77%) of almost entirely the late type coincided with the main peak at M_r 56 to 60kD (fig. 3.2.2b). Only two other fractions of M_r between 100 to 125kD and 60 to 80kD from the entire run were weakly protective; each fraction protected less than 40% of the vaccinated mice.

The whole IEF pH6 batch was used for further purification by HPLC as the main protective peak was not available in sufficient quantity.

3. 2. 3 Protection by S.200 purified IEF pH8 pooled fraction

Sephacryl S.200 separated fractions from six different IEF pH8 batches were tested as vaccines. The elution profile from a typical experiment is shown in figure 3.2.3a. Once again the protective activity was recovered in the main protein peak of M_r between 175kD and 150kD where more than 80% of the protection was of the early type (fig. 3.2.3b). Protection induced by this fraction was even stronger than that of the intact pH8 pool. Four fractions of weaker protective activity were seen at M_r between 100kD and 80kD, 80kD to 60kD, 60kD to 40kD and <10kD,where less than 40% of the animals were protected.

Further purification of the main protective peak by HPLC was not possible due to low protein yields. The whole IEF pH8 batch was therefore used for this purpose.

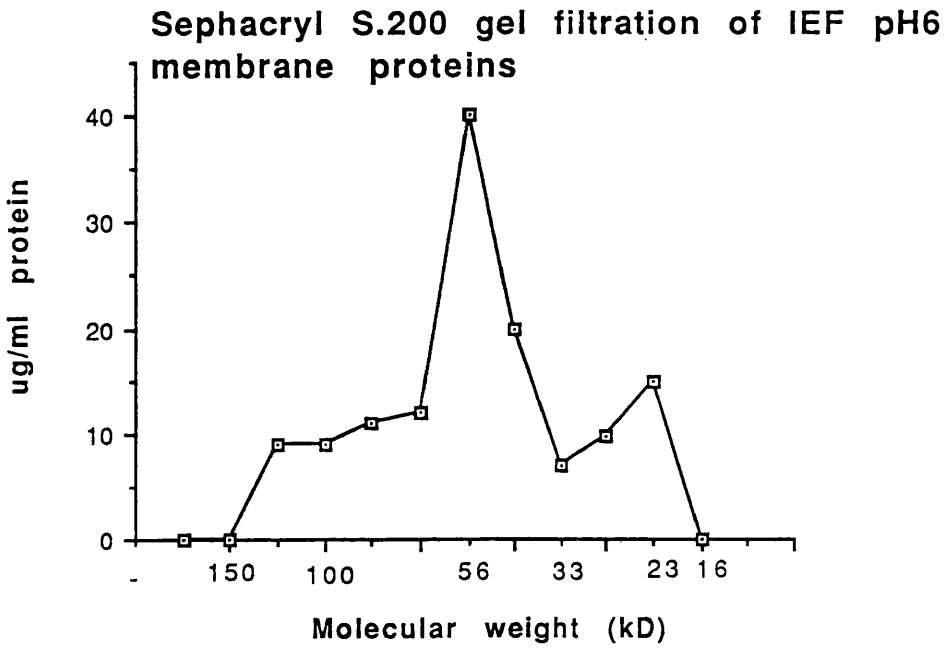


Figure 3.2.2a. S.200 profile of an IEF pH6 batch from a single representative experiment

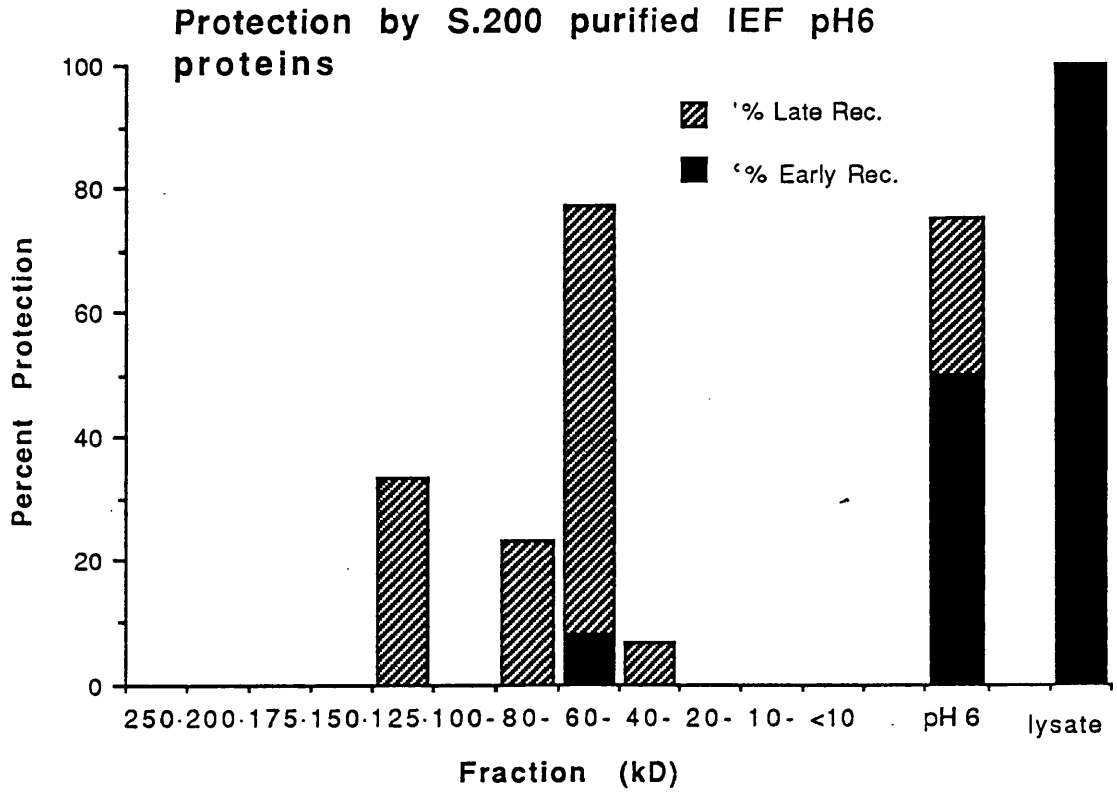


Figure 3.2.2b. Protective effect of S.200 separated IEF pH6 proteins; pooled data from 6 experiments

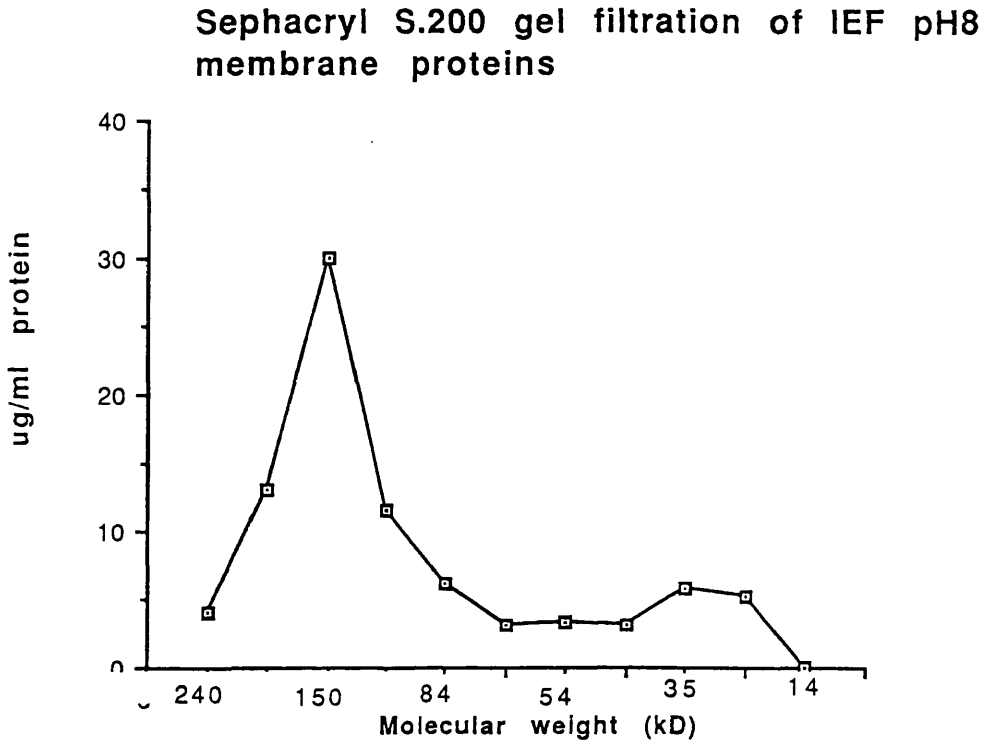


Figure 3.2.3a. S.200 profile of an IEF pH8 batch from a single representative experiments

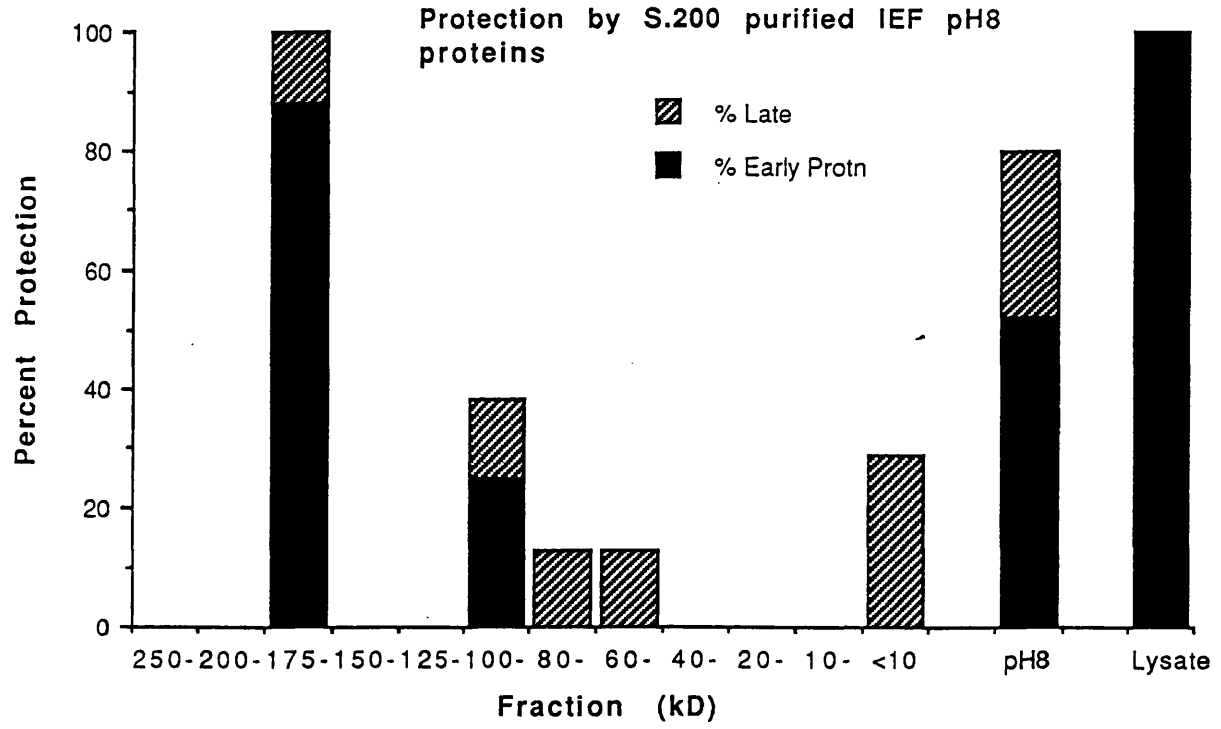


Figure 3.2.3b. **Protective effect of S.200 separated IEF pH8 proteins; pooled data fron 6 experiments**

3.3 HPLC purification of IEF pH6 and pH8 fractions (Dr. B. Coles)

This technique was used to further purify the IEF pH6 and pH8 fractions. As described before, each IEF batch consisted of pooled fractions from 3 to 4 focussing runs. The re-dissolved pH4 precipitate was not suitable for fractionation by this method as erratic elution profiles were always obtained; FPLC was used as a suitable alternative (described later).

3. 3. 1 Protection by HPLC purified IEF pH6 pooled fraction

It was not possible to use the 56kD protein from S.200 column for preparative HPLC purification owing to low yields. Hence the whole pH6 fraction was used instead.

Each IEF pH6 batch was further purified by HPLC and the eluted fractions were used in vaccination experiments. Two mice were vaccinated with between 5-10μg protein of each fraction. Larger groups were not possible owing to the limited amounts of protein in each fraction (0.5μg/ml to 10μg/ml). Experiments were repeated three times and a different IEF pH6 batch was used for each experiment. Two further confirmatory experiments were carried out only with the most protective fraction; thus each bar in figure 3.3.1b represents a total of between 8 to 12 mice. Figures 3.3.1a shows an elution profile of a typical experiment where the IEF pH6 pool was resolved into 8 peaks. The data in figure 3.3.1b clearly shows that protective activity was not related to the protein concentration of the fraction. This relationship was clarified by measuring the protein concentration of each fraction using the Bio-Rad assay which is specific for protein. The results of a representative assay are shown below in Table 3.3.1.

Table 3.3.1 Bio-Rad protein estimation of HPLC purified IEF pH6 proteins

HPLC fraction	μg/ml protein
a	3
b	3
c	1.8
d	5.75
e	2.5
f	3
g	4
h	3
i	1

Whilst the Bio-Rad assay protein concentrations of most HPLC fractions agreed fairly well their absorbance at 214nm, fraction **f** remained the only exception. This fraction had a high absorbance at 214nm but a relatively low protein concentration in the Bio-Rad assay. The most likely explanation for its high absorbance at 214nm is that it contains small amounts of TX-100; a major peak resulting from the passage of a 0.05% solution of TX-100 (50μl) through the column appears in the same position as fraction **f**.

Fraction **h** which had a low protein content was the most protective. Over 75% of the vaccinated mice were protected, although only 20% of the protection was of the early type. By contrast fraction **d** which had the highest protein concentration, was totally ineffective as a vaccine. Fractions **b** and **e** showed a weak protective effect where 20-25% of the mice recovered late and fraction **f** induced 34% late recovery.

Therefore the strongest protective antigen, fraction **h**, was selected for SDS-PAGE analysis and amino acid sequencing.

3. 3. 1a SDS-PAGE analysis and amino acid sequencing of HPLC fraction h (IEF pH6).

Antigens of interest were first analysed by SDS-PAGE and the protein was transferred to a Pro-Blott PVDF membrane by Western blotting. After staining with Coomassie blue, the relevant band was carefully cut out for amino acid sequencing. SDS-PAGE analysis of all eight HPLC purified pH6 fractions are shown in plate 3.3.1a. Only fractions **c**, **d** and **h** appeared to contain protein bands. Fraction **c** contained bands at 38kD and 30kD, fraction **d** contained a band at 20 kD and fraction **h** contained a 56kD band together with two unusual bands at 66kD and 48kD which are not normally present; these were probably contaminants.

The 56kD band was transferred to a PVDF membrane and cut out for amino acid sequencing using the Applied Biosystems sequence analyser (Dr. B. Coles). The N-terminal amino acid sequence of the first 25 amino acids obtained so far is shown below; amino acids in brackets indicate uncertainties:

NH2 - **Pro-Ser-Ile-Val-Gly-Asn-Glu-Ala-Phe-Ser-Phe-(Lys)-Ala-Gln-Ala-(Val)-Phe-Gly-Asp-Asn-Thr-Phe-(Gly)-(Gln)-Val** - COOH

Confirmation of this sequence is necessary before comparisons are made with sequences from a protein data base. This work is in progress.

HPLC purification of IEF pH6 membrane proteins

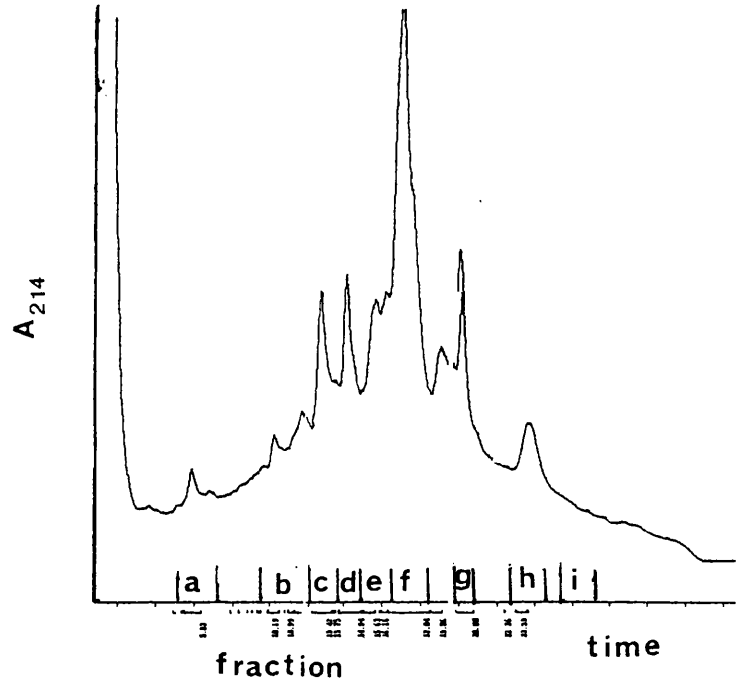


Figure 3.3.1a. HPLC elution profile of an IEF pH6 batch from a single representative experiment

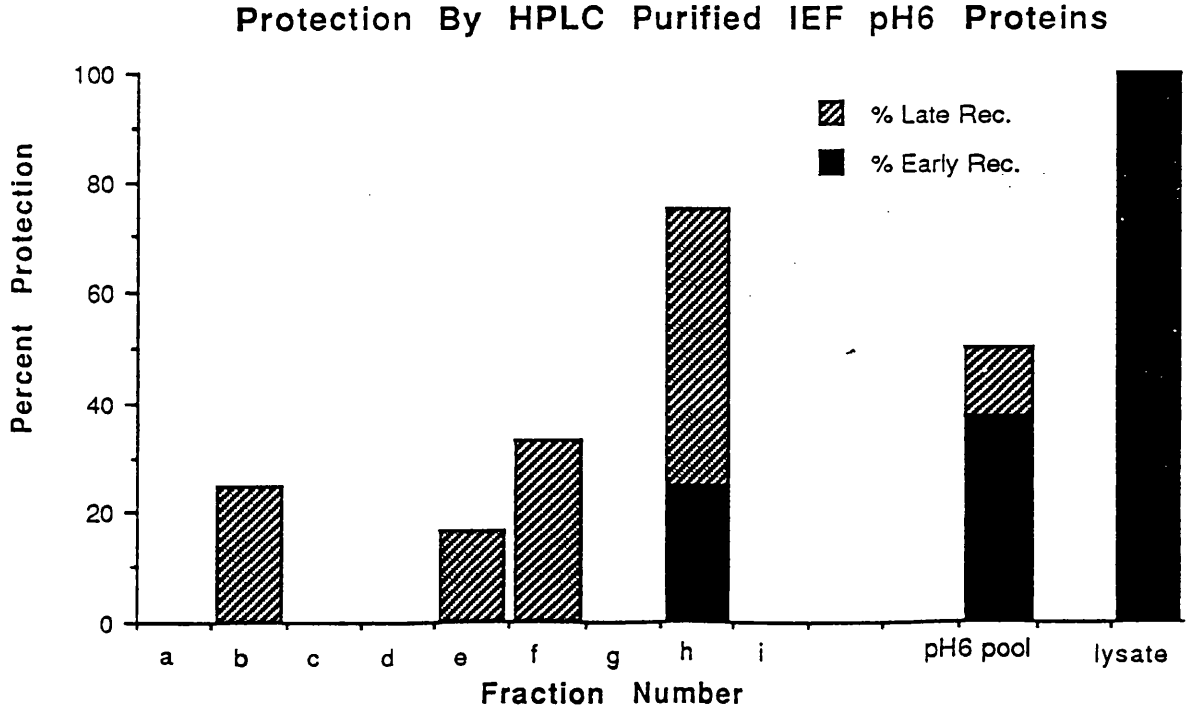


Figure 3.3.1b. Protective effect of HPLC purified IEF pH6 proteins; data from 4 experiments

SDS - PAGE analysis of HPLC purified IEF pH6 proteins

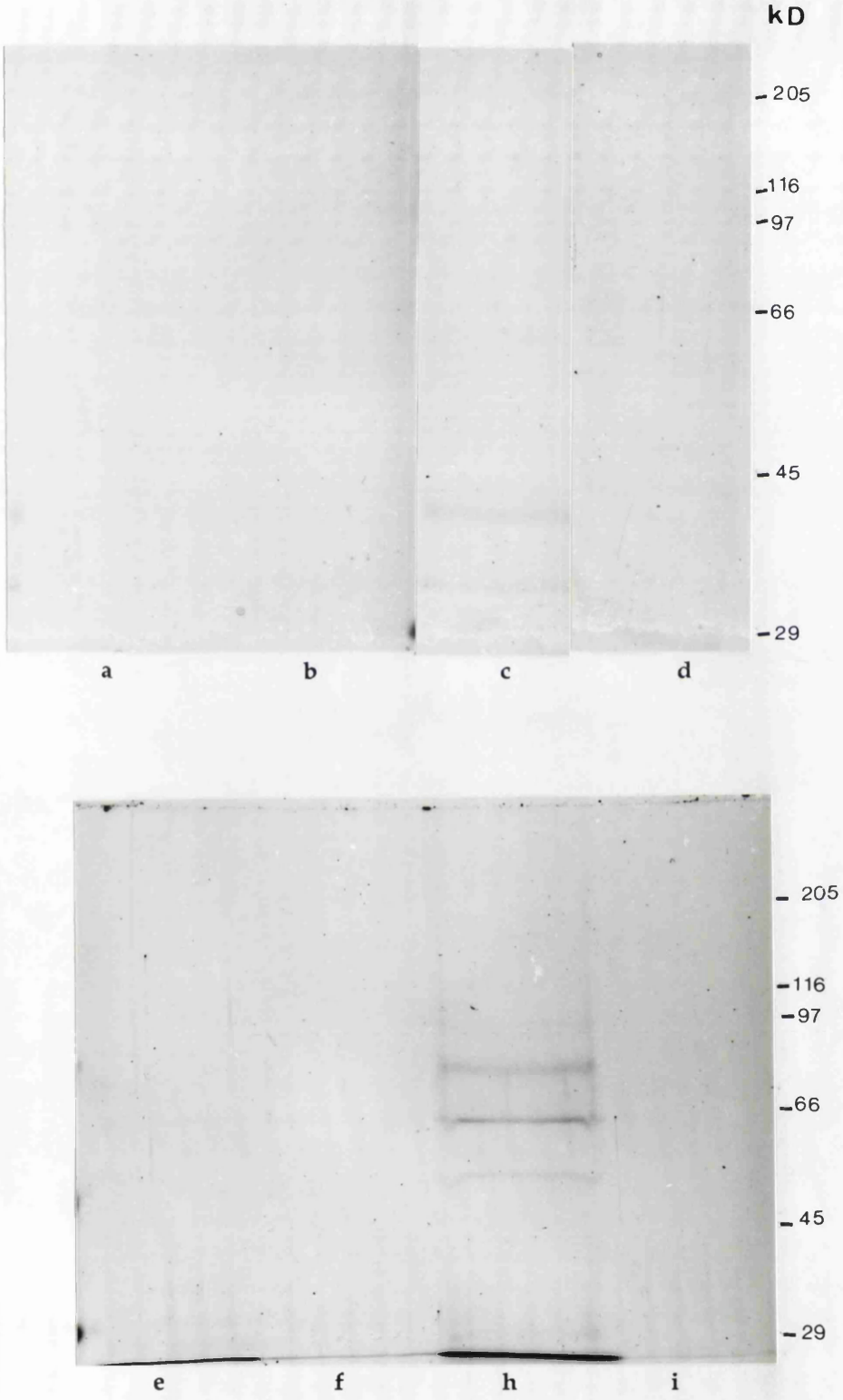


Plate 3.3.1a Coomassie blue stained gel of 10-20µg protein of HPLC purified fractions.

Fraction g - not shown but did not contain bands in previous test.

3. 3. 2 Protection by HPLC purified IEF pH8 pooled fraction

The majority of the protective activity of the IEF pH8 fraction appeared to be concentrated in only one peak of M_r between 175kD to 150kD. It was therefore important to further resolve this peak by HPLC. Four repeat experiments were set up with HPLC purified fractions from four different IEF pH8 batch preparations as described above. The elution profile of a typical experiment is shown in figure 3.3.2a. The pH8 fraction was resolved into 5 peaks. However, since neither the absorbance at 214nm nor the protein amount directly correlate with protection, each run was divided into 8 fractions and between 5-10μg protein of each was used to vaccinate a group of 2 mice. A total of 8 to 12 mice were vaccinated with each fraction. Protective activity was observed in 7 fractions (figure 3.3.2b). Fractions **b** and **c** induced the best protection in vaccinated mice where over 65% were protected. Fractions **e**, **f** and **h** protected 50% of the animals. Fraction **d** protected 30% and fraction **g** protected less than 20%. Less than 20% of the mice vaccinated with fractions 3,7 and 8 recovered early. All other recoveries were of the late type.

Although five HPLC fractions gave 50% or more protective activity, none were as effective as the intact pH8 fraction itself. Synergy between some of these fractions may be necessary to increase their protective effect or it is possible that reverse phase HPLC is not ideal for resolving the IEF pH8 proteins. Probably a molecular weight-based separation such as FPLC should be attempted.

HPLC purification of IEF pH8 membrane proteins

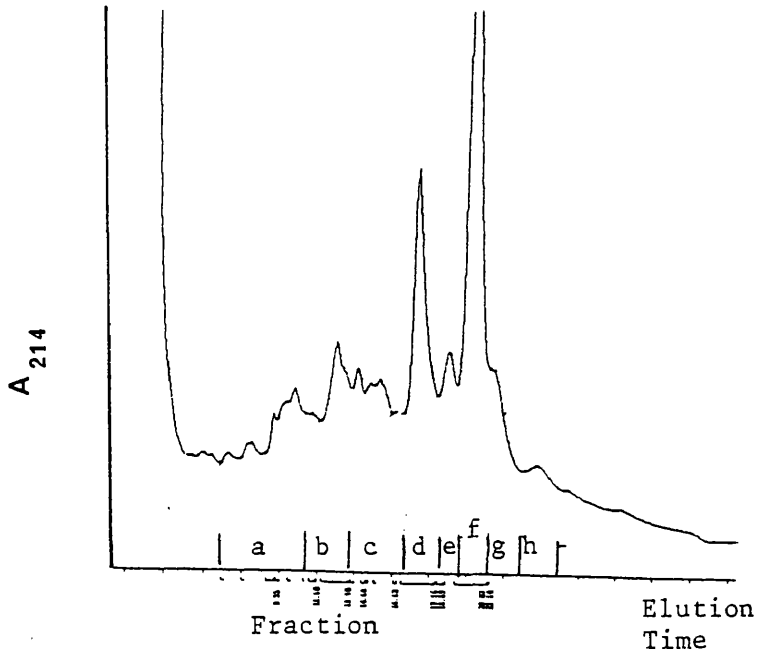


Figure 3.3.2a HPLC elution profile of an IEF pH8 batch from a single representative experiment.

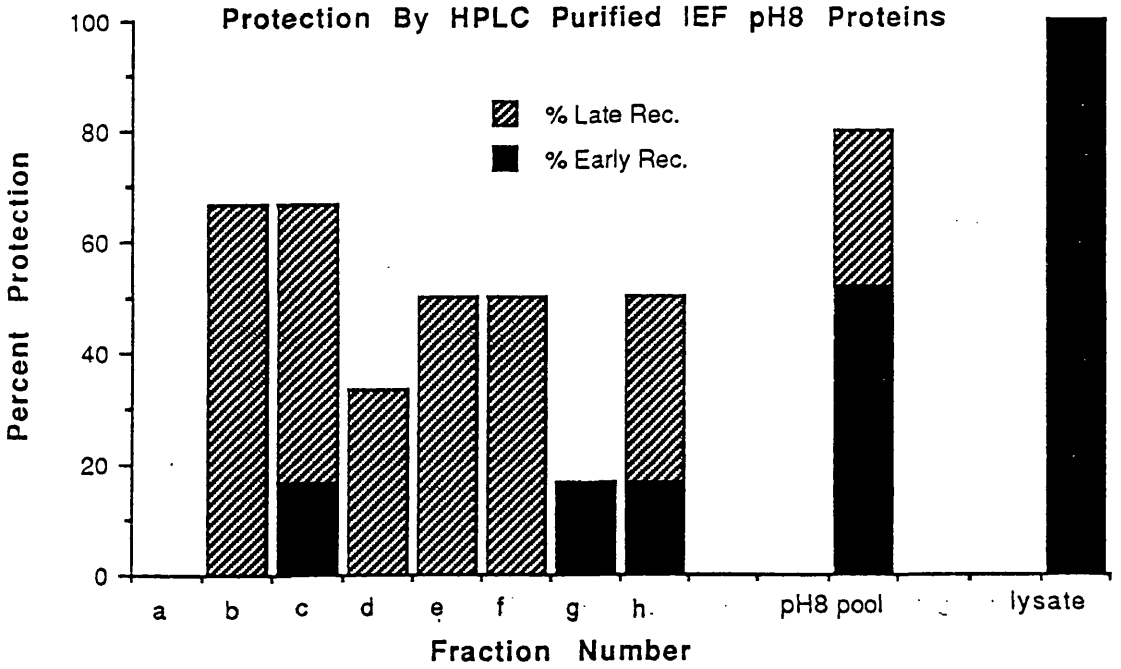


Figure 3.3.2b. Protective effect of HPLC purified IEF pH8 proteins; data from 4 experiments

3.4 FPLC purification of IEF pH4 pooled fraction

When the pH4 precipitate was re-dissolved in 0.05% SDS it was suitable for purification by FPLC. Two preliminary experiments were carried out with FPLC purified fractions from two different IEF pH4 preparations. Three mice were vaccinated with each eluted fraction. A typical elution profile is illustrated in figure 3.4a. Two well resolved peaks were seen at molecular weights 300kD and 14kD. Four other peaks which were less well resolved, were seen at molecular weights of 150kD, 80kD,45kD and 29kD. The two well resolved peaks and six other fractions between them, were tested for their protective activity. These fractions and their respective molecular weights are shown in figure 3.4a. All peaks were equally protective except for fractions 17 and 21. The results are shown in figure 3.4b where each bar represents a group of 6 mice. Clear differences were observed in the parasitaemias of the vaccinated animals following infection (data not shown). Parasitaemias of mice vaccinated with the 300kD peak never exceeded 1% and were cleared between day 6 and day 8 after challenge. Parasitaemias of mice vaccinated with fractions 11 to 15 (molecular weight range 170kD to 29kD) never exeeded 5% and were cleared by day 8 or 9. Animals vaccinated with fraction 17 experienced higher peak parasitaemias (10-35% on day 7), but nevertheless cleared their infection; 50% of these animals recovered late. Fraction 21 (14kD) was not effective as a vaccine; all these animals experienced parasitaemias in excess of 50% on day 7 and they died by day 9 or 10.

The main peaks await analysis by SDS-PAGE and Western blotting. In addition, the most protective peaks will be passed through the HPLC column to see if they bear any similarity to the fractions purified by procedure II (described below - 3.5).

FPLC PURIFICATION OF IEF pH4 P. YOELII MEMBRANE PROTEINS

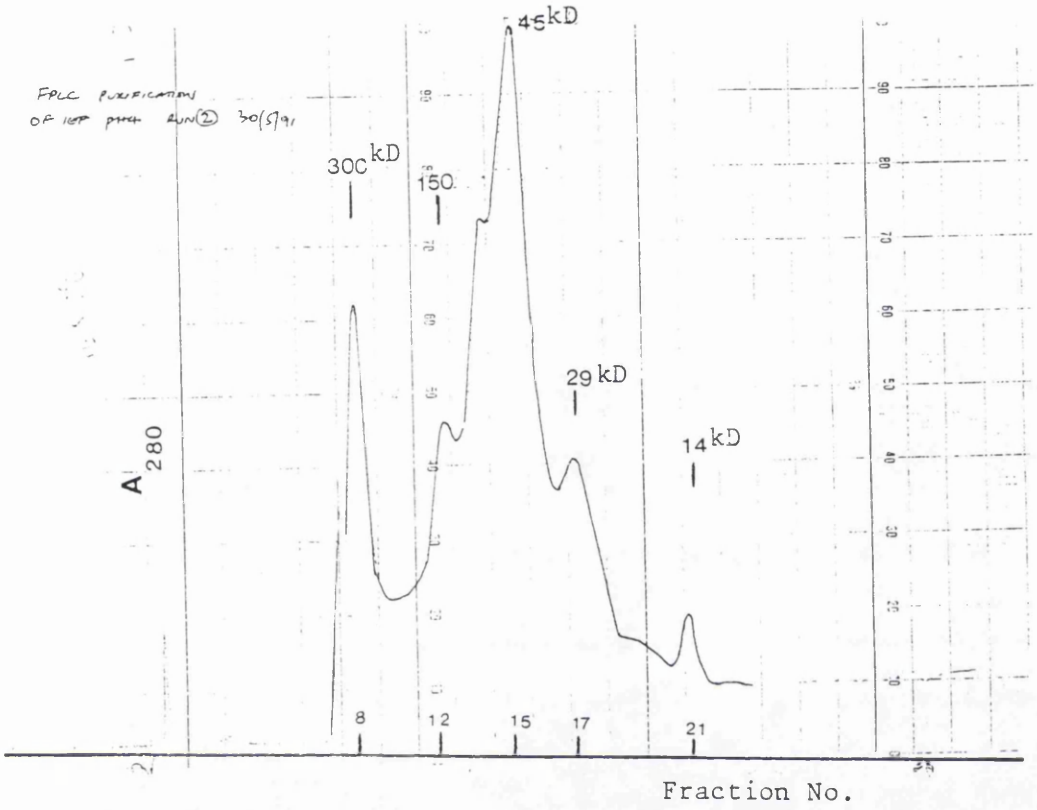


Figure 3.5a FPLC chromatograph from a representative experiment

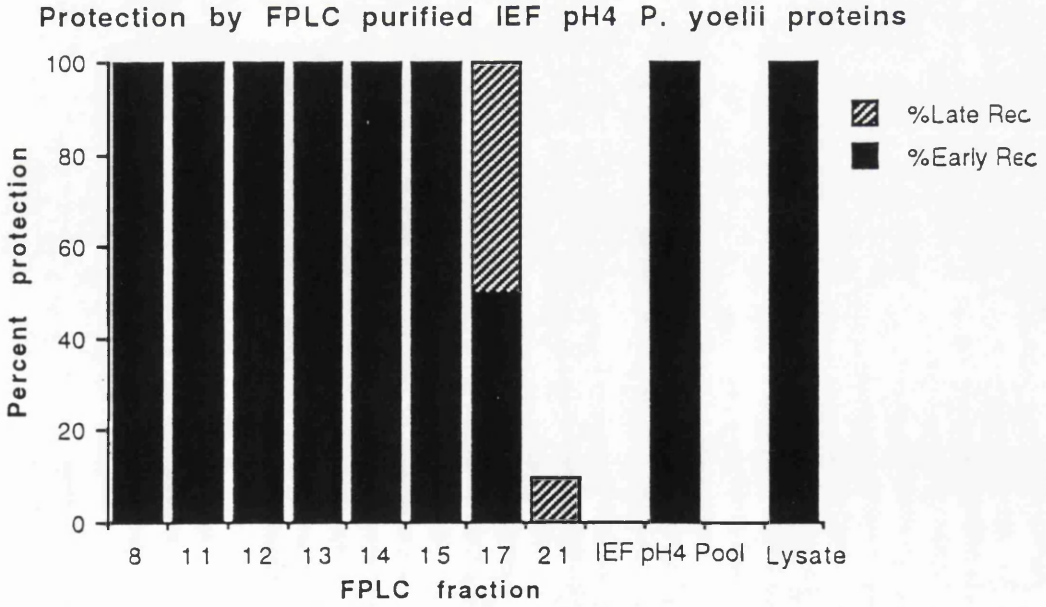


Figure 3.4b. Protective effect of main peaks from above chromatogram. Pooled data from 2 experiments.

3. 5 Purification procedure II for *P. yoelii* (YM) TX-100 lysate

The difficulties experienced with the IEF pH4 precipitate which contained 90% of the protein in the lysate prompted the use of Sephacryl S.200/S.300 gel filtration chromatography as an alternative initial purification procedure. All the gel filtration profiles shown in this section represent the protein concentration in μg/ml (Bio-Rad) of each fraction. The profiles shown in Section 2 are the actual traces obtained from the chart recorders which represent the absorbance at 280nm of each fraction.

3. 5. 1 Protection by S.200 purified TX-100 lysate

Three different lysates were millipore-filtered (0.22μ pore size) and 1.5mls (5mg protein) were fractionated through the S.200 column. The largest peak corresponding to a high molecular weight protein (M_r >250kD) appeared at the void volume of the column and two other broad peaks were also present, one of M_r between 150kD and 70kD and another of M_r between 50kD and a shoulder at 20kD (fig. 3.5.1a). Individual fractions of M_r ranging from 250kD down to 5kD were tested as vaccines in groups of 3 mice. Three experiments were set up with fractions eluted from three different lysates; thus each bar in figure 3.5.1b represents nine mice. The most strongly protective fractions (figure 3.5.1b) emerged at the high molecular weight region - M_r ranging between >250kD to 100kD. Fractions of M_r ranging from >250kD to 125kD gave the best protective response where recovery was 100% and of the early type. Fractions of M_r between 100kD-40kD protected over 75% of the vaccinated mice some of which recovered late. Fractions of M_r 20kD or less were not effective. As protective activity was detected over a wide range of fractions only the major peaks were used for further purification by HPLC. These were

termed Peak I (M_r >250kD to 190kD), Peak II (M_r 150kD to 70kD) and Peak III (M_r 50kD-20kD) as shown in figure 3.5.1c; their protein concentrations were as follows:

Peak I - 250μg/ml

Peak II - 300μg/ml

Peak III - 150μg/ml

Five different lysates were millipore-filtered and fractionated on the S.200 column. The major peaks eluted, Peak I, Peak II and Peak III were tested in groups of 3 mice. Experiments were repeated five times and the protection data is shown in figure 3.5.1d where each column represents a group of 15 mice. In terms of total recovery Peak I and Peak II were similar with 100% of vaccinated mice recovering early. Peak III was less effective with 68% (50% early recovery) of the vaccinated animals clearing their infection. Vast differences were noted in the parasitaemias of the variously vaccinated mice following challenge. All groups were vaccinated with the same dose, 10μg of antigen. Figure 3.5.1e clearly shows that mice vaccinated Peak I had the lowest parasitaemias, <10% on day 5 dropping sharply to <1% on day 7, when a small number have already cleared their infection. The rest of the group cleared their parasites on day 8. Crisis forms were always seen on blood films of these mice on the day before recovery. Mice vaccinated with Peak II showed a similar trend except on day 6 when their parasitaemias were higher and they usually recovered on day 9, although a few recovered on day 8. Animals vaccinated with these fractions had significantly lower parasitaemias and faster clearance of infection than those injected with the lysate (positive control). Parasitaemias of mice vaccinated with Peak III peaked at 25% on day 6.

Some animals died on day 8 but others were clear of infection by day 10 to 12.

All the main peaks were analysed by SDS-PAGE and Western Blotting and Peak I the most protective fraction, was selected for final purification by HPLC.

When 2mls (4mg protein) of a control mouse red blood cell (MRBC) lysate were fractionated through the same column, the largest peak eluted was Peak III of M_r ranging between 40kD and 15kD (figure 3.5.1c). This fraction contained a reddish colour and probably represents monomer and dimer units of haemoglobin. The first peak was about a tenth of the size of Peak I from the *P.yoelii* lysate and the region between M_r 150kD and 70kD, representing Peak II of the *P.yoelii* lysate was not present in the MRBC lysate. With the exception of one unexplained recovery from a group of eight mice vaccinated with Peak I (MRBC), there were no recoveries in groups vaccinated with the other MRBC fractionated peaks (data not shown).

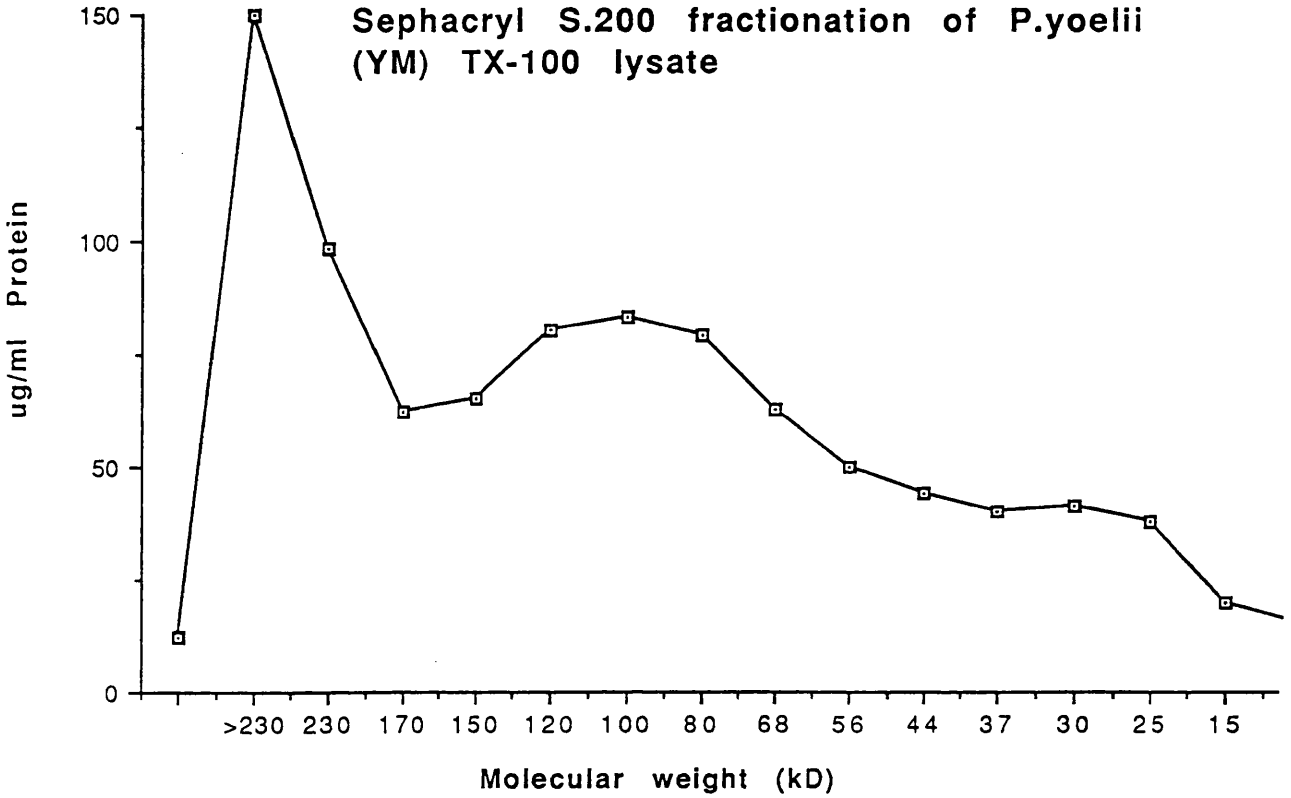


Figure 3.5.1a. S.200 profile of a P.yoelii lysate from a single representative experiment

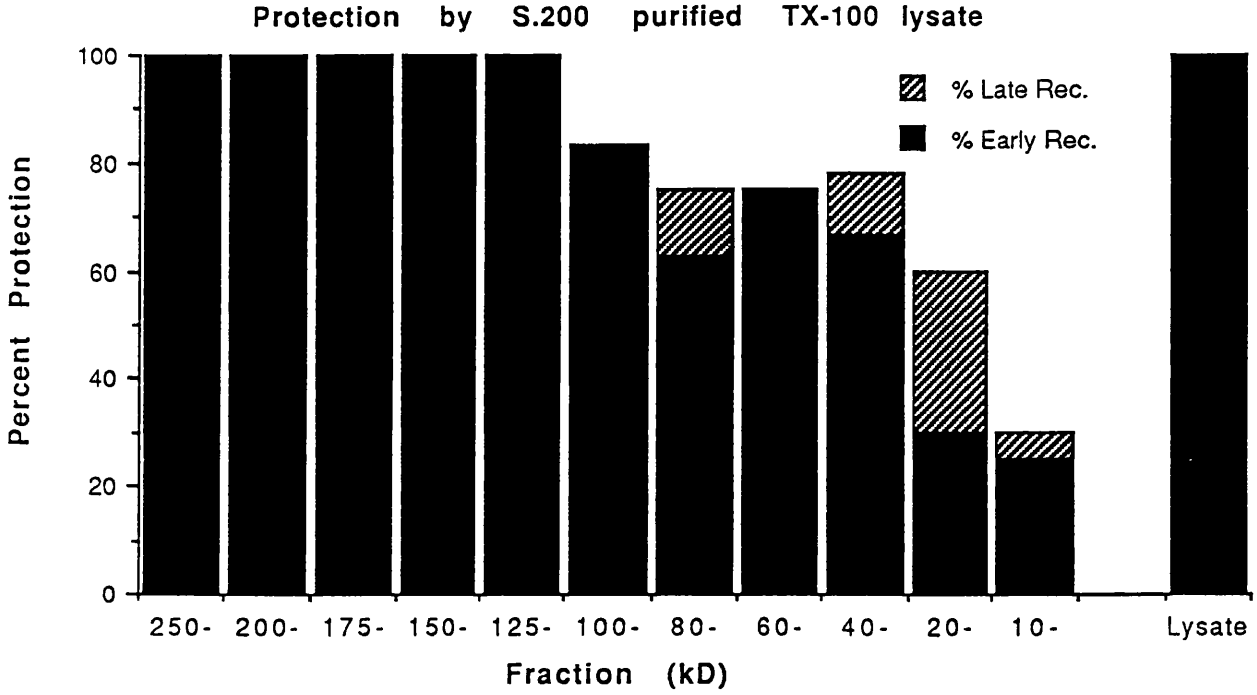


Figure 3.5.1b. Protective effect of S.200 purified lysate proteins; data from 3 experiments

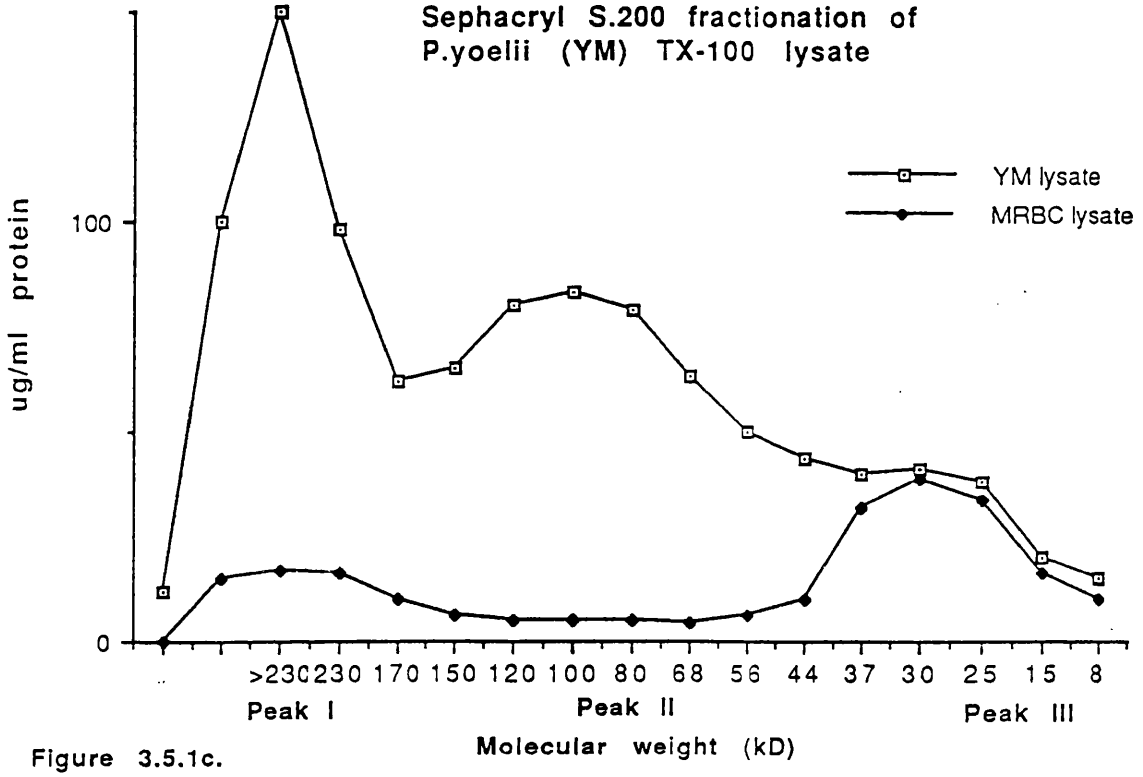


Figure 3.5.1c.

S.200 profile of P.yoelii lysate showing main peaks I II III

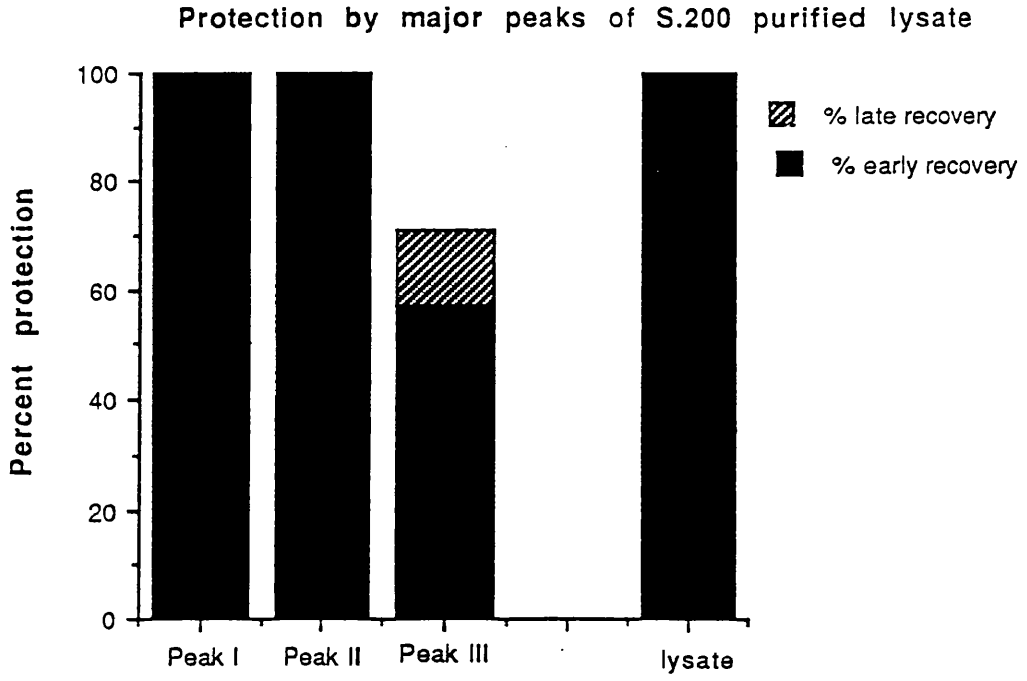


Figure 3.5.1d.

Protective effect of major peaks of S.200 separated lysate; data from 5 experiments

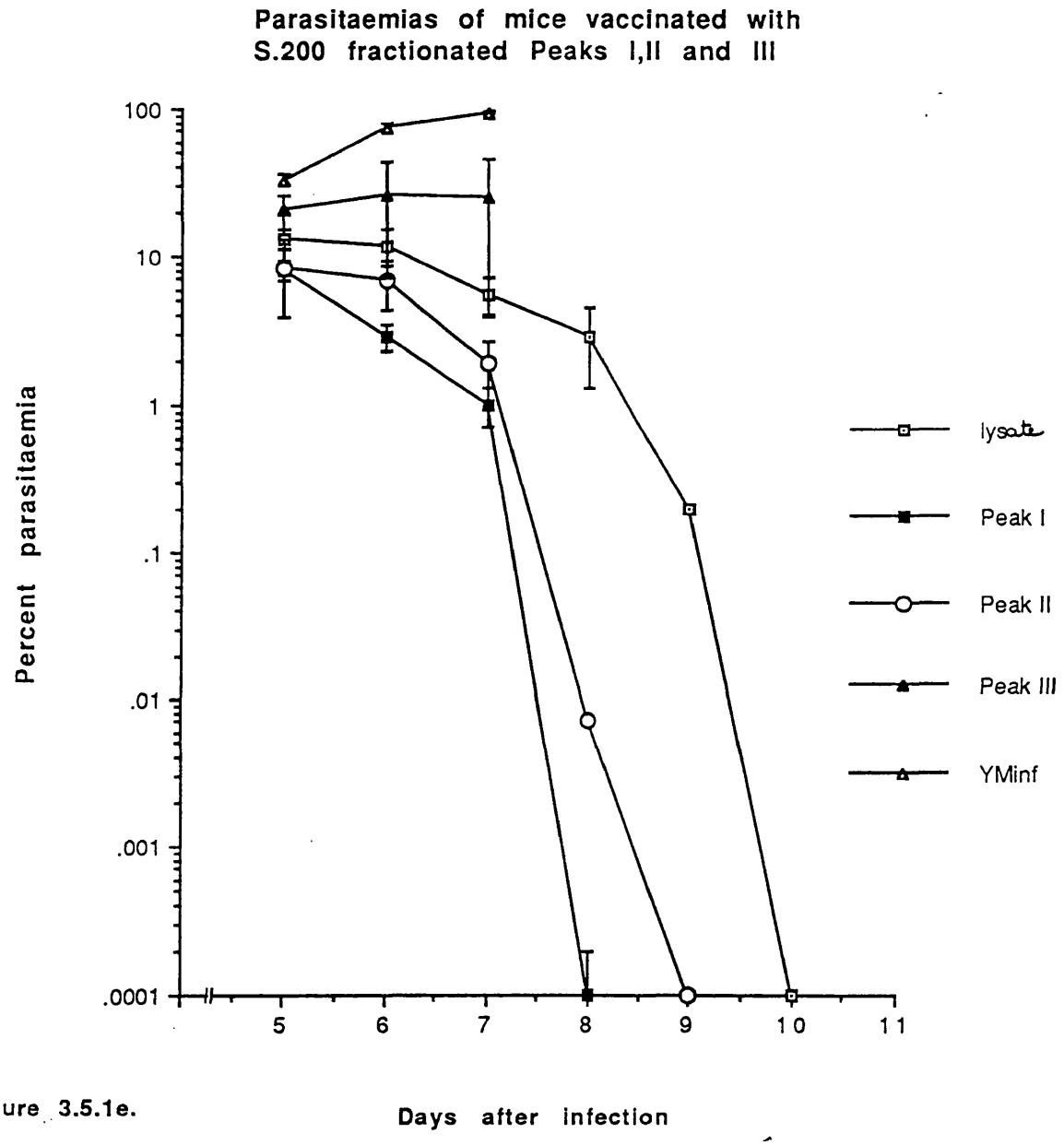


Figure 3.5.1e.

Course of parasitaemia in mice vaccinated with 10µg of protein of each fraction. Each point represents the mean ±SE of a group of 15 mice

3. 5. 2 Protection by major peaks from S.300 purified lysate

As Peak I from the S.200 fractionation appeared at the void volume of the column, an attempt was made at improving the resolution of this peak by running the lysate through an S.300 column which has a separating range from 1000kD down to 20kD. The results from a typical experiment are shown in figure 3.5.2a where 1.5 ml of millipore-filtered lysate were passed through the column and 3ml fractions were eluted. The data clearly showed that this column had further resolved the peak which appeared at the void volume of the S.200 column; it was now resolved into a peak of M_r between 900kD and 600kD and distinctly away from the 250kD region. This peak was termed Peak Ia. A region between M_r 600kD and M_r 250kD was omitted from subsequent protection studies as it contained very little protein and was not protective in preliminary studies. This appeared to be the main difference between the two gel-filtration columns in terms of their elution profiles. The other eluted fractions were divided into three regions; Peak Ib between 250kD and 105kD, Peak II between 80kD and 46kD and Peak III between 35kD and 10kD. Each peak was tested in a group of 3 mice and the experiment was repeated three times. Each bar in figure 3.5.2b represents a group of 10-12 mice.The group vaccinated with Peak Ia was the only one in which 100% early type protection was achieved. In the group vaccinated with the lysate, one mouse recovered late but the recovery rate was 100%. The group vaccinated with Peak Ib had a recovery rate of 68%, but this was largely due to three deaths in one experiment, however recovery was of the early type. Vaccination with Peak II resulted in 88% recoveries (77% of the early type) and vaccination with Peak III gave 68% recovery, 57% being of the early type. Parasitaemias of these animals reflected the difference in the strength

of protection between the four peaks (figure 3.5.2c). Unvaccinated controls had high parasitaemias on day 5, which increased progressively and deaths occurred on days 8 to 10. By contrast, parasitaemias of mice vaccinated with Peak Ia never exeeded 6% and animals recovered by day 6 to 8 after challenge. Only one mouse in the entire pool recovered on day 9. This group of mice had significantly lower parasitaemias than the group vaccinated with the lysate, particularly on days 5 and 6, and the animals recovered by day 7 or 8. The group vaccinated with the lysate recovered by day 8 or 9. Mice vaccinated with Peak Ib, were higher on days 5 and 6 and animals recovered (except the three mice mentioned above) by 6 to 10. Similar parasitaemias were seen in the group vaccinated with Peak II, except that on day 7 and 8 their parasitaemias were lower. Parasitaemias of the group vaccinated with Peak III were similar to those of Peak II on day 5 and 6 but were higher on days 7 and 8; a few deaths occurred on day 10.

Although Sephacryl S.300 resolved the first peak of high molecular weight (Peak Ia) it was not as effective as S.200 in terms of protective activity of the other major peaks; Peak Ia was the only consistently protective peak.

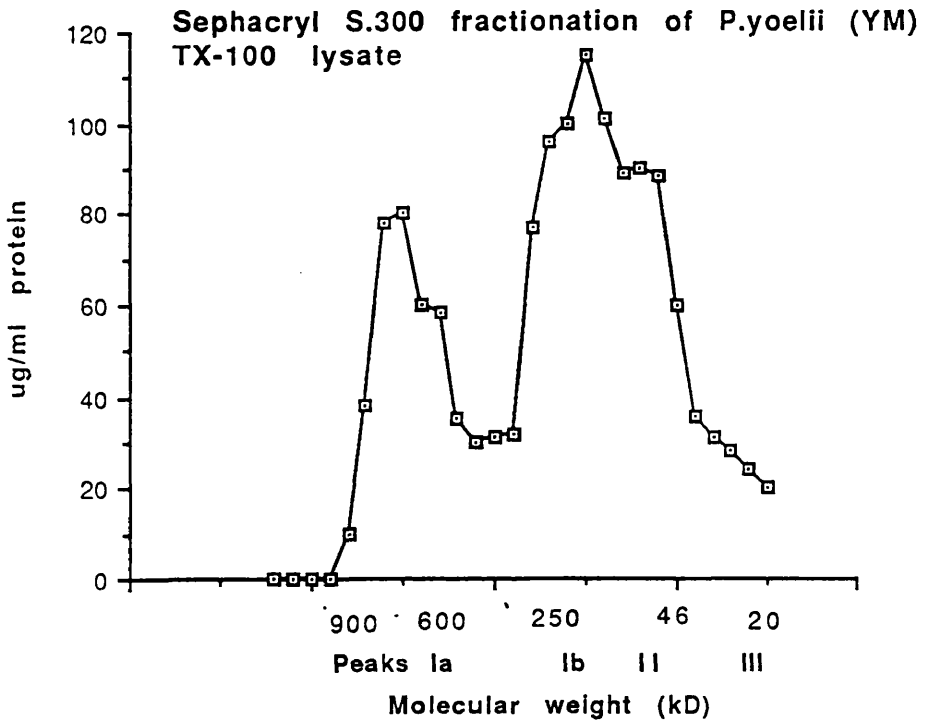


Figure 3.5.2a. S.300 profile of P.yoelii lysate from a single representative experiment showing Peaks Ia,Ib,II and III

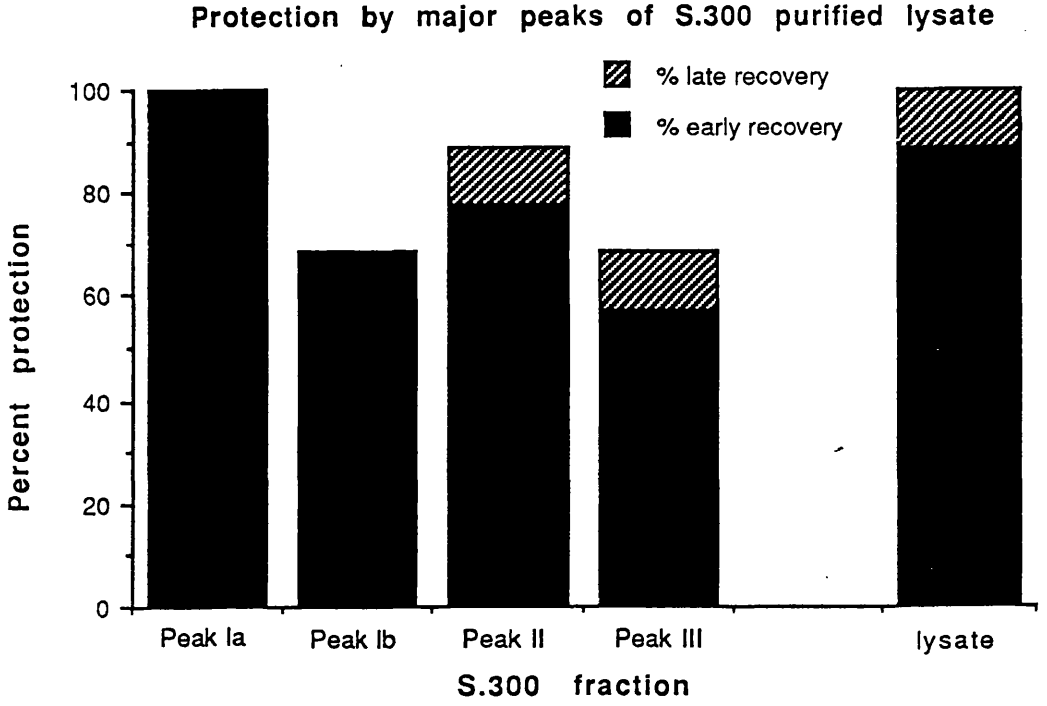


Figure 3.5.2b. Protective effect of peaks from S.300 fractionated lysate; data from 4 experiments

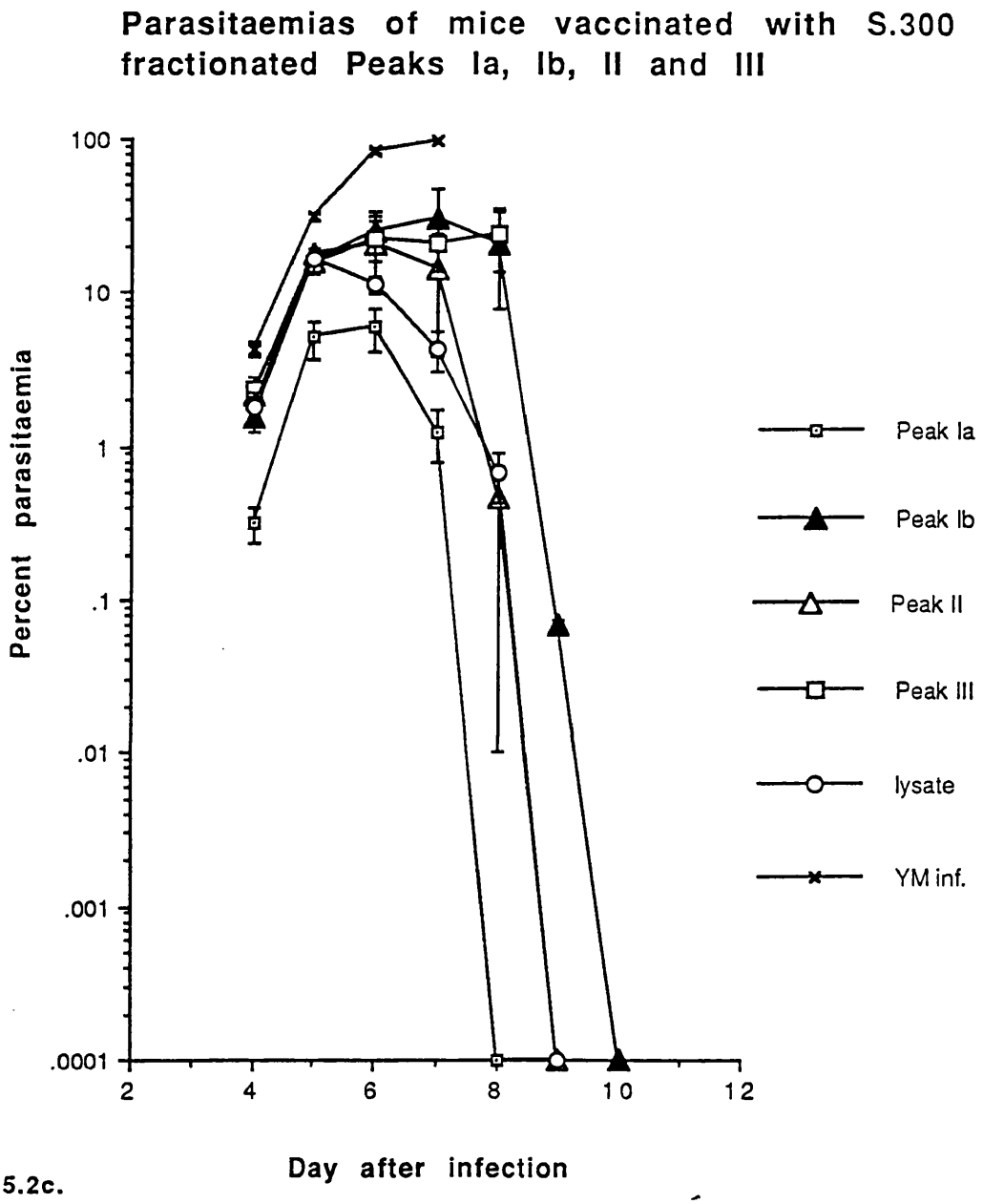


Figure 3.5.2c.

Course of parasitaemia in mice vaccinated with S.300 fractionated main peaks. Each point represents the mean ±SE of 10-12 mice

3. 5. 3a SDS-PAGE and Western Blot analysis of S.200 main peaks

The number of components or immunoreactive components within the main peaks were analysed by SDS-PAGE and Western blotting and experiments were repeated at least four times. Plate 3.5.3a shows the results of a representative experiment in which the same samples were used in both assays.

SDS - PAGE and Western blot analysis of :

Peak I

Bands on SDS-PAGE gels were always seen at M_r 140kD, 68kD, and 45kD. When the loading concentration was doubled to 50µg additional bands were seen at M_r 85kD, 54kD, 50kD, 48kD, 46kD, 40kD and 30kD. Western blots of this fraction always showed bands at 140kD, 65kD (faint diffuse), 46kD (broad diffuse) and 38kD regardless of the loading concentration.

Peak II

Several bands between M_r 120kD and 25kD were seen on the SDS-PAGE gel of this fraction with two heavily stained bands at 35kD and 43kD. Nine of these bands, in the molecular weight range 69kD to 30kD, reacted with antibody in the Western blot assay. Staining with the 35kD and 43kD bands was stronger by comparison with the others.

Peak III

One very prominent band was present at M_r 40kD and four very faint bands were seen at 60kD, 45kD, 38kD and 30kD. In the Western blot assay only the 40kD band reacted with antibody.

SDS - PAGE and Western Blot analysis of Sephacryl S.200 main peaks

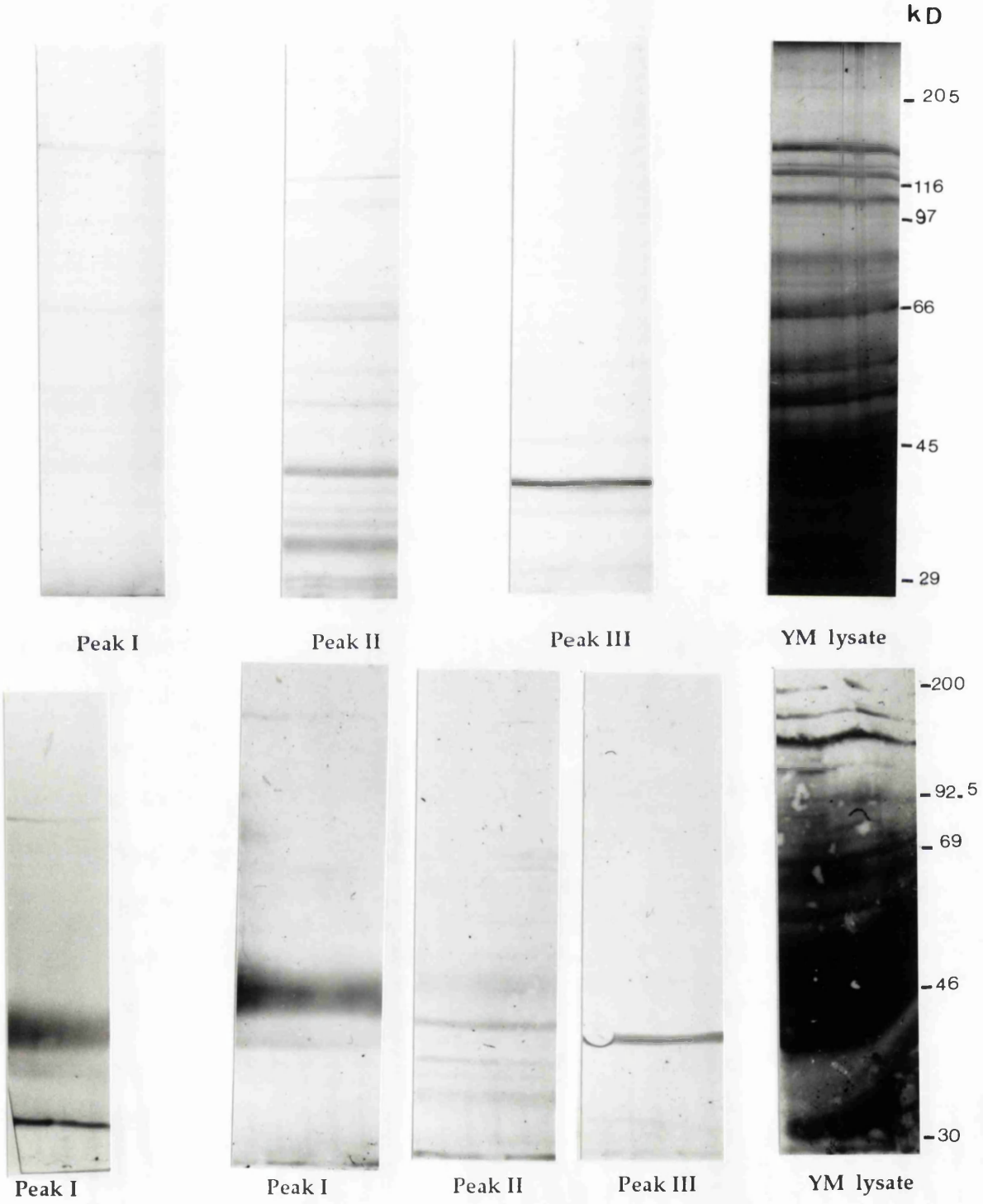


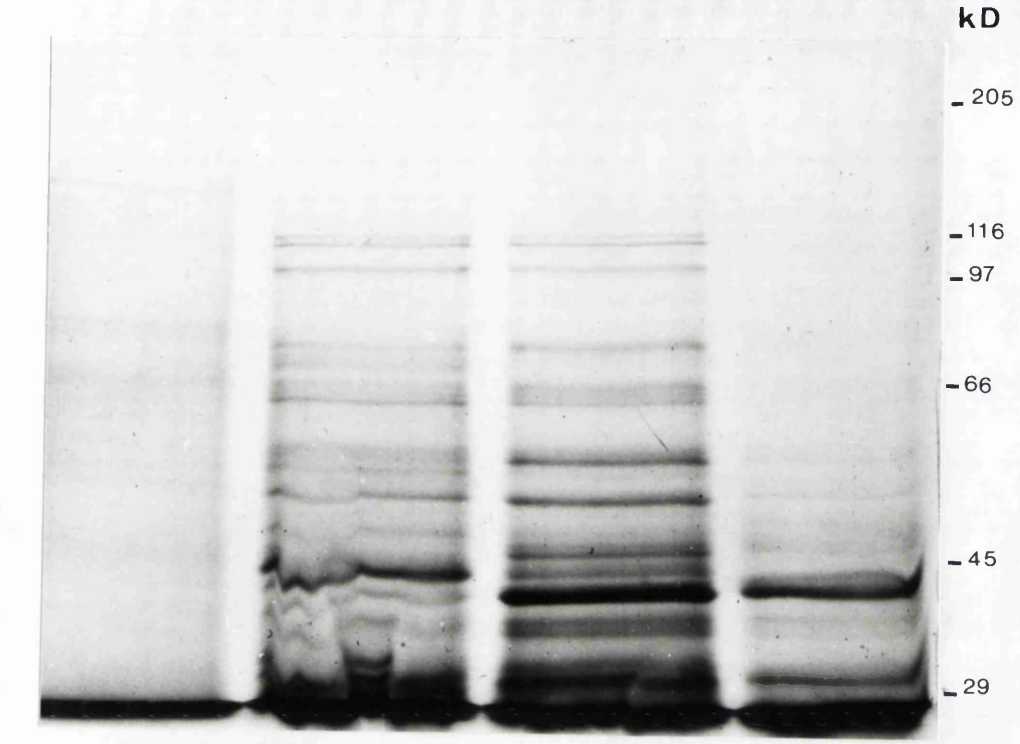
Plate 3.5.3a. Top photo - SDS-PAGE analysis of S.200 Peaks I,II,III and lysate
Bottom - Western Blot of above samples

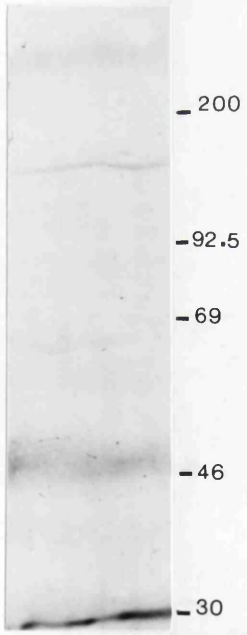
3. 5. 3b SDS-PAGE and Western blot analysis of S.300 main peaks

These results are shown in plate 3.5.3b. Peak Ia contained the sane bands as Peak I (S.200) in both SDS-PAGE and Western blot assays. PeakIb and Peak II appear to contain similar bands between M_r 97kD and 30kD. Peak III contained faint bands between M_r 66kD and 35kD and stronger bands at 30 and 20kD. These latter three peaks were not tested in the Western blot assay as they were not of immediate interest.

One clear conclusion from these analyses was that the molecular weight based separation by Sephacryl S.200/S.300 gel filtration chromatography was fairly reliable. The first high molecular weight peak of M_r >250kD to 190kD appeared to be fairly well separated. It contained one large molecular weight band and five smaller bands. These smaller molecules and possibly all the molecules within Peak I may be present within micelles which may account for its elution at the high molecular weight region of the S.200 column. The second peak Peak II did not contain molecules larger than 120kD according to SDS-PAGE which is consistent with its estimated molecular weight on the column. The presence of smaller molecules may be due to the reducing conditions of SDS-PAGE. Finally, SDS-PAGE of peak Peak III showed that the majority of this protein was of M_r 40kD agreeing with its estimated molecular weight on the S.200 column. Fractionation on the S.300 column showed a similar trend except that there seemed to be no difference between the SDS-PAGE staining pattern of Peak Ib and II.

SDS - PAGE and Western Blot analysis of Sephacryl S.300 main peaks





Western Blot of Peak Ia

Plate 3.5.3b. Top photo - SDS-PAGE analysis of S.300 Peaks Ia,Ib, II & III
Bottom - Western Blot of Peak Ia. Other peaks not done

3.6 Protection by HPLC purified Peak I (S.200) and Peak Ia (S.300) fractions

Peak I (S.200) and Peak Ia (S.300) induced the strongest protective response when compared with the other fractionated peaks as judged by significantly lower group parasitaemias (figures 3.5.1e and 3.5.2c). Furthermore they showed similar bands in the SDS-PAGE and Western blot analyses, however Peak I (S.200) was chosen for the majority of the HPLC purification experiments as it had a higher protein content than Peak Ia (S.300).

Fractions from at least four HPLC purification runs (of S.200 Peak I) were pooled for vaccination. Vaccinated animals received between 2-5µg of protein from HPLC fractions **a** to **j**. Experiments were repeated three times with 2-3 recipients vaccinated with each fraction. Two confirmatory experiments were carried out with the protective fractions; thus each bar in figure 3.6b represents a total of between 8 to 14 recipients. An elution profile from a representative experiment is shown in figure 3.6a and the protection data is shown in figure 3.6b. An important point to note in figure 3.6a is that the absorbance of the eluted fractions at 280/214nm is not a true indication of their protein concentration. This was confirmed by the Bio-Rad protein assay shown in table 3.6. Fraction **h** showed a high absorbance at 280nm but a low Bio-Rad protein concentration. Conversely, fraction **c** with a significantly lower absorbance at 280nm, had the highest Bio-Rad protein concentration. The high absorbance of fraction **h** at 280nm is consistent with the presence of detergent micelles to which a (hydrophobic) protein is bound. An impurity of TX-100 with a high 280/214nm ratio elutes at this position.

Table 3.6 Bio-Rad protein estimation of HPLC purified Peak I fractions

HPLC fraction	μg/ml protein
a	**0.0**
b	**3.8**
c	**6.0**
d	**1.6**
e	**1.6**
f	**1.5**
g	**1.5**
h	**3.2**
i	**1.5**
j	**0.01**

The data in figure 3.6b quite clearly shows that fraction **h** was the major protective fraction which induced 100% protection of the early type, similar to that of the lysate and the intact Peak I. Fraction **h** from HPLC purified Peak Ia (S.300) gave identical results (data not shown). The protection and type of recovery induced by the other fractions were as follows:-

fractions **g** and **i** - 60% mainly early

fractions **c** and **f** - 60% mainly late

fraction **j** - 50% early

fraction **d** - <40% half early, half late

fraction **e** - 20% late

fractions **a** and **b** - 0%

Figure 3.6c shows the parasitaemias of mice vaccinated with the lysate,

Peak I and fraction **h**. The course of infection in mice vaccinated with Peak I and fraction **h** was very similar, the only difference being that a few mice recovered as early as day 6 in the group vaccinated with Peak I. The group vaccinated with the lysate had higher parasitaemias from days 4 to 7 after infection and the animals usually recovered by day 8 to 10.

Fraction **h** was in fact stronger than Peak I as recipients received only 1-5μg protein which is between 1/5th - 1/10th of the normal vaccination dose as the yield was always low. This was confirmed by studying the kinetics of the protective response to Peak I. Groups of six mice were vaccinated with 1μg, 5μg or 10μg of Peak I(S.200) and between 1-2μg of HPLC fraction **h**. The data is shown in figure 3.6d where each point represents the mean + SEM. Clearly, the protective effect of fraction **h** had been concentrated since 1-2μg of this antigen was just as effective as 5 or 10μg of Peak I. Parasitaemias of challenged mice never exceeded 5% and they were cleared by day 8. The group vaccinated with 1μg of Peak I had significantly higher parasitaemias and recovered a day later.

Fraction **h** was selected for further analysis and N-terminal amino acid sequencing.

HPLC purification of S.200 Peak I *P.yoelii* membrane proteins

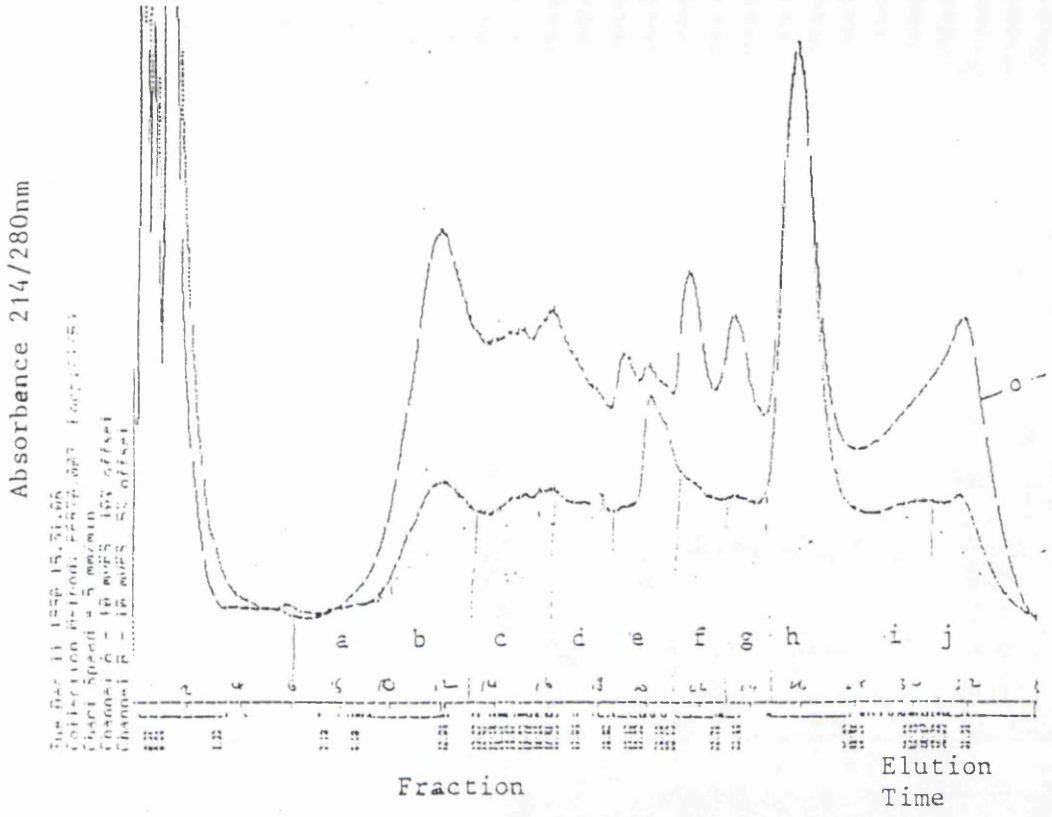


Figure 3.6a. HPLC elution profile of a batch of S.200 Peak I from a single representative experiment

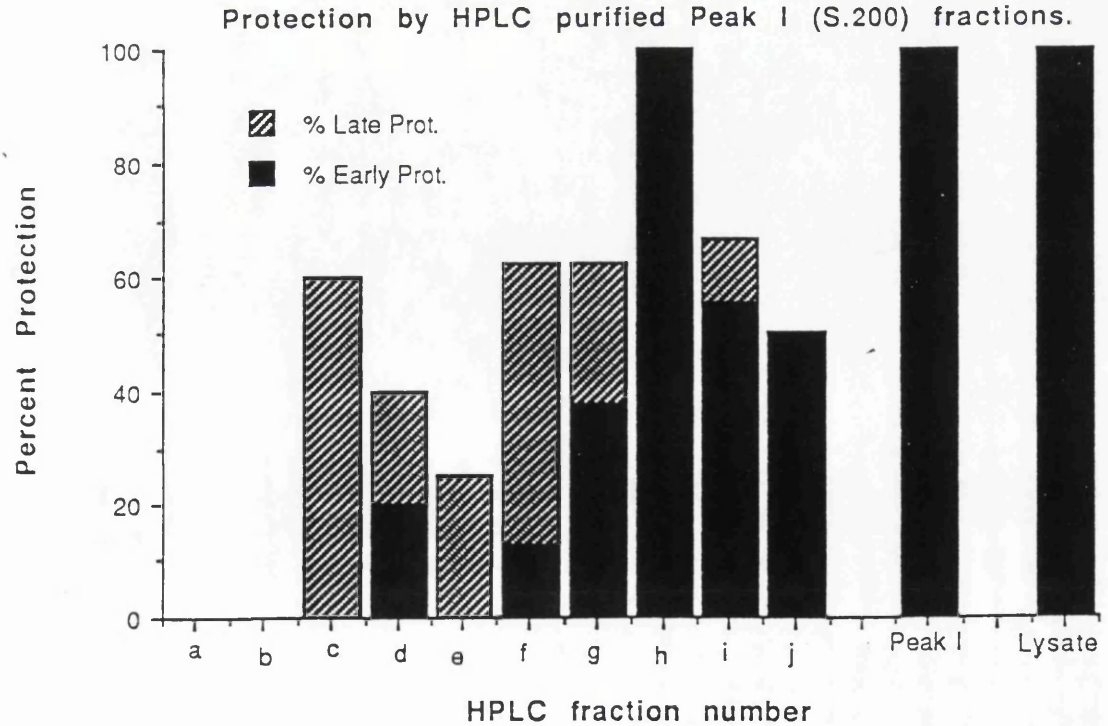


Figure 3.6b. Protective effect of HPLC purified Peak I (S.200) proteins; data from 4-6 experiments

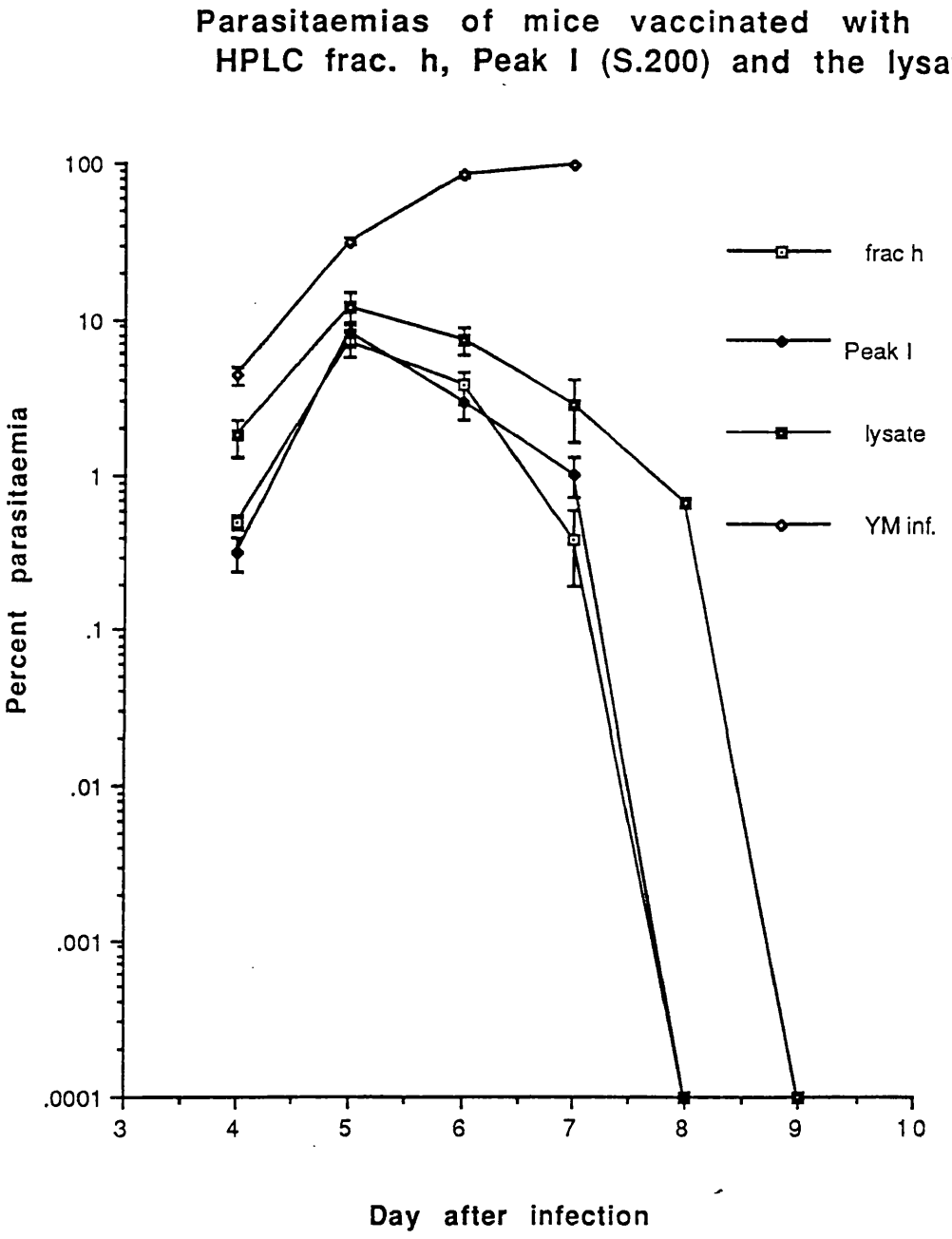


Figure 3.6c. **Comparison between the course of parasitaemia in mice vaccinated with HPLC frac.(h), S.200 Peak I and the lysate**

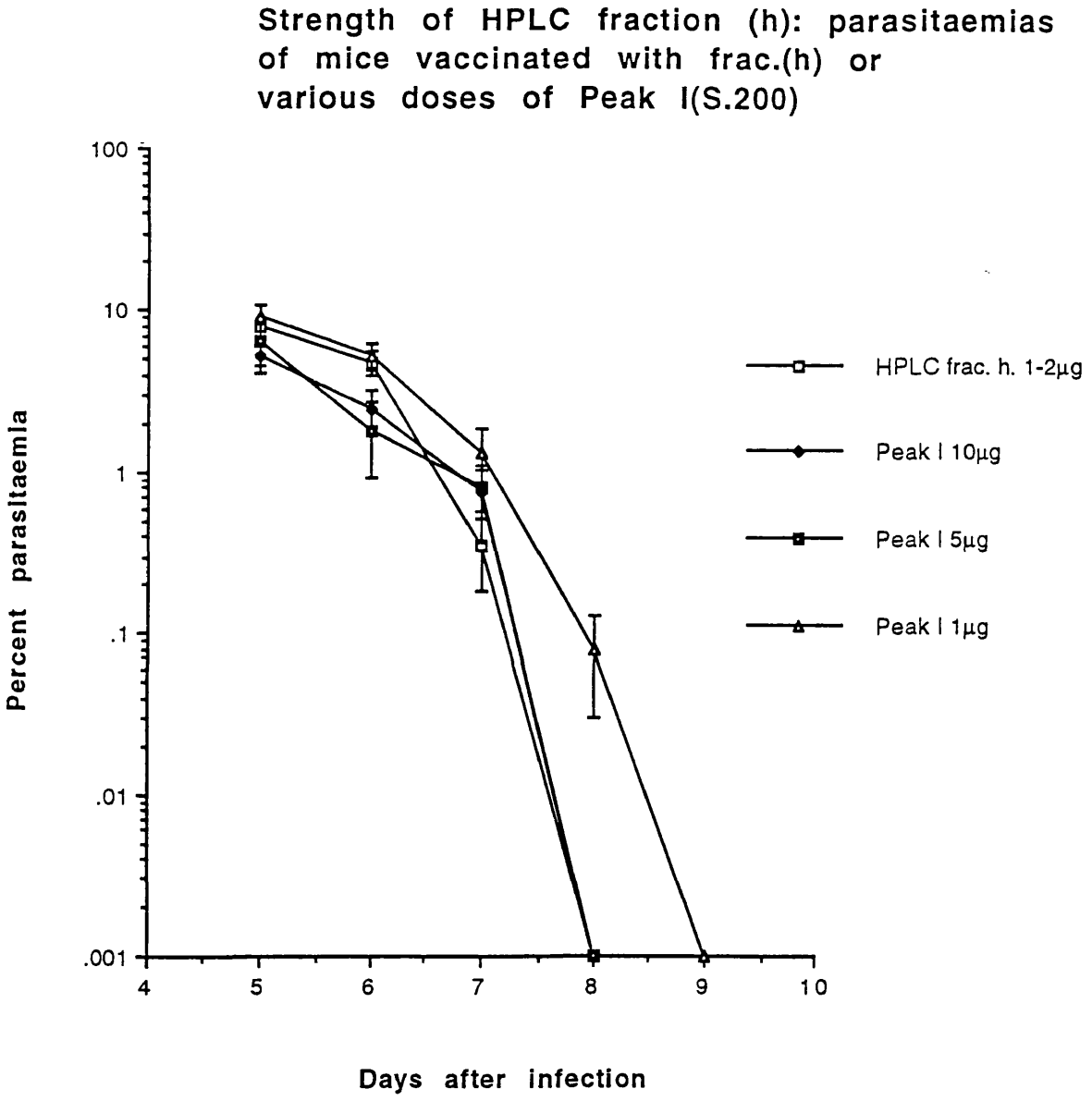


Figure 3.6d.

Dose response characteristics of Peak I (S.200); comparison with protective effect of 1-2µg HPLC frac.(h)

3.7 Further analysis of HPLC fraction (h)

a. Fraction (h) re-run through HPLC column

Fraction **h** appears to be homogeneous when re-analysed by HPLC (figure 3.7a), however the absorbance at A_{214} or A_{280} of this fraction is anomalously high for the protein content. The appearance of a single peak (figure 3.7a), suggests that the protective activity might be due to a single component.

b. Absorbance spectrum

The HPLC instrumentation permitted the scanning of the UV absorption spectrum of fraction **h** during purification. This spectrum (figure 3.7b), is not typical of that of a protein; in particular the high absorption at around 230 and 280nm. It appears that the active moiety of fraction **h** is associated with other non-protein material. The elution pattern is consistent with the association of a hydrophobic (? membrane derived) protein with a micellar component, possibly derived from TX-100. Therefore the activity of the non-protein moiety was tested - see **4.8.**

c. SDS-PAGE and Western Blotting

A silver stained SDS-PAGE of this fraction revealed a band at 140kD and two smaller bands at 63kD and 65kD; two separate batch preparations are shown in plate 3.7 top photo. Feeble staining of the same bands were seen on a Coomassie Blue stained gel. Western blots probed with immune serum were negative but a dot-blot of the fraction reacted strongly with antibody; this was consistent with its low protein yield (data not shown). However, stronger staining was obtained when blots were probed with an antibody raised against fraction **h** and interestingly, the reactive bands

were of molecular weight 63kD and 65kD (plate 3.7 bottom photo). Peak Peak I and the lysate were included for comparison. It was interesting to note that several bands in Peak I ranging from 200kD to 30kD were stained when it was probed with anti-fraction **h** antibody. By contrast only a few bands were identified with a hyperimmune serum (plate 3.5.3a). On the other hand only a few bands (M_r 60kD-30kD) of the lysate were identified by the anti-fraction **h** antibody.

d. Amino acid sequence analysis (carried out by Dr. B. Coles)

For amino acid sequence analysis using the Applied Biosystems analyser, the sample has to be subjected to SDS-PAGE followed by transfer (blotting) to a PVDF membrane which is rapidly stained and destained, and the band of interest is cut out and introduced into the analyser. The advantage of this approach is that the precise band of interest, of known molecular weight, can be selected.

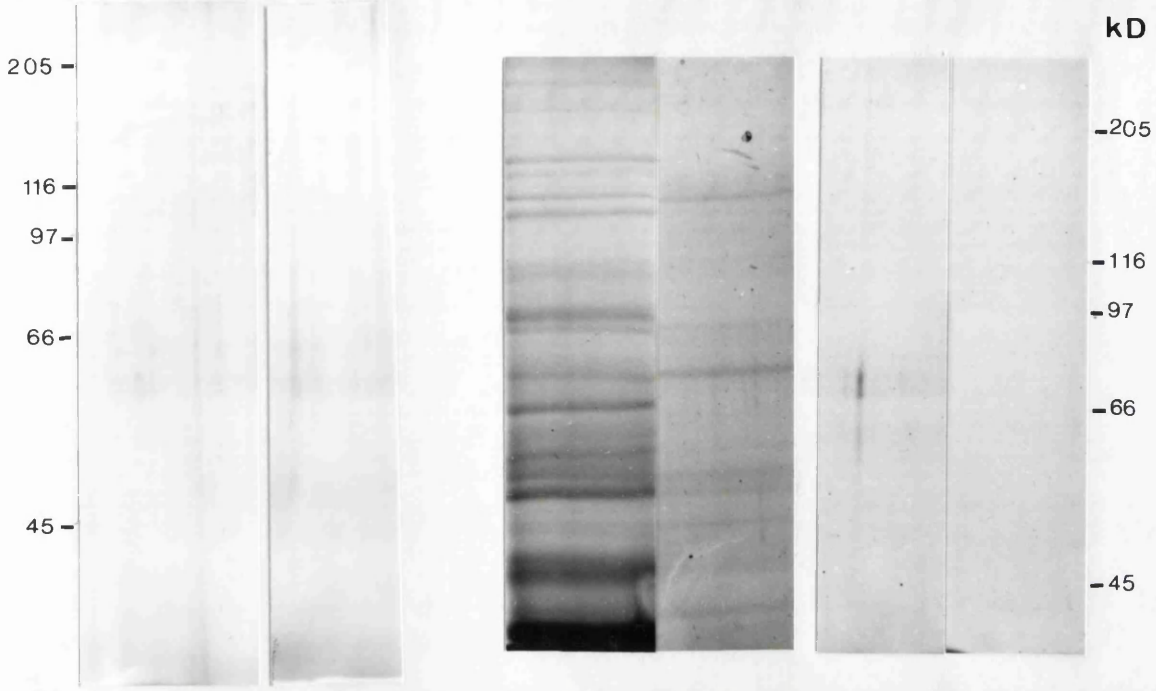
From the above analyses (a to c) it was clear that the protein concentration of fraction **h** would be a limiting factor in these sequencing studies. Even a ten fold concentrated batch of the fraction failed to produce clear bands on SDS-PAGE. Therefore amino acid sequence analysis was carried out by transferring a concentrated batch of the fraction directly to a PVDF membrane. Two short sequences in equimolar ratio, containing the following amino acids were identified.

Sequence 1. ? - ?- Lys-(Phe)- Gln- (Asp)-(Ile)-Pro-Try-(Ile)

Sequence II. ?-?- Ile-?-Asp-Asn-Ile-?-Lys-

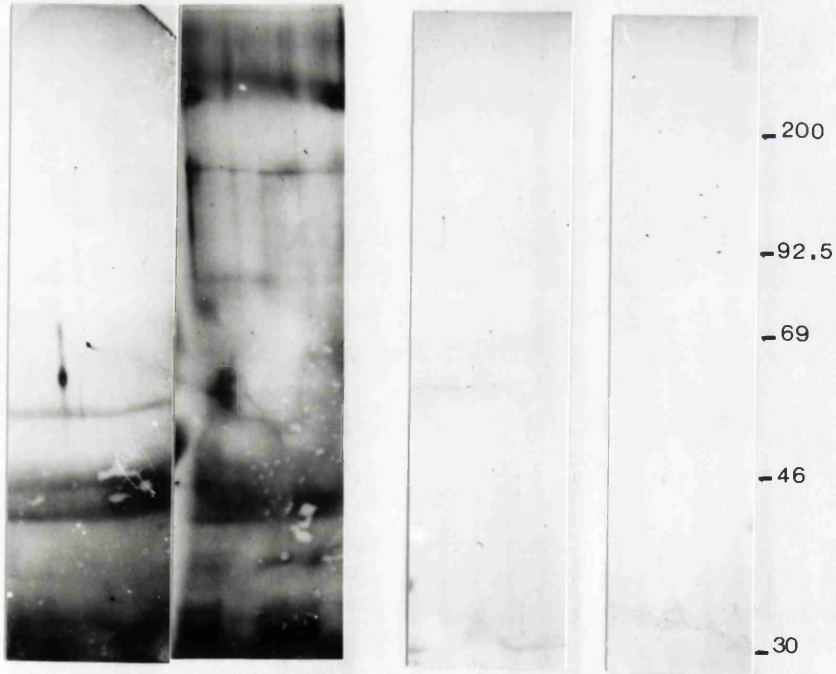
Uncertainties are indicated by brackets or the ? symbol. This sequence is currently being confirmed.

SDS - PAGE and Western Blot analysis of HPLC fraction (h), Peak I (S.200) and the lysate



HPLC fraction h
Stained with Bio-Rad Silver stain

PY lysate Peak I (S.200) fraction h MRBC lysate
Stained with Coomassie blue stain



PY lysate Peak I (S.200) HPLC fraction h MBBC lysate
Western blot probed with anti-fraction h antibody (1:50 diln.)

Plate 3.7 Analysis of HPLC fraction h. Top photo - SDS-PAGE of frac. h (X10 conc.), Peak I & *P.yoelii* and normal rbc lysates
Bottom photo - Western blot of above samples

The apparent purity of fraction (h) determined by HPLC

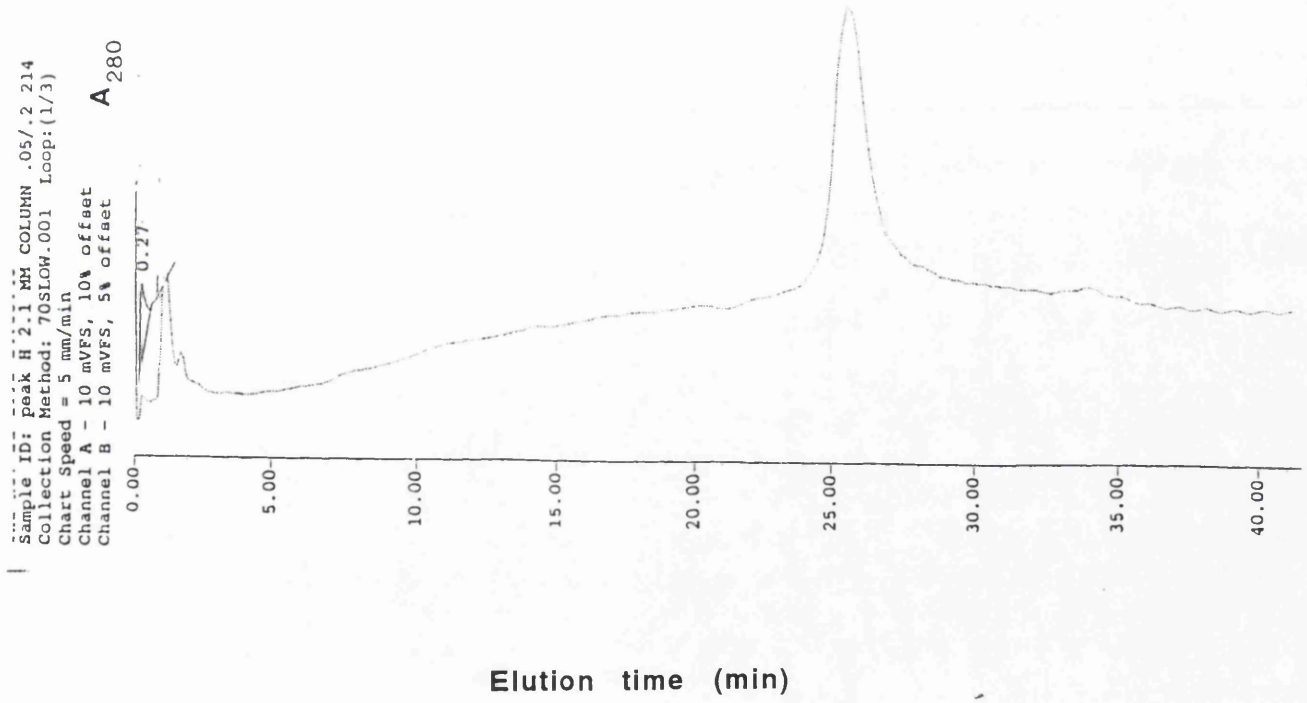


Figure 3.7a HPLC chromatogram of 100µl of fraction (h) from a representative experiment. A single peak at an absorbance of 280mnis seen at an elution time of 25 min.

The HPLC absorbance spectrum of HPLC fraction (h)

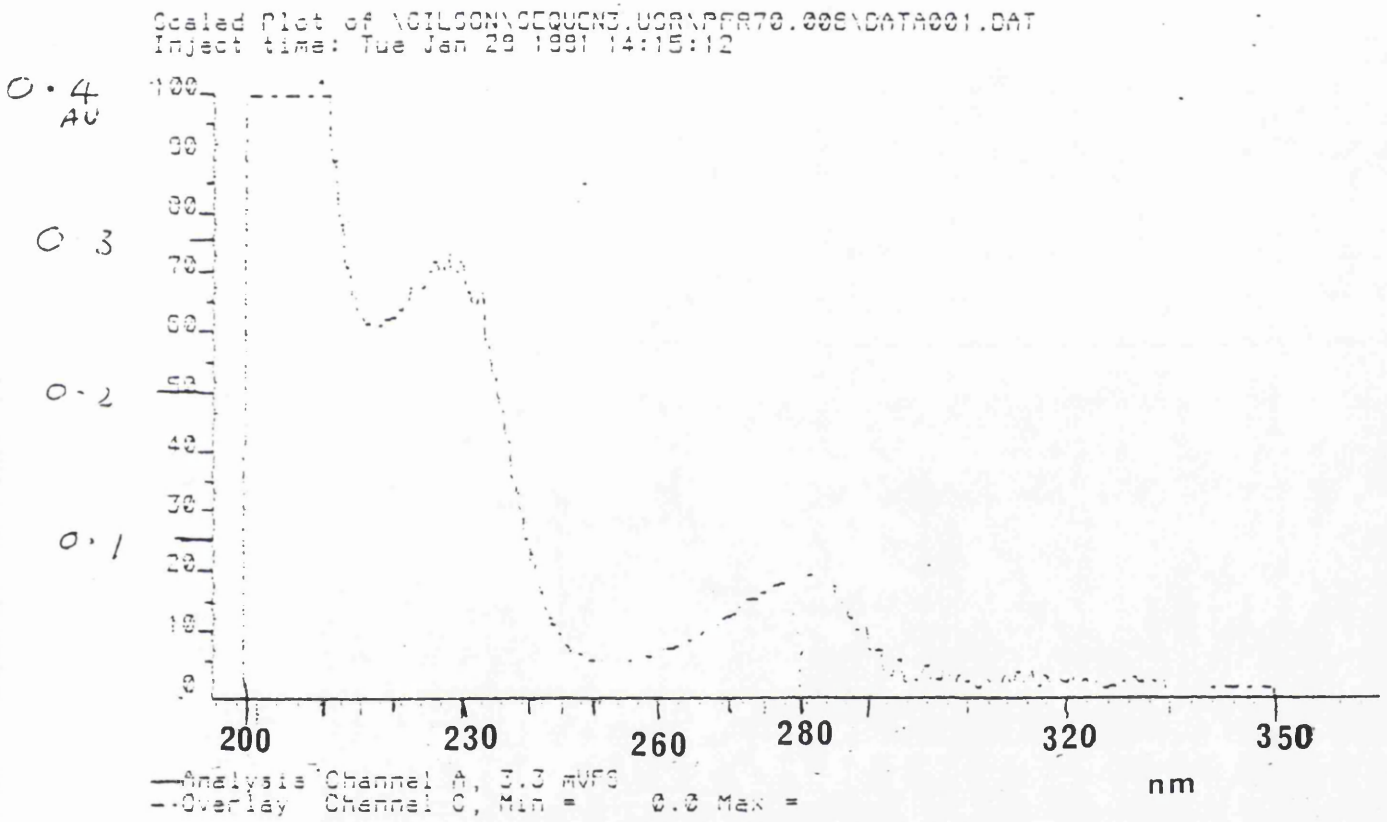


Figure 3.7b **Scan of the absorbance spectrum between 200nm and 350nm of a 0.4ml sample of HPLC fraction h. This spectrum was obtained from the Gilson UV monitor during a standard purification experiment.**

3.8 Summary of isolated antigens and their protective effect

A summary of the main protective fractions isolated by the various procedures is shown in table 3.8. It is important to note that the two HPLC purified fractions (derived from IEF pH6 and S.200 Peak I) that have been partially sequenced, are both coincidentally eluted at fraction **h** in their respective chromatographs. However, the two fractions are entirely different molecules which are eluted at the hydrophobic end of the column but differ in the following ways:

a) Each fraction has a distinct elution time. Fraction **h** from IEF pH6 is eluted at 23.5 minutes while that of S.200 Peak I is eluted at 27.4 minutes.

b) Fraction **h** from IEF pH6 has a molecular weight of 56kD and contains more protein than fraction **h** from Peak I. The latter HPLC fraction contains two polypeptides of molecular weight 65 and 63kD and appears to be associated with TX-100 micelles.

c) Both fractions have different N-terminal amino acid sequences.

Table 3.8 Protective *P.yoelii* antigens isolated by various procedures

Purification Procedure	Main protective fraction	Apparent Mol. Wt. (kD)	Protective effect (%)
	pH 4	Range 200-20	100
I. IEF of lysate	pH6	100-30	70
	pH8	70-30	82
S.200 frac. of IEF proteins			
	one main peak-	Range 300-250	80
	7 fractions	250-200	100
		200-175	89
IEF pH4		175-150	100
		150-125	100
		125-100	100
		100-80	100
IEF pH6	one main peak	60-40	78
IEF pH8	one main peak	150-175	100
II. Sephacryl frac. of lysate			
S.200	Peak I	Range >250-190	100
	Peak II	150-70	100
S.300	Peak Ia	Range 900-600	100
Final purification by HPLC			
IEF pH6	fraction **h**	56	78
IEF pH8	fracs. **b, c, e, f, h**	ND	50-75
S.200 Peak I	fraction **h**	65-63	100
Final purification by FPLC			
IEF pH4	fracs. 8, 11-15, & 17	Range 300-29	100

3.9 Discussion

These studies have confirmed the feasibility of using physical separation techniques for identifying protective antigens in *P. yoelii* malaria. They can now be extended to identify protective antigens of other *Plasmodium* species and indeed of various other parasitic diseases as well. It should however be stressed that these techniques are only suitable for small-scale preparation and identification of antigens. Yields of protective antigen tend to be very low but adequate for vaccination studies in experimental mice. Batch preparation for testing in larger mammals can be obtained by monoclonal antibody technology, where a monoclonal antibody to the antigen of interest is prepared and later used to affinity purify the antigen from a total membrane extract or lysate. Alternatively, recombinant DNA technology may be applied; a plasmid containing the gene encoding the antigen is prepared from an oligonucleotide probe and introduced into a suitable bacterial vector from which large quantities of the recombinant antigen can be recovered.

In terms of protective activity the IEF pH4 fraction was by far the strongest. Early protection was achieved with doses as low as 1μg - 5μg of protein. By contrast, the pH6 and 8 fractions lost their activity at these doses and increasing the dose to 25μg made no difference to their protective effect (data not shown). These antigens were never as consistently protective as the pH4 antigens and although 50% of the vaccinated animals recovered early, there were always a fair number that recovered late or died. A striking feature of the animals that recovered late was their ability to sustain high levels of parasitaemia (50%-70%) for as

long as 10-12 days in some cases, without appearing too distressed. This suggests that they were probably protected against early mortality. Those that died did so substantially later than the unvaccinated controls, suggesting that the pH6 and pH8 antigens may act by inducing an "anti-disease" type of immunity which enables the animals to overcome the toxic effects of the infection. However, eventual recovery is dependent upon the induction of a specific anti-parasite response. These two different patterns of recovery (early and late) might represent anti-parasite and anti-disease immunity respectively, and antigens inducing the latter may be similar to the exoantigens present in *P. yoelii* culture supernatents (Bate, Taverne & Playfair, 1989; Playfair *et al.*, 1990). Preliminary experiments suggest that the molecules are not similar as the active component of the pH6 and 8 antigens appears to be protein in nature (discussed in section 4.0) while the activity of the supernatent antigen has been shown to be dependent upon a phospholipid molecule (Bate *et al.*, 1992), although it may contain protein in the native form. Two other features of the exoantigens that are not shared by antigens fractionated by IEF, gel filtration chromatography or reverse phase HPLC, are as follows:

a) Exoantigens are T cell-independent antigens. T cell-independent antibody made against them blocks TNF secretion *in vitro* (Taverne, Bate & Playfair, 1990). The lysate and its fractionated antigens are T cell-dependent. The vaccination effect of the lysate is greatly reduced in recipient mice that have been pretreated with an anti-CD4 antibody (Heath *et al.*, 1989).

b) Vaccination with exoantigens does not require boosting or the use of adjuvants and TNF blocking activity is present in the serum of vaccinated nude or T cell-depleted mice. Effective vaccination with the

lysate and its fractions requires adjuvants and boosting.

Protective antigens of IEF pH4 *P. yoelii* proteins

The majority of the proteins in the lysate were focussed as a preciptate in the pH4 region. Most proteins do indeed precipitate at their isoelectric point, which is the point at which proteins or amino acid molecules carry no charge and are therefore electrophoretically immobile. It is also the pH at which the protein is least soluble in water. Precipitation also occurs as a result of the removal of TX-100 from the lysate during the IEF procedure. Analysis of this region by SDS-PAGE showed a number of clearly visible protein bands, after staining, which appeared to weaken or breakdown into smaller fragments when the fraction was subjected to repeated cycles of freezing and thawing. Nevertheless, its protective effect remained unaltered implying that the breakdown products were just as immunogenic. The reducing conditions of SDS-PAGE probably accounts for the absence of high molecular weight bands in some pH4 preparations. This was evident from the Sephacryl S.200 gel filtration studies of the re-dissolved precipitate, where the most protective components appeared in the high molecular weight region between M_r 300-100kD. Fractions of lower molecular weight were less active but this could not be related to their lower protein content since the immunizing dose was kept constant at 10μg for all fractions. It would have been possible to discriminate between the protective effect of all fractions by testing simple dose-response relationships but this was considered unnecessary as the fractions from this column were not efficiently resolved and our interests were specifically in the highly purified fractions. Attempts were therefore made at purifying the pH4 fraction by FPLC. Preliminary data suggested the

presence of at least six active antigens. One of these antigens that was eluted as a peak at the void volume of the column, and therefore of high molecular weight (>300kD), was the most protective. We are currently confirming the protection data and also verifying the purity and molecular weights of the fractionated antigens.

Protective antigens of IEF pH6 *P. yoelii* proteins

The IEF pH6 proteins account for roughly 5% of the total protein in the lysate and appear to contain only one protective antigen of M_r 56kD. This antigen (fraction **h**) was as protective as the intact pH6 fraction and was eluted towards the hydrophobic end of the HPLC run, indicating that the molecule was possibly a membrane hydrophobic bound component. The purity of this fraction has been verified by HPLC (data not shown) but its N-terminal amino acid sequence awaits further confirmation.

Protective antigens of IEF pH8 *P. yoelii* proteins

The precise number of protective antigens within the IEF pH8 proteins has not yet been determined. Sephacryl S.200 separation gave only one very active peak of protective activity between M_r 175kD-150kD which corresponded to the main protein peak (figures 3.2.3a and b). High molecular weight bands were not seen on the SDS-PAGE gels or immunoblots suggesting that the protein may have been denatured by the running conditions of SDS-PAGE. Three bands on the immunoblot (between 50kD-60kD) were not present on the SDS-PAGE gels. These may represent very low quantities of antigen that were not detected by the Coomassie blue stain on SDS-PAGE, or they may contain a non-protein immunogenic moiety. Further purification of the pH8 proteins by HPLC

was not conclusive. Several semi-protective peaks were eluted in both the hydrophilic and hydrophobic regions, none of which were as protective as the whole pH8 fraction. The pH8 fraction is therefore apparently made up of membrane and cytoplasmic (probably parasite protein) components. Perhaps synergy between peaks is necessary to restore full protective activity or probably reverse-phase HPLC is not suitable for this fraction; a molecular weight based separation such as FPLC may be more appropriate. These experiments have been temporarily postponed as priority was given to the strongest protective antigens.

Summary of IEF and further purification of IEF fractions

In summary, it is quite clear that the pH4 and pH8 IEF regions contain a number of protective components. The pH4 has a minimum of 6 highly protective antigens while the pH8 has 5 semi-protective ones. The pH6 fraction appears to be made up of just one major protective component of molecular weight 56kD.

Isoelectric focussing was therefore a useful first step procedure for isolating protective components from the lysate although the most protective, the pH4 antigens precipitated; this was easily overcome by re-dissolving in the appropriate detergent for further purification by gel filtration chromatography.

Protection by Sephacryl S.200/S.300 separated fractions of the lysate

In view of the problems experienced with the pH4 precipitate it was of interest to try an alternative initial purification procedure for the lysate. Sephacryl S.200 or S.300 was ideal for this purpose. The most

protective peaks from these columns, Peak Ia (S.300) and Peak I (S.200) which had similar SDS-PAGE and immunoblot patterns were finally purified by HPLC.

Several protective antigens were present in the S.200 separated fractions and amongst the three main peaks of protective activity, Peak I induced the strongest protective immune response. Four clear bands at 140kD, 65kD, 45kD and 30kD, were seen by SDS-PAGE analysis but only the 140kD and 45kD reacted with antibody as judged by the Western blot. The 45kD antigen appeared as a narrow band on the SDS-PAGE gel but as a dense broad band on the Western blot suggesting that it is a very immunogenic protein present in minute quantities or that it may be associated with an immunogenic non-protein, lipid or carbohydrate moiety. When Peak I was probed with an antibody raised against fraction **h** several immunogenic bands were seen (discussed later). In terms of protective activity Peak II was not quite as strong as Peak I. Although parasitaemias of mice vaccinated with the two fractions were not significantly different except on day 6, mice vaccinated with Peak II always recovered a day or two later. Peak II contained a minimum of 6 proteins; one of these of molecular weight 38kD reacted strongly with antibody. Peak III of relatively lower molecular weight, was less effective as a vaccine. Three proteins out of a total of 10 in Peak III reacted with antibody. Further purification of peaks Peak II and III by HPLC is currently in progress. The number of protective fractions within Peak II is awaited with interest. This fraction contains several protein bands according to its SDS-PAGE analysis but only a few low molecular weight bands appear to react with an anti-*P. yoelii* antibody.

The protective effect of the S.200 separated IEF pH4 fractions and those of the lysate (figs. 3.2.1 and 3.5.1) were very similar. This is not surprising as the pH4 fraction accounts for over 90% of the protein in the lysate. Furthermore the protective activity of Peak I of the lysate and the main peak of activity of the pH4 fraction appeared in the same molecular weight range of >230kD - 190kD and it is likely that these two fractions have a few important proteins in common. This was supported by the Western blot analysis in plates 3.1.2b and 3.5.3a, which suggested that three immunogenic proteins (M_r 140kD, 45kD and 38kD) were similar in both fractions. But the pH4 fraction does contain other antigens that are not present in Peak I as evidenced by additional bands on the SDS-PAGE gels and from the number of protective fractions obtained by FPLC purification. Some of these other antigens may be present in Peak II and III. An important similarity between the first peak of activity from the FPLC purification and Peak I (S.200) is that both were eluted at the void volumes of their respective columns. In addition they induced a very strong immune response in vaccinated mice who experienced significantly lower parasitaemias after challenge and cleared their infections earlier than mice vaccinated with other fractions. The only other antigen to give a comparably potent protective immune response was fraction **h**, resolved by HPLC purification of Peak I.

Protection by HPLC purified Peak I fractions

Of the ten fractions purified, fraction **h** was the most active; it induced 100% early protection in vaccinated mice. Perhaps synergy between selected combinations of the other fractions is necessary to achieve a

similar degree of protection. In fact fraction **h** was the strongest antigen isolated so far; it retained its protective activity at doses as low as 1μg of protein (figure 3.6d). This fraction has now been obtained in a highly purified form and some of its characteristics have been studied (section 4.0). Identification of the molecule by SDS-PAGE was difficult owing to its low protein content. Despite concentrating this fraction from several HPLC experiments, only faint bands were seen on gels. One of the possibilities for the low protein yield is that a fair amount was being lost during the concentration procedure. This has recently been overcome by adding small quantities of SDS to the fraction before concentration. Fraction **h** was nevertheless identified as two bands of M_r 63kD and 65kD in a Western blot assay probed with an antibody raised against itself. These two bands, in addition to several others, were also present in a Western blot of Peak I (from which fraction **h** was derived) when it was probed with the anti-fraction **h** antibody. Amino acid sequence data indicates the presence of two polypeptides present in equimolar ratio. Therefore fraction **h** may exists as a 65-63kD doublet rather like the 45-43kD doublet gametocyte antigen (Alano, 1991). Further studies on the nature of fraction **h** are described in the next section, but from these data it appears that its two polypeptides are possibly associated with micelles of TX-100 or with phospholipid-like molecules. The former explanation is more likely because firstly, the lysate is prepared from a TX-100 extraction buffer and secondly, when the fraction is frozen and thawed it has a cloudy appearance which is a characteristic of detergent-containing molecules.

Concluding remarks

The main finding from these studies was the identification of a 65-

63kD doublet-like molecule which induced a very potent protective immune response against a lethal *P. yoelii* malaria infection. Amino acid sequence data suggests that the molecule is not related to the major merozoite surface protein MSA-1. Confirmation of the amino acid sequence is necessary before comparisons are made with the N-terminal amino acid sequences from a protein data base. If this is a novel antigen the next logical step is to construct an oligonucleotide probe to identify its encoding gene in mice. It should then be possible to identify the human equivalent and more importantly to determine whether the molecule is variant amongst different isolates of *P. falciparum*. Assuming that the degree of variability is low, and that the basic mechanisms underlying anti-parasitic immunity in man and the mouse are the same, a carefully conducted vaccination trial could be organized first in *Aotus* monkeys and later in human volunteers. Experimental data from various malaria models indicates that a suitable adjuvant will be required, it is therefore important to find an alternative to Freund's Complete Adjuvant which seems to be the only adjuvant to induce protective immunity in monkeys (Siddiqui *et al.*, 1987). Encouraging results have been recently obtained with liposomes (Fries *et al.*, 1992). Liposome-encapsulated recombinant circumsporozoite protein ($R32NSI_{81}$) of *P. falciparum*, adsorbed with $Al(OH)_3$ induced high levels of specific antibody in human volunteers, with little or no systemic toxicity. Our success with saponin suggests that ISCOMS (immuno-stimulatory complexes, which contain Quil A), might also be effective. We have also shown that in mice, recombinant gamma interferon is a potent adjuvant for TX-100 soluble malaria antigens (Playfair and de Souza, 1987).

The existence of antigenic variation amongst the isolated antigens is not yet known, but on the basis of the numerous SDS-PAGE and Western blot assays carried out it is possible to say that variation might exist amongst the pH6 and pH8 antigens. This may account for their weak immunogenicity and it may also explain why some batches were more immunogenic (inducing early-type recovery) than others. The pH4 antigens showed relatively consistent staining in both assays indicating that these antigens may not be variant. It must however be pointed out that the parasites used for the preparation of vaccines and those used for challenge come from different passages; therefore 100% protection in vaccinated mice suggests that the variation is limited.

We have succeeded in isolating several antigens using physical separation techniques which are not based on the reactivity of antigen with protective antibody and although only two of these antigens have been further purified as single polypeptides and partially amino acid sequenced, a number of others (see table 3.8) await identification. These studies are currently in progress and include further purification of protective antigens from the IEF pH4 fraction and those from the S.200 Peaks II and Peak III. Other laboratories have adopted the approach of isolating antigens by affinity purification or DNA technology which are less laborious but restricted to identifying antigens that react with antibody. Dubois and colleagues (1984) electro-eluted protective *P. falciparum* proteins from SDS polyacrylamide gels, but once again these proteins were selected on the basis of their reactivity with immune serum. Similarly, Patarroyo and colleagues (1987a) isolated four molecules by preparative SDS-PAGE, some of which were immunogenic in monkeys. In a

subsequent study they showed that three synthetic peptides corresponding to fragments of the immunogenic proteins of M_r 83kD, 55kD and 35kD were partially protective in immunized monkeys (Patarroyo *et al.*, 1987b), but a combination of these three gave complete protection. This combination termed SPf 66 together with the circumsporozoite antigen NANP (added as a universal T cell epitope) was polymerized and checked for purity by HPLC. It was used for the first clinical trial (Patarroyo *et al.*, 1988), and subsequent field trials which have not yet been published.

Although the physical separation techniques described here are laborious when compared with the above methods of antigen identification, they provide a panel of antigens which can then be tested for various immunological activities. Some of these antigens are likely to be overlooked by methods which rely entirely on antibody reactivity, particularly if they are present in the parasite in very small amounts. The isolated antigens can then be tested in protection studies which have already been discussed in this section, and T cell helper and delayed type hypersensitivity (DTH) reactions. The latter studies including characterization of the antigens isolated, form the basis of the next section.

4.0 Immunological characteristics and nature of protective *P.yoelii* YM antigens

Introduction

Immunity to malaria depends upon a combination of effector mechanisms including antibody, cell-mediated immunity, macrophage activation and various cytokines (discussed in section 1.0). Candidate vaccine antigens must therefore be able to stimulate both T and B cells, and possibly activate macrophages. The putative vaccine antigen MSA-1 which protects mice against *P.yoelii* YM (Holder and Freeman, 1981), has also been shown to stimulate specific helper T cells and T cells responsible for delayed hypersensitivity (DTH) (Playfair, de Souza, Freeman and Holder, 1985), implying that at least one kind of T-helper cell stimulation correlates with protective immunity. Therefore to further test this correlation of protection with T-helper cell stimulation, all fractions separated by IEF including the most protective HPLC purified fractions were tested for T-helper activity and DTH. Antibody levels were also checked and in some cases the ability to activate macrophages *in vitro* was also tested (collaboration with C. Bate).

The substantial number of late type recoveries obtained by vaccination with the IEF pH6 and 8 antigens recalls the "anti-disease" type of immunity obtained with exoantigens described by Playfair *et al.* (1990). The activity of these antigens was recently shown to be dependent upon phospholipid molecules (Bate *et al.*, 1992). Thus it was of interest to determine whether the protective effects of the pH6 and 8 antigens and indeed the most protective antigens the pH4, Peak I (S.200) and its HPLC

purified antigen, fraction h were also associated with lipid molecules. Protective antigens were accordingly treated with either proteases or lipases and then tested as vaccines.

RESULTS

4.1 T-helper cell priming

This assay is based on the fact that B cells from normal mice respond naturally to the hapten trinitro-phenyl (TNP) when it is coupled to a suitable carrier molecule; thus when TNP-carrier conjugates are injected into carrier-primed recipients, a greatly increased TNP response is obtained. This anti-hapten response has been shown to be due to cooperation between T cells and B cells (Mitchison, 1968).

In these experiments fractionated malarial antigens act as carrier molecules which stimulate T cells in immunized mice. When these animals are challenged with TNP-labelled parasitized erythrocytes the activated T cells recognize the parasite on the hapten-carrier conjugate and "help" the B cells to respond to TNP. The resulting TNP responsive plaque- forming plasma cells in the spleen are detected with TNP-coupled sheep erythrocytes (target cells) in the *in vitro* Cunningham assay.

In all these experiments mice were immunized with a single discriminating dose of 1μg protein of each fractionated antigen plus 25μg saponin intraperitoneally and challenged with TNP-labelled parasitized red blood cells as described in section 2 (Methods and Materials). As the response to different batches of TNP-coated *P.yoelii* parasitized erythrocytes was variable, the TNP plaque-forming cell responses in mice

vaccinated with fractionated antigens in each experiment were expressed as a percentage of the plaque-forming cell response induced by the lysate from which they were derived.

4.2 Induction of T-helper cells by IEF fractions

All fractions within the focussed range of pH 3.8 to pH9 were tested for their ability to induce T-helper cell priming. The results of twelve separate experiments are shown in figure 4.2a where each point represents the mean of a group of three mice vaccinated with 1μg of the corresponding IEF fraction plus 25 μg saponin. The mean TNP-plaque forming cell count for each group is expressed as a percentage of the the result from a group of mice given 1μg of the lysate plus saponin. All fractions showed a variable degree of T-cell helper activity but only fractions in the pH region around 4.2, 6.5 and 8 induced T-cell priming that was equivalent to or greater than that induced by the lysate. Antigens within these regions were also the most protective - see figure 4.2b. Fractions from the pH regions 5 to 5.8 showed poor T-cell helper priming and this group of antigens were not protective. However, fractions in the pH range 6.6 to 6.8 and 7 to 7.2 were comparatively more protective but failed to induce respectable T-helper cell priming. Therefore the correlation between T-helper cell priming and protection was tested.

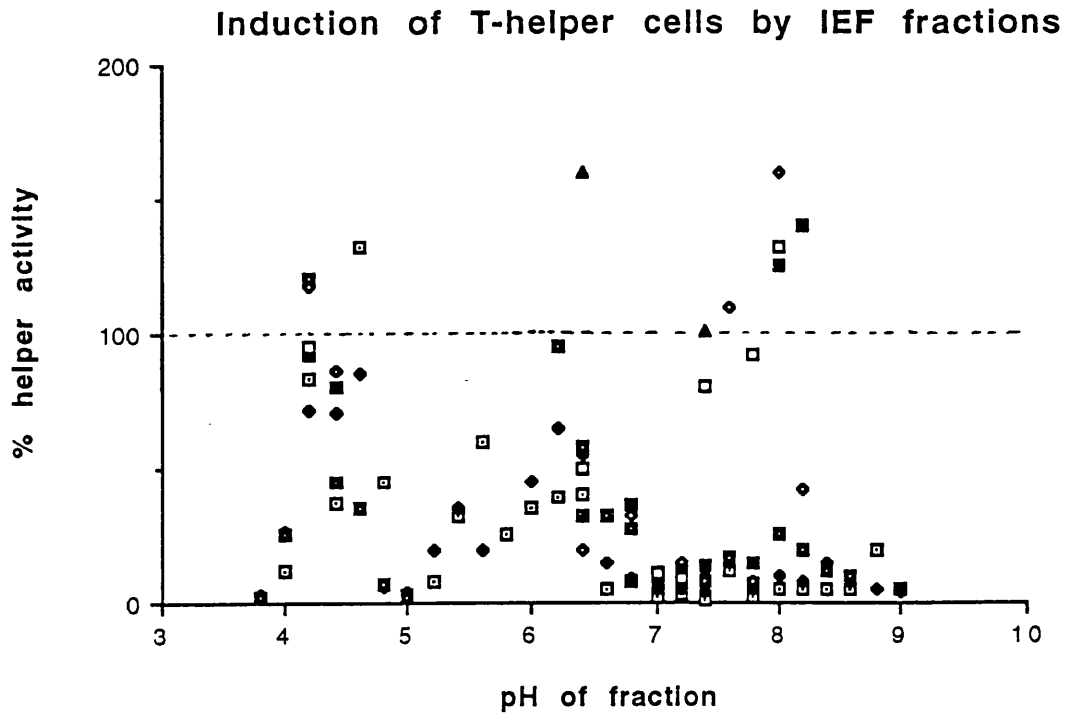


Figure 4.2a Data from 12 separate expts. Each point represents the mean of a group of 3 mice vaccinated with 1ug antigen plus 25ug saponin

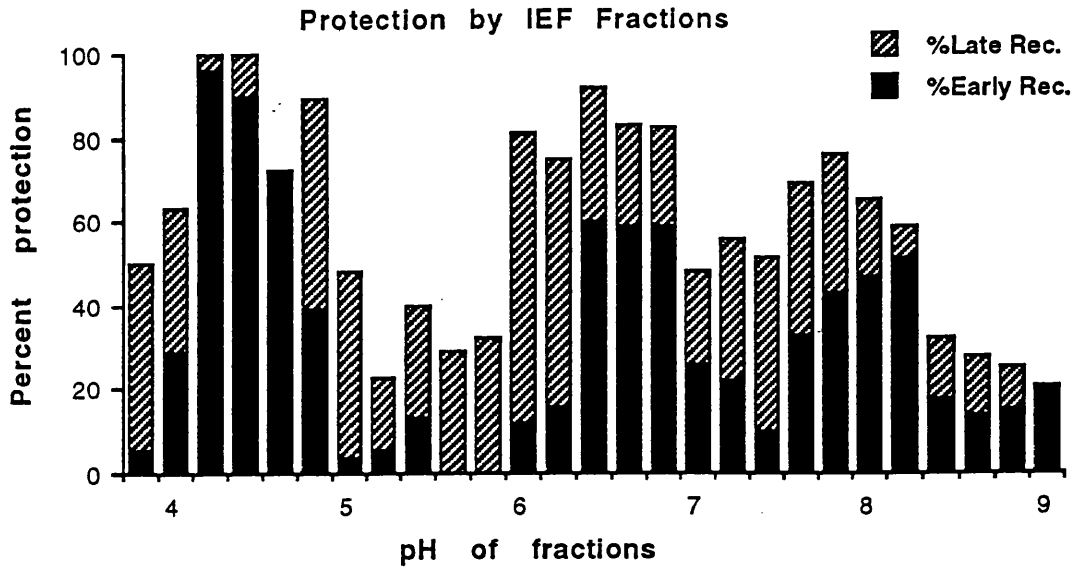


Figure 4.2b Protective effect of fractions from 7 separate experiments using the Ampholine of range pH3.5 to10. Each bar represents 17 to 21 mice.

4.3 Correlation of T-helper priming with protection

In order to correlate T-helper cell priming with protection two sets of experiments were set up in parallel. One set (three mice per group) were primed with the usual dose of IEF fraction for a T-helper cell assay while another group of three mice were vaccinated with the same fractions for protection studies (figure 3.1b; this figure has been shown again in this section as figure 4.2b, for convenience). Antibody titres were also checked.

Data from several assays representing the T-helper cell response for 132 separate IEF fractions was classed into seven groups according to the type of protection induced. In a group of three vaccinated mice the protection patterns were as follows:

3E - three mice recovering early

2E 1L - two early, one late

1E 2L - one early, two late

3L - three late

2L 1D - two late, one died

1L 2D - one late, two died

3D - three died

The data in figure 4.3 (A) shows the correlation of T-helper priming with protection; strength of protection is plotted against T-helper cell activity of 132 separate IEF fractions. Each point represents a group of three vaccinated mice tested for T-cell priming as described for figure 4.2. The adjacent set of data in figure 4.3 (B) shows the mean± standard error of each of the seven groups of protection analysed by two different statistical tests. By analysis of variance, $F = 21.76 (p < 0.001)$; Spearman's rank coefficient $R = 0.683$ ($p < 0.001$). Clearly there is a good correlation between T-helper activity and protection in the most protective groups, 3E to 3L. As the protective effect

decreased, in the groups where late protection (2L 1D to 3D) predominated, this correlation, though highly significant overall, was not so strong.

A number of poorly correlated points came from the pH6.5 to 8 region. This was clearly seen when the results from the main protective regions, pH3.8 to 5.2, pH6.0 to 7.4 and pH7.6 to 8.4 were plotted separately as shown in figure 4.3 (C). The statistical analysis was as follows:

pH3.8 to 5.2	F=16.71 (p<0.001)	R=0.843 (p<0.001)
pH6.0 to 7.4	F= 4.92 (p<0.002)	R=0.626 (p<0.001)
pH7.6 to 8.4	F= 4.63 (p<0.005)	R=0.771 (p<0.001)

The correlation between T-helper priming and protection was excellent in fractions from the first group of pH range 3.5 to 5.2. In the other groups of range pH6 to 7.4 and pH7.6 to 8.4 the correlation was lower but significant. Some protective antigens in these groups did not stimulate T-cell priming and by contrast the moderately strong T-cell stimulating antigens were not always protective.

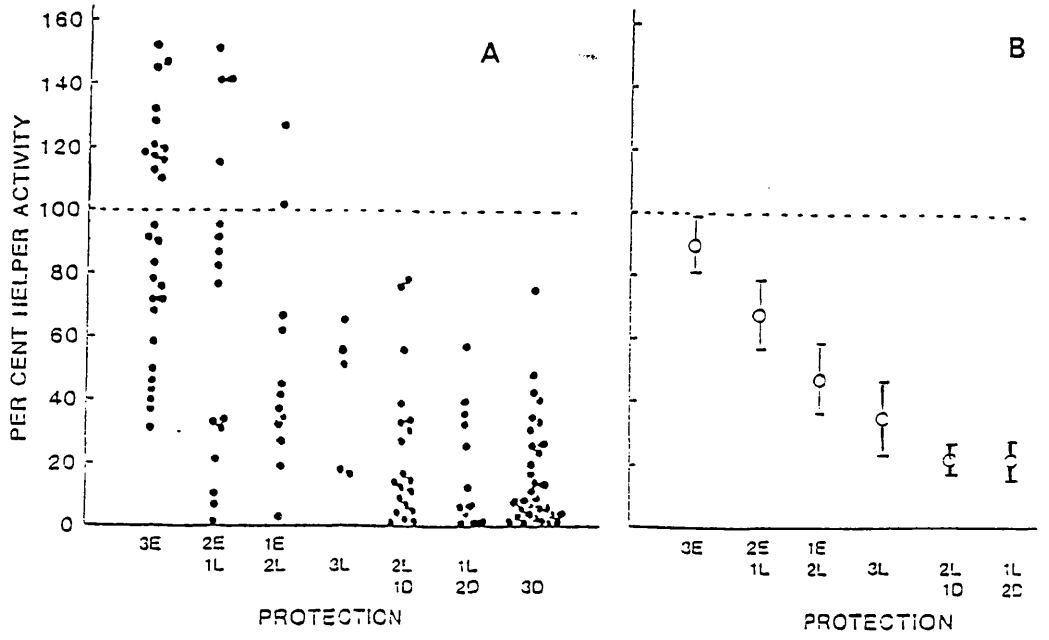


Figure 4.3 A - Each point represents the mean %helper activity of a group of 3 mice.
B - Mean ±SE of the seven grades of protection.

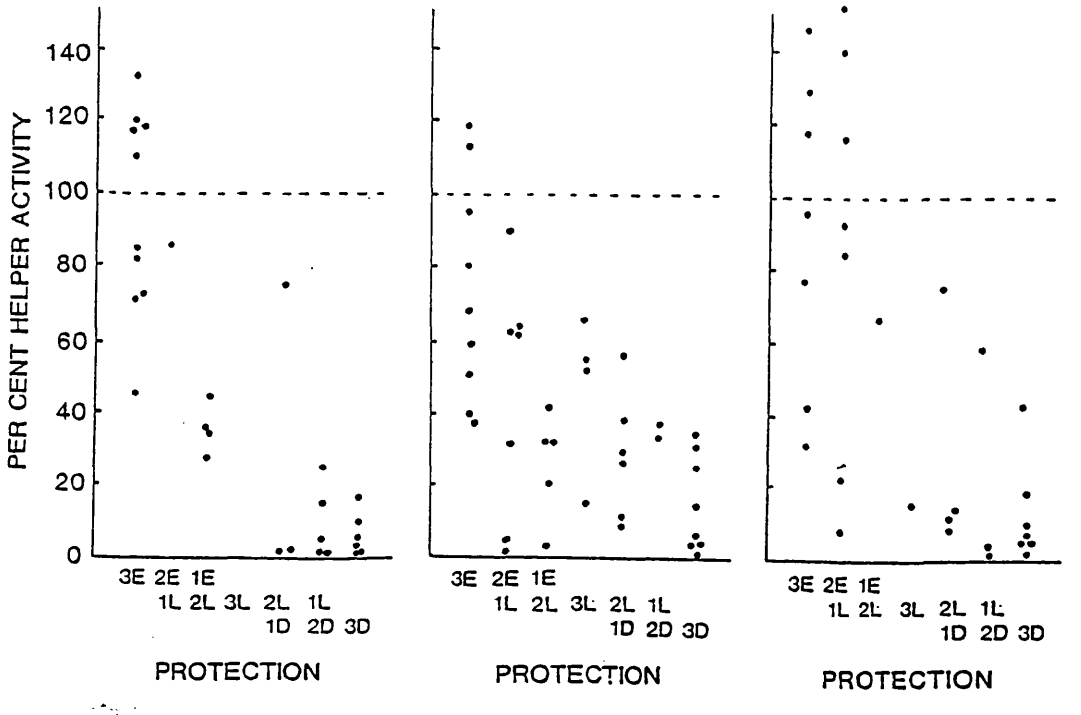


Figure 4.3C Each point represents the mean %helper activity of a group of 3 mice vaccinated with IEF fractions from the three major protective regions: pH3.8 to 5.2; pH6.0 to 7.4; pH7.6 to 8.4.

T-helper cell activity of HPLC purified IEF pH6 antigens

Two experiments were set up with two separate preparations of HPLC purified fractions. In each experiment three mice were vaccinated with 1μg protein of each fraction and assayed for T cell helper activity as described before. The results shown in Table 4.3 represent pooled data from these experiments.

Table 4.3 T-helper cell activity of HPLC purified IEF pH6 antigens

HPLC fraction	% Helper activity (mean ± SE)
a	0
b	59.8 ± 12.2
c	55.9 ± 21.2
d	44.8 ± 15.8
e	37.5 ± 15.4
f	47.4 ± 12.1
g	44.9 ± 10.0
h	87.3 ± 22.5
i	78.9 ± 29.1
j	22.4 ± 11.1
IEF pH6 fraction	59.9 ± 15.4

Clearly all the fractions except **a** and **j** were capable of inducing T helper activity that was between 30 and 60% of the activity of the control lysate. The IEF pH6 fractions in these experiments induced T helper activity that was only 60% of the control. However, this was not unusual as in the previous experiments with individual IEF pH6 fractions the T cell helper activity was variable and showed both high and low levels of

activity.

The highest T-helper cell activity was obtained with HPLC purified fractions **h** and **i**. Fraction **h** was also the most protective antigen of the HPLC purified IEF pH6 antigens. Fraction **i** had an equally good T-helper cell response but was not protective. The most likely explanation here is that fraction **i** probably contains trace amounts of fraction **h** as it is eluted directly after this fraction, and whilst these trace quantities are not sufficient for inducing protective immunity they are detected by this highly sensitive T cell assay.

4.4 T- helper cell priming by HPLC purified Peak I (S.200) fractions

The first major fraction Peak I from the S.200 column and its HPLC purified fractions were tested for their ability to stimulate T-helper cells. Three experiments were set up and the results are shown in figure 4.4a where each bar represents the mean percent helper activity ±SD (standard deviation) of a group of nine mice. By comparison with figure 4.4b (shown below) it is evident that the strongest protective antigens induced the best T-cell stimulation. Both Peak I and fraction **h** induced helper activity that was greater than that induced by the lysate from which they were derived. All the other fractions induced significantly weaker T-cell help which was consistent with their weak protective effect. Although the correlation of T-helper activity and protection was not tested statistically, it seems likely that a correlation similar to that obtained with the IEF fractions would be expected. Nevertheless, it is quite clear that the correlation is excellent for fraction **h** and Peak I. The other fractions resembled the IEF pH6 and 8 antigens in terms of correlating their T-helper activity with protection. Fractions **f** and **i** induced poor T cell stimulation but reasonable protective activity. Conversely, fractions **d** and **e** gave reasonable T cell stimulation

but relatively weaker protective activity.

Perhaps reducing the immunizing dose of antigen to below 1μg of protein might more clearly show the difference between protective and non-protective antigens.

Summary of T-helper cell activity of fractionated antigens

In summary, the most protective fractionated antigens which included IEF pH4, S.200 Peak I and the highly purified HPLC fraction **h** (derived from S.200 Peak I) induced the best T cell priming and there was an excellent correlation between protection and T cell helper activity for these antigens. With the less protective pH6 and 8 fractions the correlation between these two parameters was significant but not as strong.

The correlation between T cell priming and protection was excellent with antigens that induced 100% protection. Therefore T cell priming may be an essential requirement for the induction of protective immunity to *P. yoelii.*

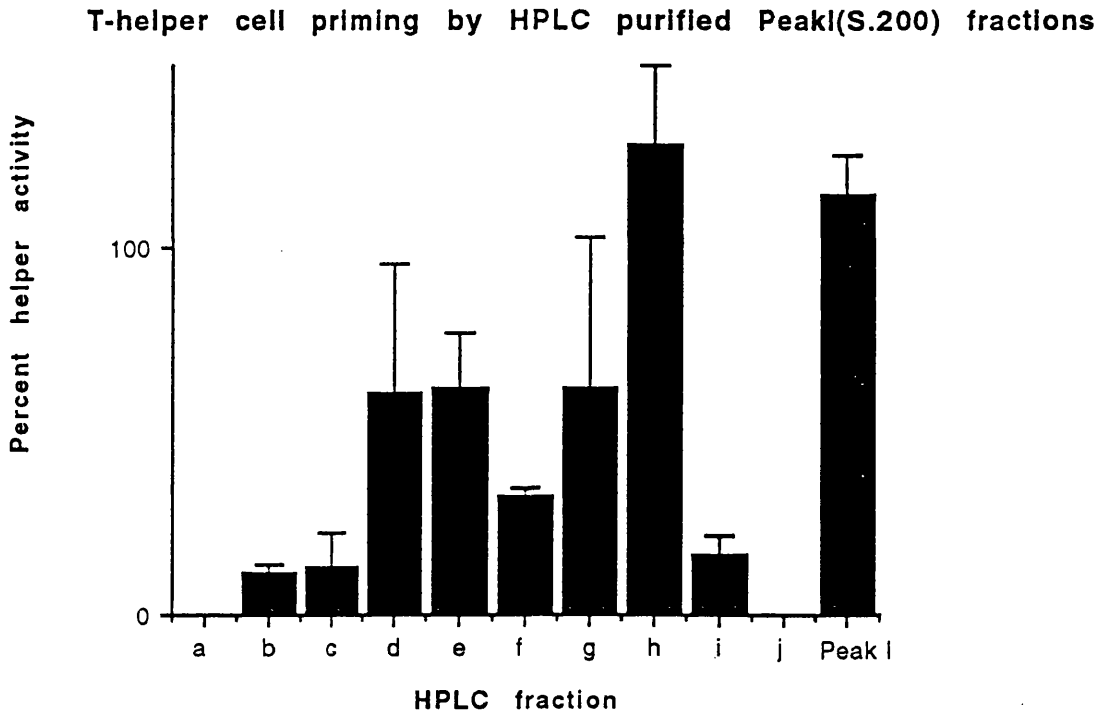


Figure 4.4a Helper activity of fractions shown as a percentage of the PFC response of the lysate from which they were derived

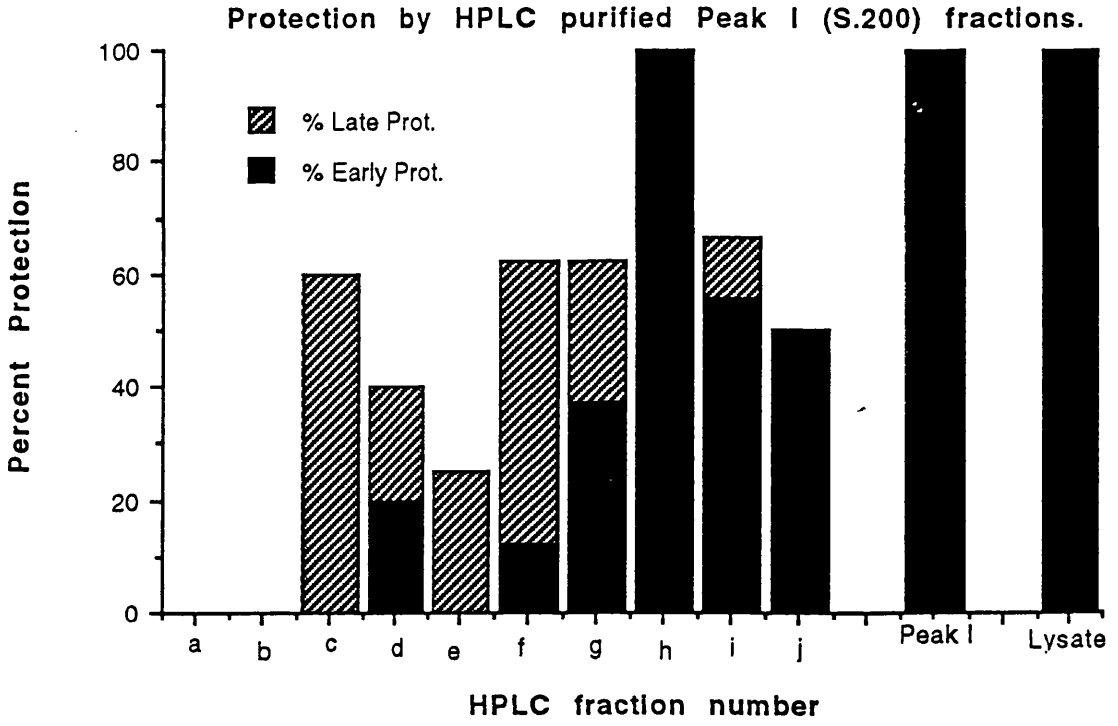


Figure 4.4b. **Protective effect of HPLC purified Peak I (S.200) proteins; data from 4-6 experiments**

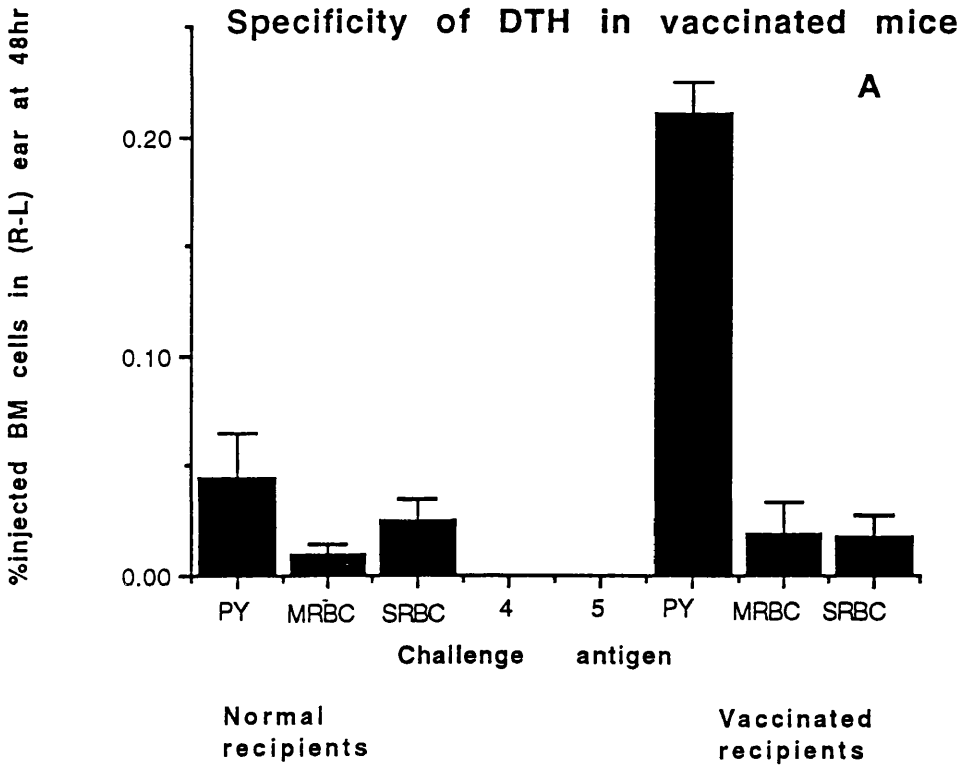
4.5 Induction of delayed-type hypersensitivity by IEF fractions.

For practical reasons experiments were not carried out with individual IEF fractions. Large numbers of recipient mice were required for both vaccination and as donors for bone marrow cells in the DTH assay. Furthermore, only the most protective purified fractions were of interest. Therefore pooled fractions (as shown in table 3.1.1a) that were used for protection studies (described in 3.1.1), were also tested for DTH activity. Groups of three mice were vaccinated with each fraction as described before (3.1.1). Two to three weeks later vaccinated and control mice were challenged in their left pinnae with 10µl PBS and in the right pinnae with 10µl (3 X 10^6 cells) *P.yoelii* infected erythrocytes or control red blood cells, and after 48 hours the degree of ^{51}Cr-labelled bone marrow cell homing into the site of antigenic challenge was measured. Three experiments were set up with pooled IEF fractions. The data in figure 4.5 represents the mean ± SE of bone marrow cell homing shown as percentage increases or decreases of the controls vaccinated with the lysate. The specificity of the DTH response is shown in figure 4.5A where challenge with *P.yoelii* antigen induced a parasite specific response in vaccinated recipients. Vaccinated animals did not respond to mouse (MRBC) or sheep red blood cells (SRBC). It is interesting that normal mice responded better to parasitized red cells than to MRBC or SRBC (discussed later). The DTH response of mice vaccinated with the IEF fractions was expressed as a percentage of the DTH response induced by the lysate from which they were derived. This accounted for the slight differences in the response to the various preparations of isotope-labelled bone marrow cells. The pH4 fraction clearly induced the best DTH response (150% of control - figure

4.5B) which correlated with protective activity. The pH6 and 8 fractions were relatively weaker inducers of DTH (50% - 60% of control) which is consistent with their weaker protective effect. All the other fractions, 3,5,7 and 9 induced a DTH response that was less than 10% of the control. All these fractions except the pH7 were poor inducers of protective immunity.

These data suggested that protective immunity correlates with DTH, and the correlation could be further tested by diluting out the fraction with the strongest response. For example, a good correlation would exist if the lowest protective dose of the pH4 fraction was capable of inducing a good DTH response. But testing this was not necessary as the pH4 fraction was by far the most potent inducer of DTH when compared with the other fractions.

These results suggest that a good DTH response is required for the induction of protective immunity, but as shown later, this may not always be the case.



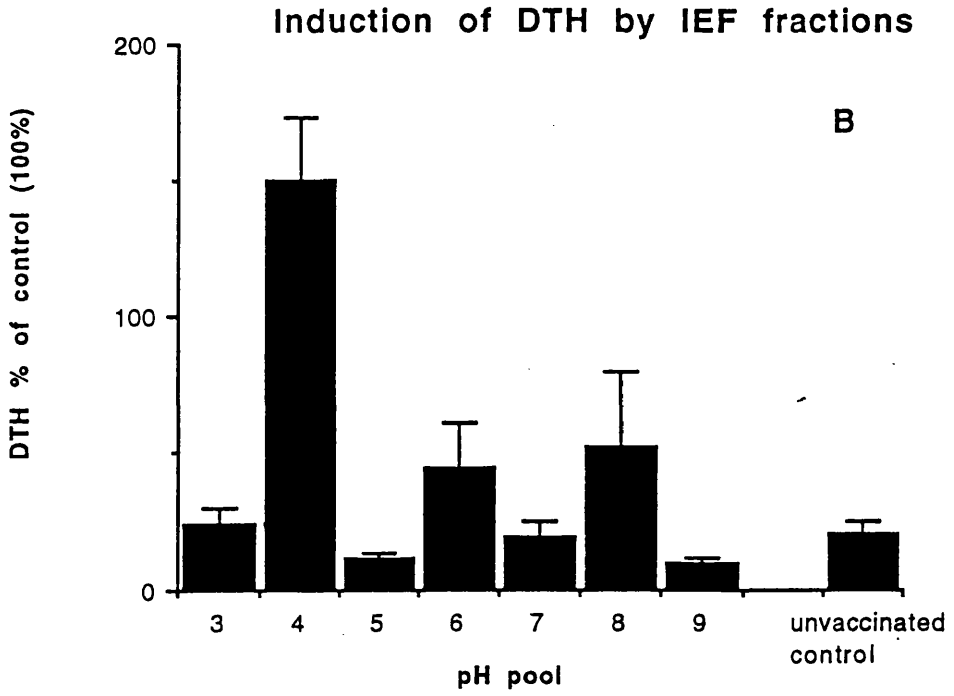


Figure 4.5 A - Shows mean ±SE specific homing of inj. BM cells to challenged ears

B - DTH as a percentage of controls vaccinated with the lysate

4.6 Induction of delayed hypersensitivity by Peak I(S.200) and fraction (h)

Fractionated antigens used in these studies were tested in parallel for T-helper cell activity and protection. Three experiments were carried out in groups of three mice vaccinated with each fraction, and challenged as described in paragraph **4.5**. The results are shown in figure 4.6 where each bar represents the mean ±SE of a group of nine mice. Both these fractions induced DTH responses that were weaker than those of the lysate (positive control), from which they were derived. This was mainly due to inconsistent DTH responses, in which only one third of the animals vaccinated with these fractions, had DTH values that were equivalent to or above those of the group vaccinated with the whole lysate. The fact that these fractions were also the most potent inducers of protective immunity, suggests that a maximal DTH response is not required for protection (discussed later).

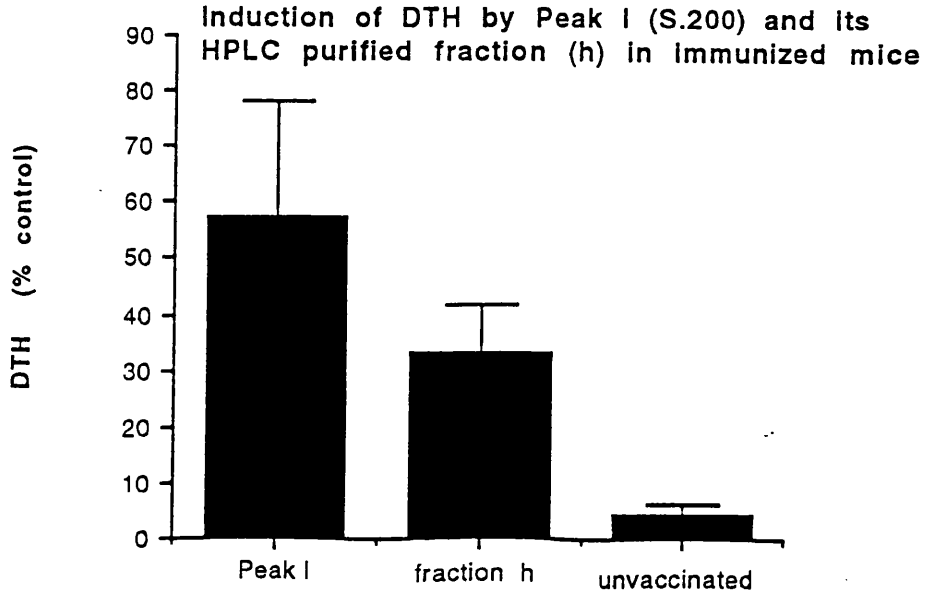


Figure 4.6 DTH is represented as a percentage of the value of the group vaccinated with the whole lysate. (Mean±SE from 3 expts.)

4.7 Antibody responses in mice vaccinated with fractionated antigens

Antibody levels were tested in all experiments with fractionated antigens. A separate group of mice vaccinated with the same antigens that were used for T-helper cell assays and DTH assays, were used for measuring antibody levels, parasitaemias and the day of recovery, judged by the absence of parasites in 10^4 red blood cells. Ten days after infection sera from each group of vaccinated mice were pooled and tested for antibody in the indirect fluorescent antibody test. The results are shown in table 4.7.

Table 4.7 **Antibody titres and survival of mice vaccinated with fractionated *P.yoelii* antigens**

Vaccination	Antibody titre (day 0)	Antibody titre (day 10)	Day of recovery mean ± SD	Day of death
IEF pH3	1:8	1:1024	16.2 ± 6.2	10 -15
pH4	1:32	1:65000	6.1 ± 0.8	-
pH5	1:16	1:1024	28.3 ± 3.2	10 -15
pH6	1:16	1:4096	11.2 ± 2.9	10 - 20
pH7	1:16	1:4096	18.1 ± 5.0	10 - 20
pH8	1:16	1:4096	12.2 ± 3.3	10 - 20
pH9	1:16	1:512	18.6 ± 3.7	10 -15
S.200 Peak I	1:16	1:32000	6.8 ± 1.8	-
HPLC fraction h (PK I)	1:16	1:32000	6.5 ± 1.6	-
P.yoelii lysate	1:32	1:65000	8.9 ± 2.6	-
unvaccinated control	1:8	1:128	-	7-10

Only groups vaccinated with the strongest antigens (IEF pH4, S.200 Peak I and HPLC fraction h) were tested on day 7 as their infections were almost resolved; all had titres of 1:32000. Animals in the other groups had high parasitaemias (20-30%), at this stage and were therefore not tested to avoid possible distress.

The most protective antigens induced high antibody titres on day 10. The data shown in Table 4.7 were taken from animals that appeared in a reasonably fit state of health on day 10. Antibody titres of the groups vaccinated with the pH5 to pH9 fractions were obtained from animals that appeared in a fit state of health. Their antibody titres varied in accordance with their parasitaemias; animals with parasitaemias above 50% had antibody titres of 1:1024 or less on day 10, but the few that eventually recovered (day 25-30) developed antibody titres of 1:32000 on recovery. In the groups vaccinated with the pH6 and 8 fractions there were clear differences in titres between the early and late recovering animals on day 10. Animals in the early recovery group, who were either clear of parasitaemia or who had parasitaemias of less than 10%, developed titres of 1:32000. The titre shown in table 4.7 represents the late recovering group who had parasitaemias of between 30-50%.

Therefore the induction of high antibody levels appeared to be essential for eventual recovery from *P.yoelii* malaria.

4.8 Nature of protective *P. yoelii* antigens

These experiments were carried out in order to determine the protective effect of the protein and lipid components of the main

fractionated antigens. Antigens included in this study were IEF pH4, 6 and 8 from purification procedure I, and S.200 Peak I and HPLC fraction **h** from procedure II. Fractions were enzymatically digested with mixed proteases to denature proteins, or with mixed lipases (immobilized on agarose) to denature the lipid components (described in section 2, Methods and Materials). Protease treated fractions were boiled for 15 minutes to denature the enzymes and dialysed extensively against PBS before use. Lipase treated fractions were microfuged to remove the enzyme-coated beads and dialysed against PBS before use. All fractions were usually diluted 1:2 before enzyme treatment and this was taken into account when final adjustments were made for vaccination. The volumes of all these fractions were adjusted to deliver the same dose of protein that was present in their corresponding untreated fractions.

Protein concentrations of all fractions were checked before vaccination. In all cases pronase treatment reduced the protein concentrations of the fractions by at least 90% and treated preparations usually contained less than 10-20μg/ml of protein; thus the immunizing protein concentrations were negligible (<1μg) after the appropriate volume adjustments were made. Fractionated antigens lost about 10-20% of their protein after lipase treatment. This method of lipase treatment has been shown to inactivate phospholipid molecules of soluble malarial antigens, that are involved in TNF triggering activity (Bate *et al.*, 1992).

Eight experiments were carried out with enzyme treated fractions and a different set of treated fractions were prepared for each experiment in which two to three mice were vaccinated per fraction. The results are

shown in figure 4.8 where each bar represents a group of 10-20 mice. Clearly the protective activity of all the fractionated antigens including the most purified HPLC fraction **h** (derived from S.200 Peak I) depended upon the protein component. Pronase treatment drastically reduced the protective activity of these fractions; the activities of IEF pH6 and 8 fractions and the most powerfully protective HPLC fraction **h** (from S.200 Peak I) were totally abolished. The lysate, IEF pH4 and to some extent S.200 Peak I, may have contained some weakly protective activity that was attributable to carbohydrate or lipid moieties; or it may be that proteolysis was not complete. Lipase treatment on the other hand did not alter the protective response in most cases. The group vaccinated with the lysate showed a slight reduction which was due to the death of one mouse. In the group vaccinated with lipase treated HPLC fraction **h** about 66% of the vaccinated animals recovered late but the overall protection level was 100%. The loss of protein after enzyme treatment probably affected this fraction more than the others as its initial protein concentration was significantly lower. The protective activity of the IEF fractions and S.200 Peak I were unaffected by the lipase treatment.

Two experiments were carried out to show the specificity of the enzyme treatment. A group of three mice were vaccinated with fractions that were treated with unrelated enzymes such as ribonuclease (RNase) or deoxyribonuclease (DNase), however these enzyme treatments did not alter the protective effect of the fractions (data not shown), thereby confirming the specificity of the pronase and lipase treatments.

Therefore the major protective effect of these antigens appeared to be specifically due to the presence of protein molecules.

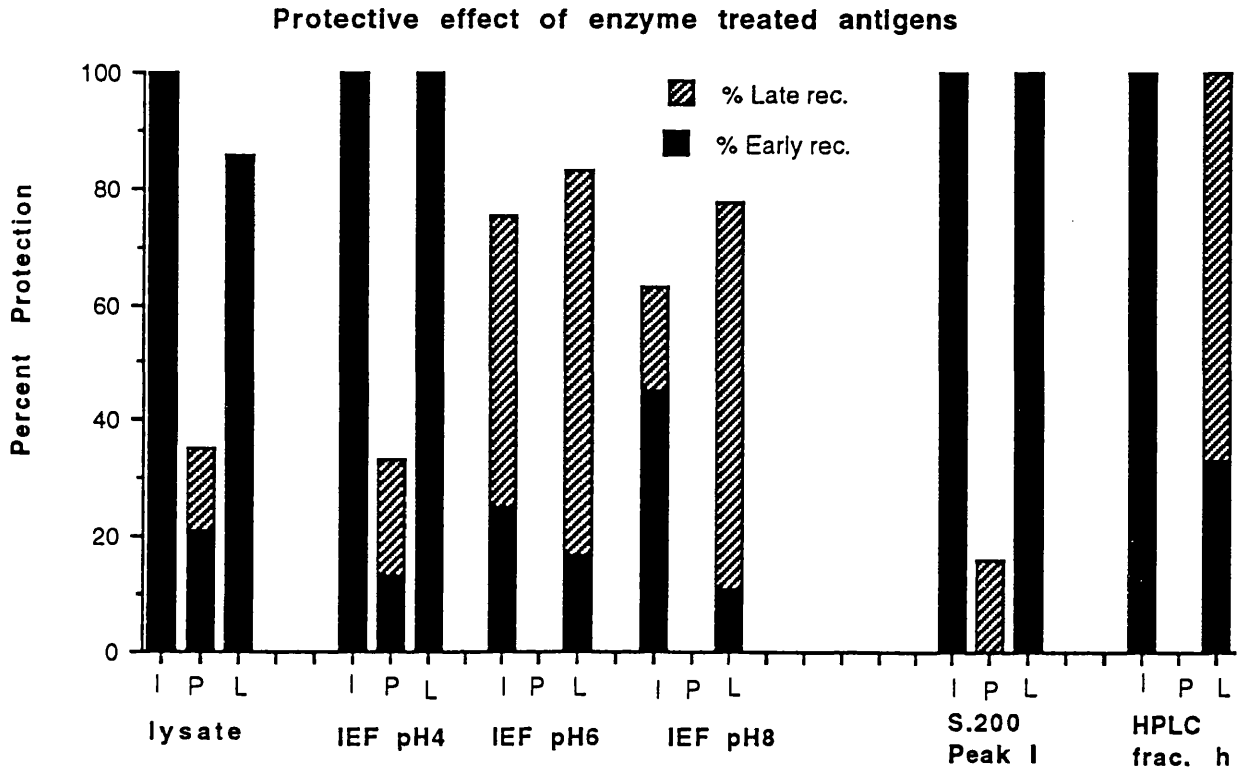


Figure 4.8 Protective effect of intact or enzyme treated antigens pooled from 8 separate experiments.

Mice were vaccinated i.p. with two doses of 10μg protein plus 25μg saponin as adjuvant. Each column represents early or late recovery after vaccination with the appropriately treated antigen. Enzyme treated fractions were adjusted to their original volumes for vaccination so that they contained the equivalent dosage of protein that was used for vaccinating with the corresponding untreated fractions.

Recipients vaccinated with HPLC fraction (h) always received between 1-5μg protein due to its low yield and for this reason only four experiments were done with this fraction.

KEY I Intact (untreated) antigen

P Pronase treated antigen

L Lipase treated antigen

A summary of the immunological responses and nature of the protective *P.yoelii* antigens is shown below in Table 4.8

Table 4.8 Summary of T cell helper and DTH activity, antibody levels and nature of protective activity of fractionated antigens

Fractionated antigen	T cell help (% lysate control = 100%) ±SE	DTH ±SE	Antibody titre day 10	Protective component
IEF pH4	99.2 ± 7.12	119.0 ± 17.3	1: 65000	Protein / lipid
IEF pH6	65.0 ± 13.03	58.0 ± 16.5	1: 4096	"
IEF pH8	67.0 ± 16.75	49.0 ± 25.3	1: 4096	"
HPLC frac. h(IEFpH6)	87.3 ± 22.5	not tested	not tested	"
S.200 Peak I	110 ± 11.5	58.7 ± 15.2	1: 32000	Protein / lipid
HPLC frac. h(S.200 Peak I)	119 ± 18.1	33.5 ± 1.2	1: 32000	"

4.9 Discussion

There are four clear conclusions from the studies described in this section.

1) The most consistently protective antigens appear to stimulate T helper cells. This has been confirmed with two highly purified fractions, HPLC fraction **h** derived from S.200 Peak I, and HPLC fraction **h** derived from IEF pH6. This type of $CD4^+$ T cell is probably equivalent to the T_H2 sub-class of T helper cells (Mossman & Moore, 1991).

2) T cells responsible for DTH (equivalent to T_H1 sub-class) are also stimulated by these fractions, but to a lesser extent in some cases.

3) Eventual recovery from infection appears to be associated with the development of high antibody titres.

4) The majority of the protective activity of the fractionated antigens appears to be mediated by their protein moiety.

T- cell helper and DTH activity, and antibody responses

$CD4^+$ T cells in mice were recently proposed to fall into two functional subsets, the T_H1 and T_H2 cells (discussed in section 1), the former being responsible for inflammatory (cell-mediated) reactions while the latter provide help for antibody synthesis. T helper cells detected by the TNP assays used in this study would accordingly be classified as T_H2 helper cells and it would be of further interest to confirm that the cytokine secretion patterns of mice vaccinated with fractionated antigens were indeed characteristic of this subset of helper T cells. Experiments currently being planned involve culturing spleen cells, from animals injected with various fractionated antigens, in the presence of the priming antigen *in*

vitro and four days later assaying the culture supernatants for the presence of IL-4 or IL-10. Alternatively it should be possible to block T cell priming *in vivo* by treatment with antisera directed against IL-4 or by the administration of IFN-γ which inhibits the proliferation of T_H2 cells responding to IL-2 or IL-4 (Mossmann and Moore, 1991). It should also be possible to enhance T_H2 cell priming by treating with anti-IFN-γ antibody. In terms of T helper cell priming there is no doubt that the most protective fractions including the HPLC purified antigens appear to be the strongest stimulators of T helper cells.

T cells responsible for DTH responses belong classically to the T_H1 subset of $CD4^+$ T cells. In the initial studies with the IEF antigens DTH appeared to correlate with protective activity. The most strongly protective fractions (pH4) induced the highest DTH responses while the pH6 and 8 fractions induced comparatively lower DTH responses which were consistent with their weaker protective effect. However, when the highly purified antigen HPLC fraction **h** and S.200 Peak I (from which it was derived), were tested it was clear that protective activity did not always correlate with DTH. It is therefore important to determine the cytokine secretion patterns of animals vaccinated with these antigens in order to confirm their apparent predilection for T_H2 rather than T_H1 cells.

High antibody levels were always associated with the final elimination of parasitaemia even in animals vaccinated with the less protective fractions. Total antibody levels (IgG and IgM classes) were measured in our immunofluorescence assay, but it is quite likely that the majority were IgG antibodies as we have previously shown that in animals vaccinated with

the formalin-fixed vaccine (which induces a similar T-helper cell response) there was a massive increase in the IgG sub-classes following infection (Playfair and de Souza, 1979). This suggests that T_H2 cells may be important at this stage of the infection where antibody may be involved in blocking parasite re-invasion of red blood cells (Butcher & Cohen,1972), as well as for coating killed parasites for their eventual removal by the reticuloendothelial system.

The results can be explained **speculatively** in terms of the cytokine secretion patterns for each type of fractionated antigen.

***P.yoelii* lysate and IEF pH4** - These antigens induced 100% early type protection which correlated with excellent T helper cell stimulation and priming for T cells responsible for DTH. Identical responses were induced with a crude formalin-fixed preparation of *P.yoelii* parasites (Cottrell, Playfair and de Souza, 1978). It is very likely that these antigens induce an initial T_H1 cell response which leads to the secretion of IL-2, IFN-γ and TNF-β which mediate DTH and cytotoxicity. The response probably lasts for seven days or less because antibody titres begin to rise on day 7 and reach peak levels by day 10. This indicates that by day 7 a T_H2 cell response has already been established. Therefore these fractions appear to be capable of sequentially stimulating both subsets of $CD4^+$ T cells probably by the different antigens present within them. This will be clarified by determining the T cell subset response induced by single-component FPLC purified pH4 antigens.

IEF pH6 and 8 fractions - These fractions induced a comparatively weaker DTH and T helper cell response but in terms of the T cell subset activation

they probably behave in a similar manner to the lysate and pH4 fraction. The weak responses induced by these antigens may be due to reduced levels of cytokine secretion. Determination of cytokine levels in mice vaccinated with these antigens will be required to confirm this.

S.200 Peak I and HPLC fraction (h) - These antigens were amongst the most protective of the fractionated antigens, inducing 100% protection of the early type. However, whilst they were excellent at T helper cell priming they were not consistently effective at inducing good DTH activity. The protective effect of these antigens might be due to a combination of a rapid T_H1 response which is soon replaced by a T_H2 response, leading to a very strong immune response in favour of antibody. The relatively weaker DTH responses may be explained by the inhibition of the T_H1 cells by IL-4 or IL-10 secreted by T_H2 cells. The fact that at least some mice showed a good DTH responses, might indicate that there is an initial but transient T_H1 response which cannot establish itself for any length of time due to the inhibitory effects of the cytokines IL-4 and IL-10 secreted by the strongly activated T_H2 subset. IL-10 is thought to inhibit cytokine release from T_H1 cells (Moore, *et al* 1990). This hypothesis can be tested by examining the cytokine secretion responses immediately after vaccination with these antigens, for example, from days 1 to 5 after the final vaccination dose.

It is interesting that the correlation between T cell stimulation and protection was stronger with antigens from the IEF pH4, S.200 Peak I and HPLC fraction **h**, than with those from IEF pH6 and 8. The induction of DTH by the IEF fractions correlated closely with the priming of T helper cells, however DTH did not correlate any better with protection than T help did. In fact our most protective antigen HPLC fraction **h** induced a

weak DTH response. Immune T or B cells can be used to passively transfer immunity against *P.yoelii* (Fahey and Spitalny, 1986) which is consistent with the findings of protection without strong T cell help in some fractions. Therefore antigens of the pH6 and 8 fractions may activate B cells or T cells alone, while the pH4 antigens might act by stimulating T cells for both antibody and DTH responses such as macrophage activation and activation of non-specific cells to produce factors that are toxic to the parasite (Playfair, Dockrell and Taverne, 1985). HPLC fraction **h** may act by activating T helper cells for antibody production and it may also be an efficient activator of B cell priming. We do not yet know whether this antigen has both T- and B- stimulating regions present on its surface.

Taken together, it appears that most of the fractionated antigens can activate both T_H1 and T_H2 cells; the former being activated first and the latter second. Antigens from S.200 Peak I and HPLC fraction **h** were strong activators of T_H2 cells, but possibly weaker activators of T_H1 cells. This preferential activation of T_H cells is shown in figure 4.9 where the relationship between DTH and T-helper activity was analysed for each set of fractions. The data used in this figure were obtained from experiments in which the same fractions were studied for both these activities. Clearly the IEF pH4 antigens can activate both T_H1 and T_H2 cells sequentially, perhaps by subset-specific cytokine regulation. The induction of T_H subsets is probably followed by cytokine regulation; IFN-γ inhibiting the proliferation of T_H2 cells while IL-4 would inhibit IL-2 receptor expression and IFN-γ production. The pH6 antigens show a similar trend while the pH8 antigens do so occasionally. In some experiments the pH8 antigens stimulated either one sub-set or the other and not both. These variable T_H

cell responses may be due to the variable antigenic composition of different batches of the pH8 fraction. S.200 Peak I and HPLC fraction **h** appear to activate mainly T_H2 cells. However, in two experiments with S.200 Peak I and in one with HPLC fraction **h**, there was a good correlation between the two types of T cell activity. This is consistent with my earlier suggestion that a strong T_H1 response may have occurred transiently, possibly shortly after the booster dose of antigen; it may also be a convenient explanation for the lower DTH responses of mice vaccinated with these fractions (as shown in figure 4.9).

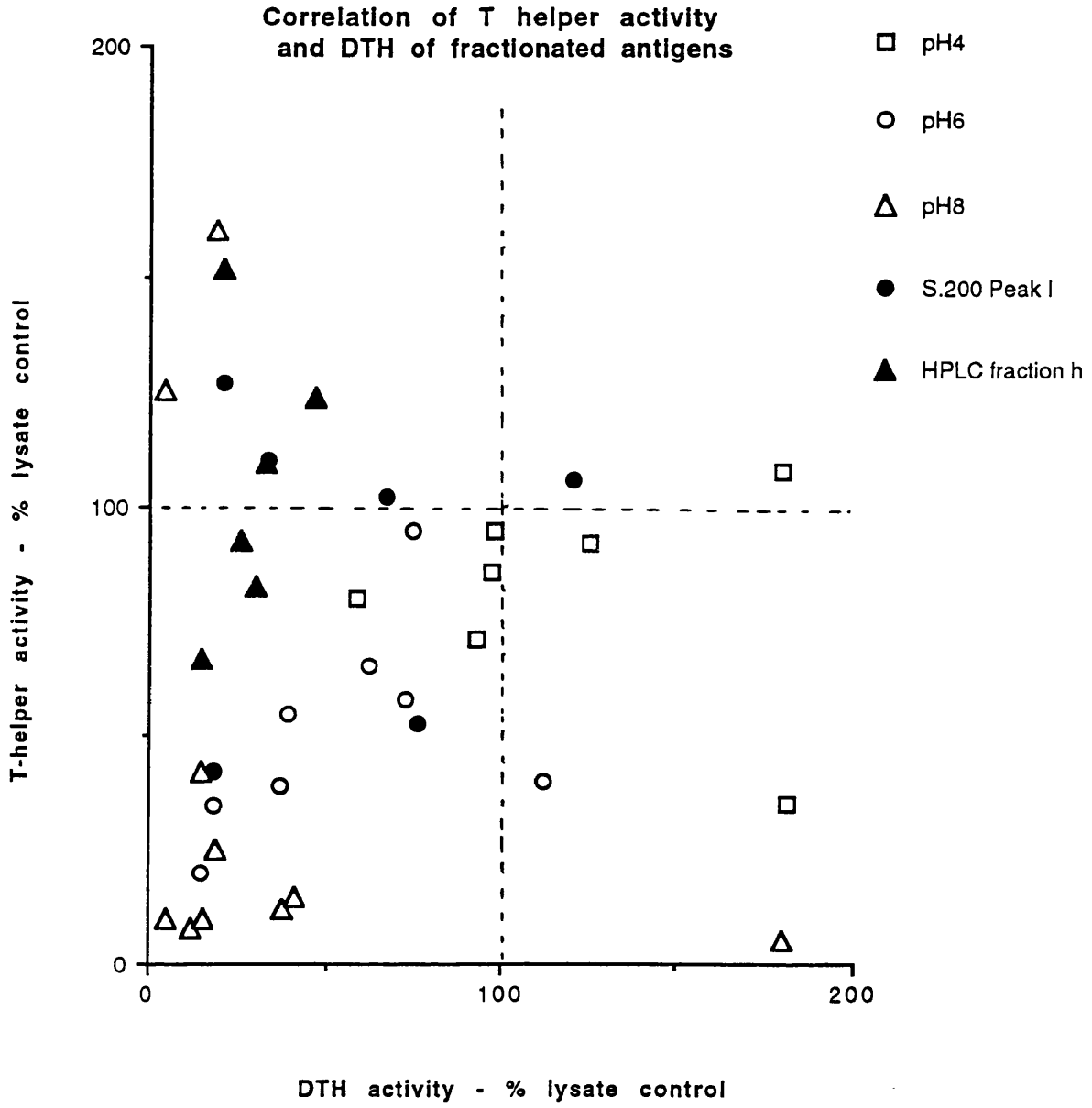


Figure 4.9 Correlation of T-helper cell activity with DTH of fractionated antigens. Each point represents a group of 2-3 vaccinated mice tested for T-cell priming (as for fig.4.2) and another group of mice vaccinated with the same antigen and tested for DTH (as for figs. 4.5 and 4.6). Both types of T cell activity are expressed as a percentage of the control *P. yoelii* lysate.

Macrophage activation by fractionated antigens (Dr. C. Bate)

Only a few macrophage activation experiments were possible therefore the data are not shown. Briefly, all the fractionated antigens were able to activate macrophages to produce TNF *in vitro*. However, the triggering activity was never as strong as that of the exoantigens (Taverne, Bate and Playfair, 1990) and protective activity did not appear to correlate with TNF triggering ability. Whilst the protective IEF fractions induced reasonable TNF triggering activity, some non-protective fractions from the pH5 and 7 and 9 regions were equally effective. Nevertheless it was interesting that HPLC fraction **h** was poor at inducing this activity.

The relatively weak TNF triggering ability of the *P.yoelii* fractionated antigens may be due to their high content of membrane antigens consisting mainly of proteins. The exact origin of the exoantigens is not known but their active TNF triggering components are phospholipid molecules (Bate *et al.*, 1992).

Protective effect of enzyme treated fractions

These experiments were carried out to determine the nature of the protective fractionated antigens. It was clear that the protein component of all the antigens tested were the most important for the induction of protective immunity. The non-protein portions of the lysate and IEF pH4 contained some weak activity and that of S.200 Peak I was even weaker. This significantly poor protective effect may have been caused by trace quantities of proteins avidly bound to lipid or carbohydrate moieties, or it may have been entirely due to the effect of the latter moieties.

These experiments show that protective activity induced by the fractionated antigens of *P.yoelii* is mediated mainly by proteins. Thus these antigens are entirely different from the exoantigens which owe their activity to the presence of phospholipid molecules.

5.0 CONCLUSIONS AND DISCUSSION

These studies have shown that a large number of both protective and non-protective antigens can be isolated by physical separation techniques. Whilst protective antigens have been the main area of interest here, the role of non-protective antigens is of equal importance particularly with regard to pathology, toxicity and immunosuppression during malaria. A similar approach may be used to identify these antigens.

It has also been shown that some highly protective antigens can stimulate both helper T cells and T cells responsible for DTH activity, while others apparently stimulate the latter type of T cell activity to a lesser extent.

5.1 Identification of protective *P. yoelii* antigens - advantages and disadvantages of physical separation techniques

The two-step procedure, IEF or S.200 followed by HPLC or FPLC, offers yet another very useful technique for the identification of protective malaria antigens. A detergent-soluble extract of membrane antigens is pretreated to remove complexes and excess detergent and finally purified by HPLC. Clearly there are advantages and disadvantages of this approach. The advantages are : a) Both protective and non-protective antigens can be extracted for further study.

b) Unlike recombinant DNA technology, this method is not restricted to the identification of protein antigens.

c) A panel of protective antigens can be obtained in an unbiased manner without prior selection based on their reactivity with immune sera or T cell clones.

d) These antigens can be tested for various types of activity associated with protection, and amino acid sequences of the interesting protein antigens can finally be determined.

e) The role of the non-protective antigens can also be studied, in particular their possible involvement in the pathology of malaria. This can be tested using a toxicity assay, for example in D-galactosamine (D-gal) sensitized mice as described by Bate and colleagues (1989). The assumption being made here is that some of the non-protective antigens will induce TNF secretion *in vivo* and therefore injection of these antigens into D-gal pretreated animals will lead to their death.

f) The role of non-protective antigens in anaemia, which is a major cause of infant mortality in the tropics (Greenwood, *et al* 1991), and hypoglycaemia can also be studied.

The disadvantages of this approach of antigen separation include :

a) Yields of the purified antigen are very low and several HPLC or FPLC experiments are required to obtain reasonable quantities of protein, particularly for SDS-PAGE analysis. Perhaps the use of larger purification columns will overcome this problem.

b) Membrane proteins tend to be hydrophobic in nature and can therefore only be extracted in detergent-containing buffers. Once the detergent is removed, for example by IEF or S.200, the proteins tend to precipitate and become difficult to manipulate. This affects only the fractions with the highest protein concentrations. Whilst the protective effect of the precipitated protein remains unaffected, their further purification by HPLC or FPLC becomes difficult, and re-dissolving the precipitate in SDS is not always satisfactory. The pH4

proteins re-dissolved in SDS behave inconsistently on the HPLC column, probably due their high protein concentration which might result in variations in the size of the micelles or the formation of aggregates; SDS can lead to physical enlargement of polypeptide chains to produce large aggregates. Although this may affect the reverse-phase HPLC purification, the molecular weight-based FPLC separation appears to be unaffected.

Undoubtedly the advantages of this approach outweigh the disadvantages which are not really a cause for concern. The two main disadvantages, low protein yield and precipitation of the IEF pH4 proteins, were dealt with appropriately. Precipitation was overcome by re-dissolving in SDS, and the low protein yields did not matter in practice as sufficient quantities of the fractions were available for vaccination studies. When larger amounts of protein were required, for example for SDS-PAGE and Western blotting analyses, fractions from several chromatograms were pooled.

5. 2 TX-100 as a detergent

We previously showed that *P. yoelii* lysates prepared from TX-100 produced the best protective effect in vaccinated mice (Playfair and de Souza, 1986). The larger micellar weight (90kD) of TX-100 was probably the main factor that distinguished it from the other detergents tested, for example SDS (18kD) and deoxycholate (1.7- 4.2kD). However, artifacts in molecular weight estimation are likely. For example Peak I from the S.200 fractionation was eluted at the void volume of the column at an apparent molecular weight of >230kD, but its molecular weight estimated by SDS-PAGE showed only one high molecular weight band at M_r 140kD.

Trace quantities of TX-100 are present even in the IEF fractions where they are detected as large peaks in the HPLC purification chromatographs; fraction f in all experiments was contaminated with TX-100. Although this fraction never induced 100% protection in vaccinated mice it was considered possible that TX-100 might have acted as an adjuvant. This was tested by experiments in which *P. yoelii* lysates prepared by repeated cycles of freezing and thawing in an extraction buffer lacking in TX-100 were used for vaccination. Lysates prepared by this method were not as effective as the TX-100 lysates at the standard vaccination dose of 10μg protein; only 30% of vaccinated animals recovered late (data not shown). However, when 0.05% TX-100 (concentration of detergent in a typical lysate) was added to this type of extract, 90% of the vaccinated animals recovered late. These experiments were only preliminary and need to be confirmed, but it can be concluded firstly, that TX-100 probably does act as a weak adjuvant, and second, that it is essential for the extraction of highly immunogenic membrane components.

Another aspect of TX-100 interference is its absorbance at 280nm (A_{280}) which tends to artificially increase the peak size of fractions on the elution chromatographs leading to inaccurate protein estimation based on the A_{280}. This was noticeable with all chromatographic procedures used, but was of little significance in these studies as the protein concentration of all the fractions were estimated by the Bio-Rad protein assay before use in vaccination studies.

Triton X-100 seems to be an ideal detergent for extraction of *Plasmodium* membrane proteins. At the concentration used in these studies (0.5% w/v)

it solubilizes the membrane lipids into mixed micelles and saturates the binding capacity of proteins that bind detergent, and more importantly it interacts predominantly with those proteins which are bound to membrane lipids by hydrophobic interactions (Helenius and Simons, 1975).

5. 3 T cell assays

Although the T-helper cell and DTH assays used in these studies were very sensitive they should be replaced by the more recently developed cytokine assays which specifically assess the status of the T_H1 and T_H2 subsets of T cells. This would be advantageous as direct correlations could be made between protective activity and cytokine responsiveness.

Less encouraging results were obtained with T cell proliferation assays *in vitro* (collaboration with L. Fergusson and A. Alavi). Proliferative responses to the IEF fractions were extremely variable and did not always correlate with their protective activity. We intend repeating these experiments with our protective HPLC and FPLC purified antigens.

5. 4 Blood-stage antigens identified by other laboratories

Various approaches have been used to identify blood-stage *Plasmodium* antigens (discussed in section 1.0). Two of the more commonly used procedures include DNA technology and affinity purification chromatography using monoclonal antibodies. Several antigens have been identified by these procedures and at least three, MSA-1, MSA-2 and RESA have been considered as potential vaccine candidates. The effectiveness of MSA-1 as a vaccine has been proved in various host-*Plasmodium* systems including in mice (Holder & Freeman, 1981) and in monkeys, where

partial protection was obtained (Perrin *et al.*, 1985), and in humans (Patarroyo *et al.*, 1988). Our approach of antigen identification has been somewhat similar to that of Patarroyo and colleagues, the main difference being that we have used physical separation techniques for identifying our antigens rather than reactivity with immune sera. Patarroyo and colleagues isolated a panel of antigens on the basis of their reactivity with immune serum in Western blot assays; twenty-two different proteins were identified and tested in monkeys (Patarroyo *et al.*, 1987). The protective antigens were chemically synthesized and first tested in monkeys and then in a small trial in human volunteers. This synthetic peptide incorporating three molecules (83.1, 55.1 and 35.1) based on the structure of the 83kD, 55kD and 35kD merozoite-specific proteins of the *P. falciparum* MSA-1 molecule, showed promising results in a human clinical trial (Patarroyo *et al.*, 1988). Two other apparently successful large-scale field trials have been carried out by Patarroyo and colleagues (data not officially published). In one of these trials in Colombia, the exact results of which are not known, an encouraging 85% success rate was reported. Although this work has been widely criticised for lack of appropriate placebo controls it has nevertheless proved the feasibility and safety of synthetic vaccines against malaria.

The *P. yoelii* (YM) MSA-1 molecule is processed into seven fragments of molecular weight 197kD, 160kD, 151kD, 126kD, 90kD, 56kD and 28kD (Holder and Freeman, 1981). Our most protective HPLC purified antigen fraction **h** so far does not appear to bear any resemblance to MSA-1. An important difference is the type of immunological response stimulated by these antigens. Whilst both MSA-1 and fraction **h** are potent inducers of parasite-specific T-helper cells, potent DTH activity is exhibited only by

MSA-1 (Playfair, de Souza, Freeman and Holder, 1985). Furthermore preliminary data suggest that the partial N-terminal amino acid sequence of fraction **h** (the first 10 amino acids) and that of MSA-1 and its fragments are different. These results are currently being confirmed.

In previous studies (collaboration with J. Keen) we showed that antisera raised against the IEF pH4, 6 and 8 fractions immunoprecipitated a 230kD protein from preparations of ^{35}S-methionine labelled blood-stage *P. yoelii* proteins, which suggested that the fractions contain fragments of the MSA-1 molecule. However, when the 230kD molecule was depleted from the IEF fractions they still retained their protective activity, implying that other lower molecular weight protective antigens are present in these fractions (Keen, 1989). These findings suggest that there are other protective antigens present in the TX-100 lysate which need to be identified, particularly in view of the variable nature of the MSA-1 molecule.

Another protective *P. yoelii* antigen identified by Holder and Freeman (1981) is the 235kD rhoptry antigen. In terms of the strength of protection this antigen is not as strong as MSA-1. The possible relationships between this antigen and the ones we have identified are not yet known, but it seems unlikely to be related to HPLC fraction **h** since weight for weight it is a less powerful inducer of protective immunity.

Identification of *P. yoelii* antigens by IEF was also attempted by Kironde and colleagues (1991), who have confirmed our earlier finding that the pH4 region was the most protective (de Souza and Playfair, 1988). However, there were basic differences in approach. First, they used the *P. yoelii*

nigeriensis strain of parasite which is known to be self-resolving in about 50% of the infected animals, although in their experiments it was lethal in Balb/c mice. Second, when their immunizing lysates were injected with either Complete Freund's adjuvant (CFA) or saponin it was just as effective; in our experiments saponin was more effective than CFA. Third, their lysate in combination with either adjuvant did not induce total immunity in vaccinated mice; prolongation of survival (partial protection) was their best achievement. Finally, their main finding was that sera from convalescent animals reacted with five protein bands of the lysate on a Western blot. These were of molecular weight 150kD, 84kD, 40kD, 19kD and 16kD. Sera from partially protected animals did not recognize any of these bands. In our studies 10 bands of the lysate, of molecular weight ranging between 140kD and 30kD, reacted with immune serum. In addition, antibody to HPLC fraction **h** (from Peak I) reacted with several bands on a Western blot of Peak I. Direct comparisons between our antibody-reactive bands and those of the above study cannot be made as different strains of parasite were used.

Several *P. falciparum* antigens have been identified to date, but only a few have been tested as vaccines in the monkey model of malaria, which is the only experimental system available for testing vaccines against this strain of parasite. The drawbacks to this system are the high costs involved in the purchase and maintenance of monkey colonies. The mouse model of malaria still offers the best possible system for testing large numbers of animals with a wide range of antigens more cheaply. Once we have confirmed the amino acid sequences of our protective antigens it should be possible to compare our antigens with the *P. falciparum* antigens identified to date. Serological cross-reactions between the MSA-1 molecule of human

and rodent malaria parasites have been demonstrated (Holder, Freeman and Newbold, 1983), and this indicates that they have related amino acid sequences. Assuming that the amino acid sequences of our *P. yoelii* protective antigens are similarly related, the identification of new protective *P. falciparum* analogues can be reasonably expected.

5. 5 Carbohydrate and lipid antigens as vaccines

In our studies the non-protein moiety of the fractionated antigens was never as effective as the protein moiety in inducing protective immunity in vaccinated mice. Nevertheless, phospholipid-containing exoantigens induced partial protection in our mice (Playfair, Taverne, Bate and de Souza, 1990), which was exclusively of the late type. This is the major difference between the exoantigens and fractionated antigens of the lysate. It is quite possible that protein antigens are responsible for the early type protective response, while the lipid or carbohydrate antigens which are present in the exoantigens induce mainly late type protection. Ramasamy and Reese (1985) showed that removal of carbohydrate significantly reduced the antigenicity (binding to immune sera in ELISA assays) of *P. falciparum* blood-stage antigens. However, vaccination with carbohydrate antigens from the parasite surface membrane has not yet been attempted.

Whilst non-protein moieties may be important in stabilizing the tertiary or quaternary structure of parasite proteins, they are certainly involved in the anchoring of proteins to the plasma membrane. A well known membrane anchor is the glycosyl phosphatidylinositol (GPI) anchor which covalently links proteins such as MSA-1 to the merozoite membrane (Haldar, Ferguson & Cross, 1985). GPI anchors are also involved in binding the trypanosome variant surface glycoprotein to the parasite's

surface membrane. It is possible that the phospholipid antigens referred to above are derived from the GPI anchor of a merozoite surface molecule.

5. 6 Epitope selection for malaria vaccines

Our experiments suggest that an effective vaccine should probably contain elements which stimulate both the parasite-specific T helper cells for antibody production and the T cells responsible for the induction of cell-mediated immunity. Priming with T cell epitopes is essential for boosting the immune response during a natural infection. This is evident from the studies of animals vaccinated with protective fractions; they always had elevated antibody levels at the time of clearing their infection.

There is increasing evidence that T helper cell subsets play an important role in experimental malaria. In *P.chabaudi* malaria there is an initial T_H1 cell response, followed later on by a T_H2 cell response which is thought to be involved in the final clearing of parasitaemia (Langhorne, 1989). In *P.berghei* malaria, T_H1 responses appear to be essential; Waki and colleagues (1992) have recently shown that an anti-IFN-γ antibody suppressed immunity against an irradiation-attenuated strain (Pb XAT) of *P.berghei* NK65 (Pb NK65). This treatment also suppressed immunity in Pb XAT vaccinated animals that were later challenged with the lethal Pb NK65 parasite. Treatment with an anti-IL4 antibody did not affect recovery, suggesting the apparent lack of involvement of T_H2 cells. However, immune serum was effective in overcoming a Pb XAT infection. Thus whilst both subsets of T_H cell are probably required, the T_H1 response appears to be of critical importance. This result is in agreement with our earlier finding that a strong DTH response was essential for immunity to

P.berghei (Cottrell, Playfair and de Souza, 1978).

Assuming that the T-helper cell and DTH responses detected in this project, are equivalent to the T_H2 and T_H1 T-cell subset responses respectively, these results suggest that both subsets of T_H cell are involved in immunity against *P. yoelii* malaria. The IEF pH4 antigens stimulate both types of T_H cell but the S.200 Peak I and its HPLC purified antigen fraction **h** appear to stimulate stronger T_H2 responses. As suggested previously there may be an early but transient T_H1 response which is rapidly replaced by a T_H2 response. Fraction **h** may also be an excellent antigen because it only transiently stimulates T_H1 cells. Excessive stimulation of this subset may result in harmful responses leading to pathogenesis; this is supported by recent studies on the role of T cells in *P.berghei* in which it was shown that IFN-γ produced by $CD8^+$ T cells could stimulate TNF-α production by macrophages in the liver (Waki *et al.*, 1992) Clearly the various antigens of *P. yoelii* are capable of inducing different T_H cell responses.

Epitopes that stimulate humoral immunity alone are not likely to be effective against the blood-stage infection. This is evident from the recent vaccination trials in monkeys. *Aotus* monkeys immunized with the 190L polypeptide (from the 80kD molecule) of *P. falciparum* MSA-1 were only partially protected against a live challenge infection; however addition of the universal T cell epitope CS.T3 (from the circumsporozoite protein of *P. falciparum*) protected three out of four vaccinated animals (Herrera *et al.*, 1992). Antibody levels against the parasites and the immunizing antigens did not correlate with protection, nor did the ability of immune monkey sera to block the *in vitro* invasion of parasites. The strongest

correlate of protection was elevated IFN-γ levels. Therefore epitopes that stimulate T_H1 cells (for cell-mediated immunity) are a necessary requirement in this model. Epitopes that stimulate T_H2 cells will also be required to provide help to B cells for antibody production, which seems to be necessary for the final clearance of parasitaemia. The most protective fraction from this project, HPLC fraction **h** appears to stimulate a predominantly potent T_H2 response for antibody production; this contrasts with the 190L polypeptide of Herrera and colleagues (1992), whose protective effect does not appear to correlate with antibody. Antibody isotype may be critical in this case. Recent evidence suggests that antimalarial antibodies of the IgG2a isotype play a dominant role in controlling a *P. yoelii* parasitaemia (White, Evans and Taylor, 1991), although all four IgG isotypes recognize the same set of *P. yoelii* antigens. Gamma interferon produced by T_H1 cells enhances IgG2a production and suppresses IgG1, while IL-4 produced by T_H2 cells enhances IgG1 synthesis and suppresses other IgG isotypes (Snapper and Paul, 1987). In humans, cytophilic immunoglobulins IgG1 and IgG3, which predominate in individuals protected against *P. falciparum*, were found to be strong inducers of antibody-dependent cytotoxic (phagocytic) mechanisms *in vitro* (Bouharoun-Tayoun and Druilhe, 1992). It seems possible therefore, that HPLC fraction **h** may stimulate a similar immune response. It is therefore important to determine the antibody subclasses induced by vaccination with fraction **h**.

Taking all the above findings together, it seems that several factors will have to be considered for the design of an efficient blood-stage vaccine against malaria. Epitopes will have to be selected carefully to generate the

desirable immune response. The work of this thesis has shown that epitopes that stimulate both cell-mediated immunity and humoral immunity, as in the case of the IEF pH4 antigens, should be effective. It has also been shown that some protective antigens, such as HPLC fraction **h**, are capable of inducing a powerful antibody mediated response in very low doses, and possibly an antibody-dependent phagocytic response. Further studies are necessary to confirm this. In either case it appears that a cellular response, whether T-cell-mediated or phagocytic, needs to be coupled with a humoral response. It is also important to identify and exclude antigens that induce damaging responses. Activation of the appropriate T_H cell response will be crucial in determining whether protective or suppressive responses are generated. For example, protective immunity in *Leishmania* is associated with the activation of a T_H1 cell response, while the T_H2 response is involved in disease progression (Locksley and Scott, 1991). T cell suppression is also a feature of malaria infections (Jayawardena *et al.*, 1978). Failure to receive the appropriate T cell activation signal or the actual elimination of a crucial population of T cells by circulating anti-lymphocyte autoantibodies (de Souza and Playfair, 1983) may be responsible. It therefore appears that the ability of the parasite to suppress or modify effector T cell responses is vital to its survival. Identification of the antigens that activate and suppress T cell activity in malaria and their relevance to the development of protective immunity is vital before effective immunization is to be achieved.

5. 7 Vaccination strategy

The studies of Patarroyo and colleagues (discussed previously) have already proved the usefulness of synthetic peptide vaccines in combination with aluminium hydroxide as an adjuvant. More importantly, these

studies also showed that a combination of at least three peptides were required for effective vaccination. This approach, of using a combination of peptides, was recently used for testing recombinant vaccines in *Aotus* monkeys (Knapp *et al.*, 1992). Recombinant vaccines consisting of individual blood-stage antigens have been largely ineffective as vaccines (Holder, Freeman & Nicholls, 1988; Pye *et al.*, 1991). However, when used in combination, as in the *E. coli*- expressed hybrid proteins containing partial sequences of SERA(P) (serine repeat antigen), HRPII and MSA-1, complete protection was achieved in *Aotus* monkeys (Knapp *et al.*, 1992); in these experiments two hybrid proteins, MSA1-SERP-HRPII and SERP-MSA1-HRPII injected with polyalphaolefine as an adjuvant induced convincing protection against *P. falciparum* challenge. These results also suggest that the combined effect of two or three antigens may be necessary for effective vaccination. Although HPLC fraction **h** (S.200 Peak I) appears to be highly effective on its own, synergy between the other less protective HPLC fractions may be necessary to achieve better protection. This is particularly applicable to the partially protective HPLC purified IEF pH8 fractions.

Therefore an ideal blood-stage malaria vaccine, in the form of a synthetic peptide or a recombinant protein, would need to contain a combination of different protective antigens for inducing an effective immune response. A combination of antigens may also be useful in reducing the risk of antigenic variation in response to immunological pressure.

Vaccination against malaria began in the 1970s with an attempt to understand the immunology of the basic host-parasite interaction. By the

early 1980s there was much optimism about malaria vaccines being just round the corner, but sadly even today in the 1990s we are scarcely any nearer to experiencing any results from this optimism. However, with our strengthened understanding of the factors influencing malarial immunity in the 1990s we should be able to design an effective vaccine. Perhaps the four basic immunological requirements for a vaccine proposed by Ada (1990) should be carefully considered: (a) activation of antigen-presenting cells to initiate antigen processing and production of interleukins; (b) activation of both T and B cells to give a high yield of memory cells; (c) generation of T-helper and T-cytotoxic cells to several epitopes, to overcome the variation in the immune response of the population due to major histocompatibility complex polymorphism; and (d) persistence of antigen, probably on dendritic follicular cells in lymphoid tissue where B memory cells are recruited to form antibody-secreting cells that will continue to produce antibody.

Much work is needed to test the large number of antigens of the parasite before a successful vaccine is developed and it is likely that the murine malaria model used in this project will continue to play a significant role in future developments in the vaccination field.

There is no doubt that the task of defeating *Plasmodium* is a challenging one, but it is comforting to know that it is being tackled by the combined efforts of parasitologists, immunologists, biochemists and molecular biologists worldwide.

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BLOOD-STAGE MURINE MALARIA : IDENTIFICATION OF PROTECTIVE ANTIGENS FOR VACCINATION

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Supplement to thesis

The first two papers in this supplement contain preliminary work which led to the studies of this thesis. The third paper contains some data that is included in the thesis. A fourth publication (shown below) is currently in preparation.

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Immunization of Mice against Blood-Stage *Plasmodium yoelii* Malaria with Isoelectrically Focused Antigens and Correlation of Immunity with T-Cell Priming In Vivo

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Mice were immunized with lethal *Plasmodium yoelii* blood-stage malaria antigens that had been fractionated by isoelectric focusing using a variety of Ampholines over the range pH 3 to 10. Fractions were tested for their ability to protect against live challenge and to prime for parasite-specific T-cell help. Both activities exhibited three major peaks in the pH regions 4.5, 6.5, and 8, the pH 4.5 peak being the most consistently protective. There was a significant correlation between protection and T-helper-cell priming, particularly with antigens from the first peak, suggesting that T-cell priming represents an important component of the function of some protective malaria vaccines.

There is considerable interest in the development of a vaccine for human malaria, and a number of potential antigens have been identified (13). However, the evaluation of such antigens inevitably has to rely on indirect evidence, such as recognition by monoclonal antibodies or immune sera or reactivity with particular T-cell populations in vitro, and in the present state of knowledge none of these reactions is guaranteed to predict protection in vivo. The mouse model of malaria allows candidate antigens to be tested in vivo on a large scale for their effects both on antibody and cellular immunity and, most relevant of all, on protection against challenge infection.

We have previously shown that mice could be protected against lethal blood-stage *Plasmodium yoelii* malaria by a crude Formalin-fixed vaccine (9) and by a purified 230,000-M_r merozoite protein (10) and that both preparations also primed the mice for a strong helper T-cell response, as measured by the antitrinitrophenyl (anti-TNP) response to TNP-coated parasites. We subsequently showed that a Triton X-100 lysate of parasitized erythrocytes was also highly protective (7). Two-dimensional gel analysis of biosynthetically labeled *Plasmodium falciparum* proteins has shown that lysates of the asexual blood forms contain several hundred separate polypeptides (5, 14). In the present study we have subjected our *P. yoelii* lysate to isoelectric focusing (IEF) over the pH range 3 to 10 as a first step in identifying the antigens responsible for protection and for T-cell priming. The results suggest that the two activities are closely correlated, at least in the case of some antigens, but that several different antigens may be capable of protection in this model. This approach, which does not depend on the use of monoclonal antibodies or T cells to select potential antigens, should make it possible to identify the most suitable antigens for vaccination without prior knowledge of the immune mechanism by which they act.

MATERIALS AND METHODS

Mice. (BALB/c × C57BL) F1 hybrid mice were bred in our laboratory from parent strains supplied by the National Institute for Medical Research, Mill Hill, London, U.K. Mice of both sexes were used at 10 to 14 weeks of age.

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Parasites. The lethal strain YM of *P. yoelii* was obtained from D. Walliker (University of Edinburgh, Edinburgh, Scotland). This parasite gives rise to a rapid parasitemia in our mice, resulting in deaths by day 9. Parasites were maintained by weekly blood passage. Infections were initiated by intravenous injection of 10^4 parasitized erythrocytes, and parasitemias were counted on Giemsa-stained tail blood films.

Vaccine preparation. A Triton X-100-soluble lysate prepared from heavily parasitized erythrocytes was used as a vaccine and as the starting material for IEF. Details of the procedure were described previously (7). Briefly, a saponin-lysed preparation of infected erythrocytes (more than 90% schizonts) was solubilized in a Triton X-100 extraction buffer for 3 h at 4°C. Insoluble debris was removed by microfuging at 8,000 × *g* for 10 min. The clear supernatant was dialyzed against distilled water for IEF or against phosphate-buffered saline for vaccination.

Vaccination. Mice were given two intraperitoneal doses of 25 μg of protein (lysate or IEF fraction) combined with 25 μg of saponin HP3, 2 weeks apart, followed 3 weeks later by challenge with 10^4 viable parasites (for protection studies). Alternatively, mice were given a single dose of 1 μg with 25 μg of saponin (for T-helper studies).

IEF. Lysates prepared in a sucrose density gradient (5 to 50%) containing 4.5% Ampholine were fractionated in a 110-ml vertical LKB IEF column, following the instructions in the LKB manual (LKB 8100 Ampholine Electrofocusing Column). Usually, 2 ml of lysate, containing 30 to 60 mg of protein, was mixed with 5 ml of Ampholine, and 3 ml of this mixture was added to a dense (50%) or a light (5%) sucrose solution. Each of these solutions was finally made up to 55 ml with distilled water. A 5-to-50% gradient solution was introduced into the focusing column via a gradient former. The Ampholine mixtures used were pH 3 to 10 (broad range) and pH 3.5 to 5, 5 to 8, 7 to 9, and 9 to 11 (narrow ranges). Electrofocusing was carried out for 48 h using a power of 5 W, a current of 10 mA, and a variable voltage. The apparatus was cooled with a supply of coolant pumped through the cooling jackets. On completion, 3-ml fractions (25 to 30) were collected, and their pH was measured. Fractions were then dialyzed against phosphate-buffered saline overnight at 4°C to remove the sucrose and Ampholines, after which the

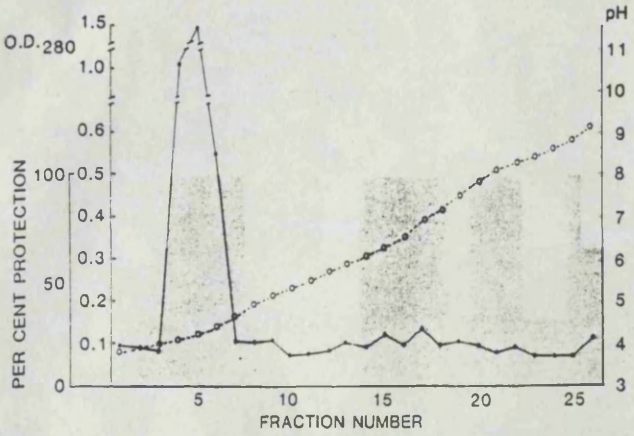

FIG. 1. Protection by IEF fractions, showing the pH (○), protein content (OD_{280}; ●), and protective effect (shaded columns; groups of three mice) for each 3-ml fraction from a single representative experiment using Ampholines in the pH 3 to 10 range. Only the linear part of the pH gradient is shown. Mice were injected twice intraperitoneally with 25 μg of protein plus saponin and challenged with 10^4 viable parasites, as described in the text.

optical density at 280 nm (OD_{280}) was read on an LKB spectrophotometer. Judged by this assay (1-cm quartz cell; 0.1 A_{280} unit equaled 0.1 mg/ml), recovery of protein from the whole procedure was 75 to 80%, while the average yield of protein from 10^{10} erythocytes was 13 mg (nonparasitized) and 105 mg (schizont parasitized). Thus about 88% of the protein in our parasite lysates was of parasite origin. However, we noticed that, while estimates of the protein content in the whole lysate by spectrophotometry agreed with those obtained with the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Hertfordshire WD1 8RP, U.K.), the IEF fractions consistently gave lower readings with the Bio-Rad method, which is based on binding to Coomassie blue (1), the difference being about sixfold. This may reflect differences in the amino acid composition of particular antigens, and we have used the OD_{280} values in calculating the doses used for vaccination, which may therefore be overestimates.

T-helper assay. Parasitized erythrocytes were labeled with TNP as described in detail previously (8). Briefly, parasitized blood with at least 50% parasitemia was washed three times in phosphate-buffered saline. The washed cells were labeled with 2,4,6-trinitrobenzene sulfonic acid at a concentration of 5 mg/ml in 0.28 M cacodylate buffer (pH 6.9). Packed cells (1 ml) were added to 7 ml of 2,4,6-trinitrobenzene sulfonic acid solution with mixing, and the reaction was allowed to continue for 30 min at room temperature. The reaction was stopped by the addition of cold phosphate-buffered saline, and the coated cells were washed until the supernatant was clear of TNP (yellow color). Four days after intravenous injection of 10^5 TNP-coated-*P. yoelii*-parasitized erythrocytes, the mice were killed, and their spleens were tested for anti-TNP immunoglobulin M plaque-forming cells, using the method of Cunningham and Szenberg (3). TNP-labeled sheep erythrocytes were used as detector cells. This assay has been shown to reflect parasite-specific helper T-cell activity (8).

Statistics. The relationship between protection and T-helper priming was tested in two ways, by analysis of variance and by Spearman's rank correlation test. Conventional regression analysis is not appropriate because of the nonlinearity of the data for protection and mortality.

RESULTS

Strength of protection by vaccines. We have previously shown that recovery in mice protected by vaccination with parasite lysates falls into two patterns: parasitemias were cleared by day 8 to 12 or around day 17 to 20. We refer to these as "early" and "late" protection, respectively (7). In each group of three mice, the protection patterns were found to be of seven types: 3E (three early), 2E 1L (two early, one late), 1E 2L, 3L, 2L 1D (two late, one died), 1L 2D, or 3D. We feel this notation offers the best way of quantitating the strength of protection in our model.

Protection by IEF fractions. In preliminary experiments, three separate lysates were focused using the broad-range Ampholines (pH 3 to 10). Figure 1 illustrates a typical experiment, showing that a substantial proportion of the protein focused in the pH 4.1 to 4.6 range, while the protective activity was found not only in this region but also in a second peak around pH 6.5 to 7.2 and a third at pH 7.8 to 8.3. These three major peaks were separated by regions with partial or no protective activity. All three experiments gave closely similar results.

Twelve lysates were then focused, using the narrow-range Ampholines (three times each), and the results (obtained with >500 mice) confirmed the existence of three main protective regions (Fig. 2). Only in the first peak (pH 4.2 to 4.4) were fractions found which always gave 100% protection, but fractions of pH 6.4 to 6.6 and pH 7.8 to 8.0 consistently gave better than 75% protection. In general, early and late protection were found in the same regions, though the pH 4.8 to 5.2 and pH 6.0 to 6.4 fractions seemed to give predominantly the late type of protection. Lysates prepared from nonparasitized erythrocytes yielded only about 12% as much protein as the same number of parasitized cells, though again much of this was in a peak at around pH 4.5 (not shown). These lysates did not have any protective activity, nor did fractions from the pH 4.2 to 4.4 region.

No differences were observed between male and female mice in protection by the lysate or by IEF fractions.

T-helper-cell priming by IEF fractions. Because of slight differences in the response to different batches of TNP-coated-*P. yoelii*-parasitized erythrocytes, the TNP plaque-

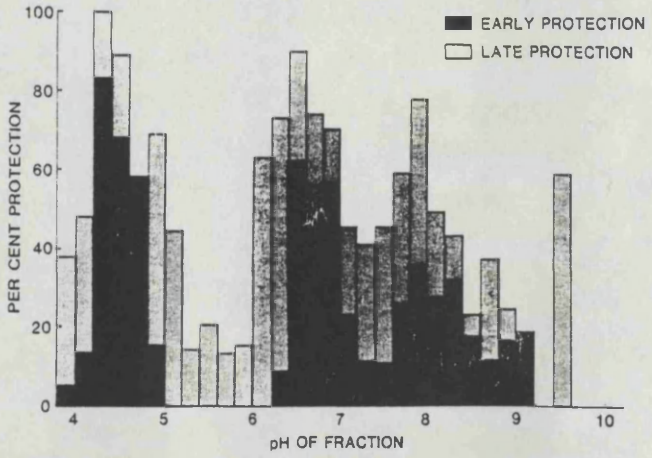

FIG. 2. Protection by IEF fractions, showing the protective effect of fractions pooled from 12 separate experiments using Ampholines in the ranges pH 3.5 to 5, 5 to 8, 7 to 9, and 9 to 11. Vaccination and challenge were as described for Fig. 1. Each column, corresponding to a range of 0.2 pH unit, represents 12 to 30 mice and shows the percent of mice with early (solid) or late (shaded) protection.

forming cell responses in mice vaccinated with IEF fractions in each experiment were always expressed as a percentage of the plaque-forming cell response induced by the lysate from which they were derived. T-cell priming close to or greater than that induced by the lysate was only induced by IEF fractions in the pH regions around 4.3, 6.5, and 8 (Fig. 3). A comparison of Fig. 3 with Fig. 1 and 2 shows that these were also the most protective regions.

Correlation of T-helper priming with protection. In Fig. 4 the strength of protection is plotted against the helper-T-cell priming response for 132 separate IEF fractions. Figure 4A shows the individual points, and Fig. 4B gives the means ± standard error of each group. It is evident that for the better-protected groups (3E to 3L) the two parameters correlated closely, but that in the less well-protected groups, where the late pattern of protection predominated (2L 1D to 3D), this correlation, though highly significant overall, was not so strong.

We noticed that many of the poorly correlated points came from fractions in the pH 6.5 to 8 range, and Fig. 5, which shows the results separately from the three main protective regions, confirms this. With fractions from the most acidic protective regions, the correlation between T-helper priming and protection was excellent, whereas the region from pH 6 to 7.4 showed slightly lower, but still significant, correlations. Evidently some of the protection induced by antigens in this region was not associated with strong T-cell priming, and conversely, some moderately strong T-cell-stimulating antigens were not protective.

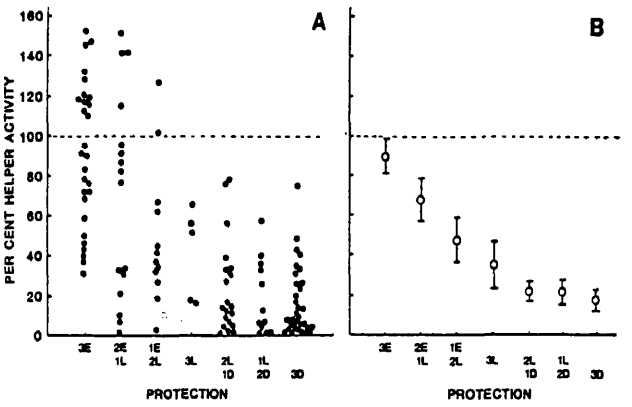

FIG. 4. Correlation of T-helper-cell priming with protection. (A) Each point represents a group of three vaccinated mice tested for T-cell priming (as for Fig. 3) and another group of three mice injected with the same IEF fraction and tested for protection (as for Fig. 1 and 2). (B) Mean ± standard error of each of the seven grades of protection (E, early; L, late; D, died; see text). By analysis of variance, $F = 21.76$ ($P < 0.001$); Spearman's $R = 0.683$ ($P < 0.001$).

DISCUSSION

One clear conclusion from these experiments is that a minimum of three different antigens are able to protect against blood-stage *P. yoelii* malaria. It is unlikely, though possible, that each of the three peaks shown in Fig. 2 contains only one protective antigen, so the total number of protective antigens is probably much higher. It is also possible that all three peaks represent processed or breakdown products of the same antigen. The fact that no fraction is consistently as effective as the same amount of the whole lysate (which always gave 100% early protection) suggests either that there are other important antigens that do not focus on our column or, more likely, that the strongest protection requires the additive or synergistic effect of more than one antigen. We have not, however, been able to demonstrate this convincingly so far between fractions, though it may well be operating within them. But, as already mentioned, the OD_{280} may be overestimating the protein content of our fractions, and it is possible that with more protein the best fractions might become as protective as the lysate. It is difficult to evaluate which of our protective fractions contain the antigens reported by others to be protective, since these are usually classified by molecular weight rather than isoelectric point (13). However, it is noteworthy that a considerable number of the parasite-specific proteins of the simian parasite *Plasmodium knowlesi* focus at around pH 4.5 (12), and soluble, heat-stable antigens of *P. falciparum* also have pI's in this region (2, 18). A soluble, heat-stable antigen has been isolated from *P. yoelii*, but its protective effect was not tested (15).

A second conclusion is that the most consistently protective antigens appear to stimulate helper T cells. The final proof of this will have to wait until our most protective

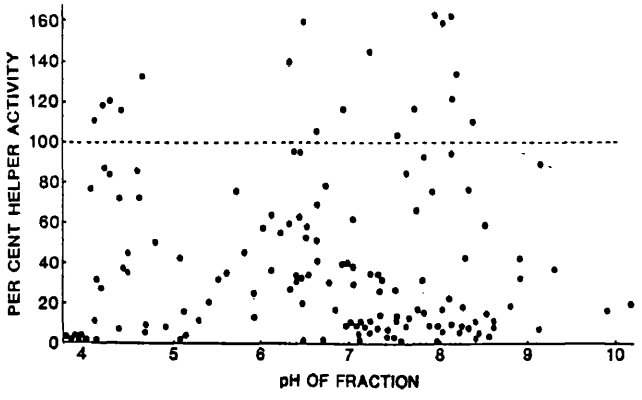

FIG. 3. T-helper-cell priming by IEF fractions. Each point represents the mean of a group of three mice vaccinated with 1 μg of the corresponding IEF fractions plus 25 μg of saponin and challenged with TNP-coated parasites as described in the text. The results are shown for 12 separate experiments, in each of which the mean TNP plaque-forming cell count for each group has been expressed as a percentage of the result from a group of mice given 1 μg of the lysate plus saponin.

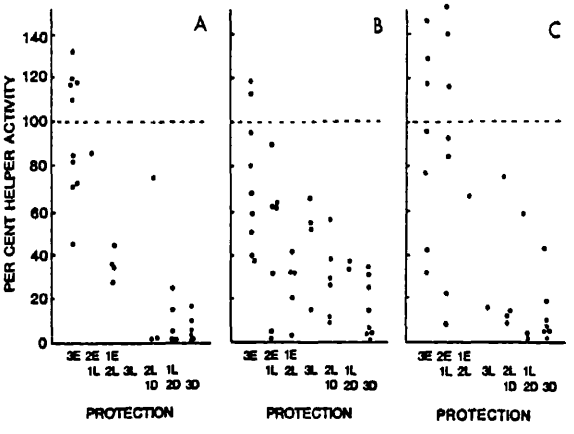

FIG. 5. Correlation of T-helper-cell priming with protection, showing the data for IEF fractions from the three major protective regions: (A) pH 3.8 to 5.2; (B) pH 6.0 to 7.4; (C) pH 7.6 to 8.4. Details as for Fig. 4. Statistical analysis was as follows: for group A, $F = 16.71$ ($P < 0.001$) and $R = 0.843$ ($P < 0.001$; for group B, $F = 4.29$ ($P = 0.002$) and $R = 0.626$ ($P < 0.001$); and for group C, $F = 4.63$ ($P = 0.005$) and $R = 0.771$ ($P < 0.001$).

fractions, which as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown) still contain up to 10 separate protein bands, have been further separated on a molecular-weight basis. These experiments are now under way. Meanwhile, it is interesting that the correlation between protective activity and T-cell stimulation was stronger with one of our groups of antigens than with the other two. In preliminary experiments (not shown) we have found that the induction of delayed-type hypersensitivity by IEF fractions correlates closely with the priming of T-helper cells as recorded here, so that the induction of delayed-type hypersensitivity is unlikely to correlate any better with protection than T help does. Passive cell-transfer experiments have shown that both immune T and immune B cells can transfer protection against *P. yoelii* YM (4), which is consistent with our findings of protection without strong T help in some fractions. It may be, therefore, that the antigens of our second and third groups (pH 6.5 to 8.4) owe their protective effect mainly to an effect on B cells or T cells alone, whereas those in the first group (pH 4.2 to 4.4) act by stimulating T cells for both the antibody response and delayed-type responses—for example, the activation of macrophages and other non-specific cells to produce parasitotoxic factors (11)—and also stimulating B cells. We do not yet know whether different antigens in this group of fractions are responsible for T- and B-cell priming, or whether, as in the case of the well-characterized circumsporozoite antigen of *P. falciparum*, T- and B-stimulating regions are present on the same molecule (6).

It seems likely, however, that the most effective antigens for vaccinating against human blood-stage malaria will also be those which stimulate both T and B cells. Screening of antigens is clearly not so easy as in the mouse model, though to some extent they can be analyzed in mice (6), and another approach that shows promise is the measurement of the response to candidate antigens in vitro by lymphocytes from immune versus nonimmune patients (16, 17). We predict that the best antigens would be those in which the response involves both T and B cells, which could be demonstrated by, for example, the degree of sensitivity of the proliferative response to inhibition by anti-T-cell sera.

ACKNOWLEDGMENT

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Recombinant gamma interferon is a potent adjuvant for a malaria vaccine in mice

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SUMMARY

Mice were protected against lethal *Plasmodium yoelii* malaria by vaccination with a Triton X-100 lysate of whole parasitized erythrocytes. For full effectiveness this vaccine required an adjuvant, and we have found that recombinant γ-interferon has strong adjuvanticity in this model when given either intraperitoneally or subcutaneously. Specific immune responses that were enhanced included antibody, T cell help, and delayed hypersensitivity.

Keywords interferon adjuvant vaccine malaria

INTRODUCTION

Current interest in the development of new vaccines centres on the use of peptides produced synthetically (Arnon, 1984) or by recombinant DNA technology (Harris, 1984), which will usually require adjuvants to enhance their immunogenicity (Allison, 1984). Safe potent adjuvants for human use are very few in number, and several, including Freund's incomplete adjuvant and *Bordetella pertussis*, are not without undesirable side-effects (Leclerc, Morin & Chedid, 1983). Recent work on the requirements for the triggering of immune responses has revealed the importance of antigen presentation and the role played by physiological mediators such as the interleukins and interferons (Allison, 1984). We report here that in a murine model of vaccination against blood-stage malaria, γ-interferon (γ-IFN) has a powerful adjuvant effect, particularly when given with antigen by the subcutaneous route.

MATERIALS AND METHODS

Mice. (BALB/c × C57Bl)F_1 mice of both sexes were purchased from the National Institute for Medical Research, London. They were used at 10–14 weeks of age.

Parasites. The virulent YM line of *Plasmodium yoelii* was obtained from Dr D. Walliker (University of Edinburgh) as described previously (Freeman & Holder, 1983), and maintained by blood passage at weekly intervals. Mice were infected by i.v. injection of 10^4 parasitized red cells. Parasitaemias were counted on Giemsa-stained tail blood films.

Vaccine. In all experiments we used a Triton X-100 lysate of parasitized red cells, whose preparation has been described in detail elsewhere (Playfair & De Souza, 1986). Briefly, schizont-rich parasitized blood was lysed in 0·01% saponin (saponin HP3; Food Industries Ltd, Lancashire, UK), washed three times, and the pellet resuspended in 0·5% Triton X-100 in 50 mM Tris-HCl with 5 mM EDTA, 20 mM iodoacetamide, 5 mM PMSF, 1 μg/ml pepstatin, 5 μl/ml Trysalol, and 20 μg/ml

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leupeptin. After 3 h at 4°C, insoluble material was sedimented by microfuging and the supernatant dialysed overnight against PBS.

Adjuvants and immunisation. As a standard, we used 25μg of the Triton X-100 lysate described above, plus 25 μg of saponin, injected i.p. on two occasions 2 weeks apart. Mice were challenged with 10^4 parasites 3 weeks after the second injection. For IFN experiments we used recombinant γ-IFN produced by Genentech Inc. and supplied by Boehringer Ingelheim, lot numbers 3209-14 and 3209-33, specific activity $1{\cdot}5 \times 10^7$ units/mg, endotoxin content $<0{\cdot}25$ EU/mg. Unless otherwise indicated, antigen and adjuvant were mixed just before injection.

Assay for T help. Mice were vaccinated as described above, and 3 weeks after the last dose of vaccine they were injected intraveneously with 10^5 red blood cells parasitized with *P. yoelii*, coated with the hapten trinitrophenol (TNP) by incubation with 2,4,6-trinitrobenzene sulphonic acid (Playfair, De Souza & Cottrell, 1977) (TNP.PY), or with TNP-coated normal mouse red blood cells (TNP·MRBC). Four days later their spleens were assayed for direct anti-TNP plaque-forming cells (PFC) in Cunningham chambers (Playfair *et al.*, 1977). Because we had previously shown that T helper cell priming is maximal with small doses of vaccine, we routinely used a single injection of 1 μg of antigen.

Assay for delayed hypersensitivity. Mice were vaccinated as described above, and 3 weeks after the last dose of vaccine they were injected in the pinna of the right ear with 3×10^6 red blood cells parasitized with *P. yoelii* in 10 μl of phosphate buffered saline (PBS) and in the left (control) ear with PBS only. One day later they were injected intraveneously with 10^7 ^{51}Cr-labelled normal syngeneic bone marrow cells and after another 24 h they were killed, their ears were cut off and their radioactivity was counted on an LKB Gamma counter (Cottrell, Playfair & De Souza, 1978). The results shown in Fig. 2 are the percentage of the total injected bone marrow cells homing specifically to the antigen-challenged ear, calculated as:

$$\frac{(\text{ct/min in right ear}) - (\text{ct/min in left ear})}{\text{Total ct/min in bone marrow injection}} \times 100$$

Antibody. Antibody responses following challenge were measured by indirect fluorescence (IFA) using the slide method of Voller & O'Neill (1971). In vaccinated mice, this assay detects predominantly IgG antibody (Playfair & De Souza, 1979).

RESULTS

Protection. For convenience in describing the degree of protection in 250 mice, we have scored protection by vaccines as 'early' or 'late', since, as described previously (Playfair & De Souza, 1986), in this experimental model, challenged mice either recover on days 8–10 (early) or between days 15–20 (late). Table 1 shows all the protection data. Unvaccinated mice given antigen (lysate) alone showed only occasional late protection, the subcutaneous route being somewhat better than intraperitoneal. As described earlier (Playfair & De Souza, 1986) antigen i.p. plus saponin protected all the mice, almost invariably by day 10.

The adjuvant effect of γ-IFN was only slightly inferior to that of saponin. With 5000 u, the dose used in most experiments, over 90% of the mice were protected including over 70% which recovered early; the intraperitoneal and subcutaneous routes were equally effective. Intradermal and intramuscular injection were also effective but somewhat less so. In a smaller series of experiments, doses of IFN down to 200 units were also effective.

An important consideration with all recombinant materials is whether the effects are due to contaminating endotoxin, particularly since lipopolysaccharide (LPS) is known to have adjuvant activity. We do not believe that the effects reported here are due to LPS since the endotoxin content (by Limulus assay) of the IFN was less than 0·25 EU/mg protein, which is equivalent to less than 0·1 pg per 5000 units of γ-IFN. As Table 1 shows, doses of LPS well above this range had only weak adjuvant activity in our model and never induced strong early protection. Saponin and γ-rIFN alone were not protective, so it is unlikely that the γ-IFN is acting directly on the blood-stage parasites, as has recently been shown for the liver stage (Ferreira *et al.*, 1986).

Antibody. Figure 1 shows the total IFA titres 10 days after challenge. Mice vaccinated with

Table 1. Effect of adjuvants on protection

Vaccine	Adjuvant	Dose	Route	Recovered		Died	Per cent protected
				Early	Late		
—				0	0	32	0
P. yoelii lysate	—	25 μg	s.c.	0	4	28	12·5
	—		i.p.	0	0	19	0
	—		i.m.	0	2	12	14·3
	Saponin	25 μg	s.c.	7	2	2	82
			i.p.	36	2	0	100
	γ-IFN	5000 u	s.c.	25	6	3	91
			i.p.	14	2	2	89
		2000 u	s.c.	2	2	1	80
			s.c.	2	2	0	100
		1000 u	s.c.	5	4	3	75
			i.p.	2	3	0	100
		500 u	s.c.	3	1	1	80
			i.p.	4	0	1	80
		200 u	s.c.	2	1	2	60
			i.p.	1	3	1	80
		100 u	s.c.	1	0	4	20
			i.p.	3	0	2	60
	LPS	20 μg	s.c.	0	3	4	43
		2 μg	s.c.	0	3	3	50
		200 ng	s.c.	1	2	7	43
		20 ng	s.c.	1	1	4	33
		2 ng	s.c.	0	0	3	0
		200 pg	s.c.	0	0	2	0
—	Saponin	25 μg	i.p.	0	0	6	0
—	γ-IFN	5000 u	s.c.	0	0	6	0
—			i.p.	0	0	6	0

Mice were given two injections 2 weeks apart of vaccine and adjuvant by the route indicated. The results are the numbers of mice which cleared their parasitaemia early (< 10 days) or late (> 10 days) or died, following a challenge of 10^4 parasitized red cells. The total per cent of mice protected is also shown.

antigen alone had titres of 1/256–1/1000 whilst both saponin and γ-IFN boosted these 8–16-fold. Intraperitoneal injection appeared marginally better.

Delayed hypersensitivity. Figure 2 shows that mice vaccinated with antigen alone gave DTH responses no stronger than unvaccinated mice, but that both saponin and γ-IFN, given subcutaneously, induced strong priming for DTH. Saponin i.p. (the most protective combination) induced the strongest DTH but, surprisingly, γ-IFN by this route did not induce significant DTH.

T helper assay. Figure 3 shows that both saponin and γ-IFN induced excellent T helper priming. In this case γ-IFN was equally effective by the subcutaneous and intraperitoneal routes.

DISCUSSION

The principal new finding reported here is the effectiveness of γ-interferon as an adjuvant, particularly with regard to T cell mediated responses such as help and DTH. We used a malaria

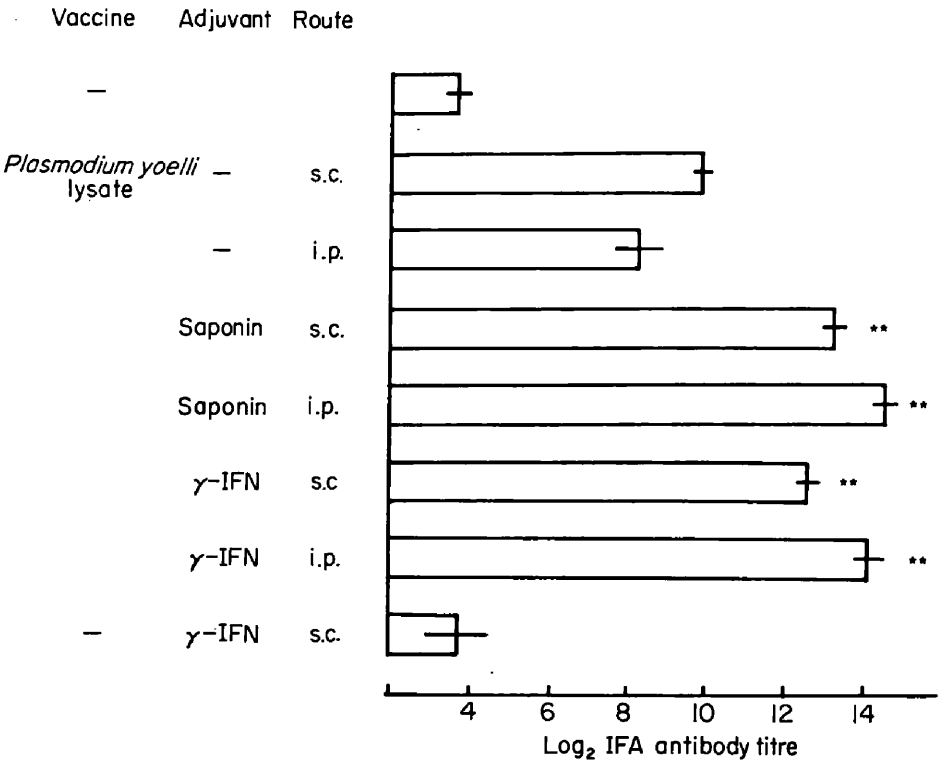

Fig. 1. Effect of adjuvants on the antibody response during a *P. yoelii* infection in vaccinated mice. Mice were bled 10 days after infection. Doses were: *P. yoelii* lysate 25 μg, saponin 25 μg, γ-IFN 5000 units. For details of vaccination see text. Five to ten mice per group. Asterisks denote values significantly different (*$P<0{\cdot}02$, ** $P<0{\cdot}005$) from the corresponding group without adjuvant.

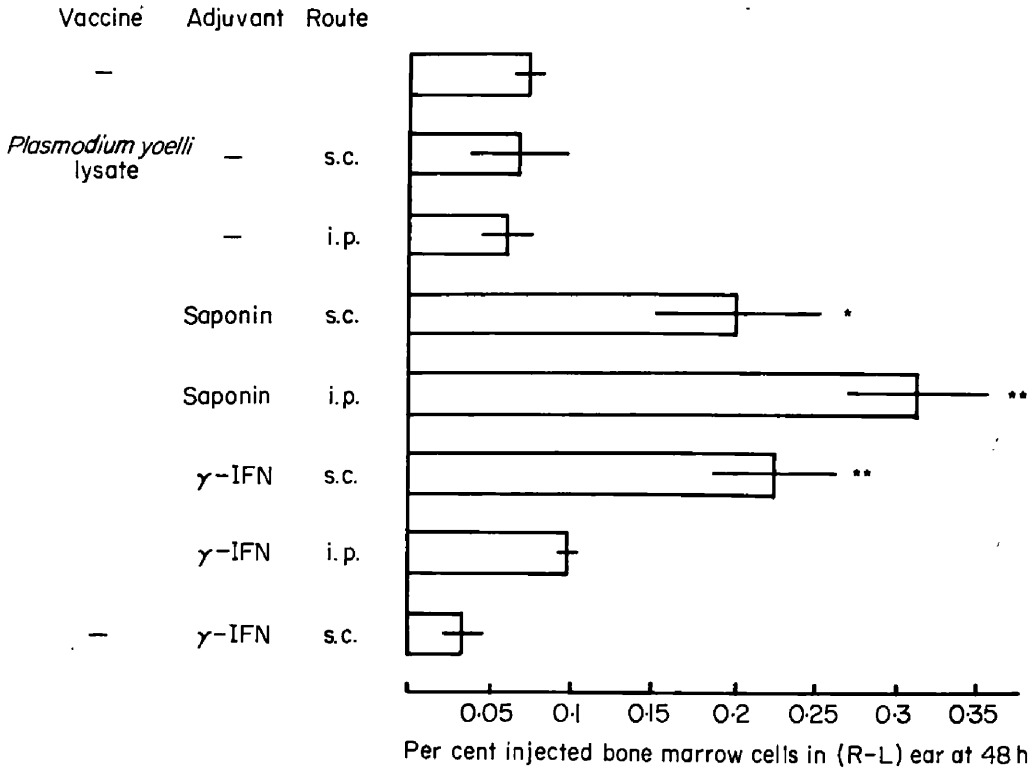

Fig. 2. Effect of adjuvants on the DTH response to *P. yoelii* antigens in vaccinated mice. Five to ten mice per group. Details and significance values as in Fig. 1.

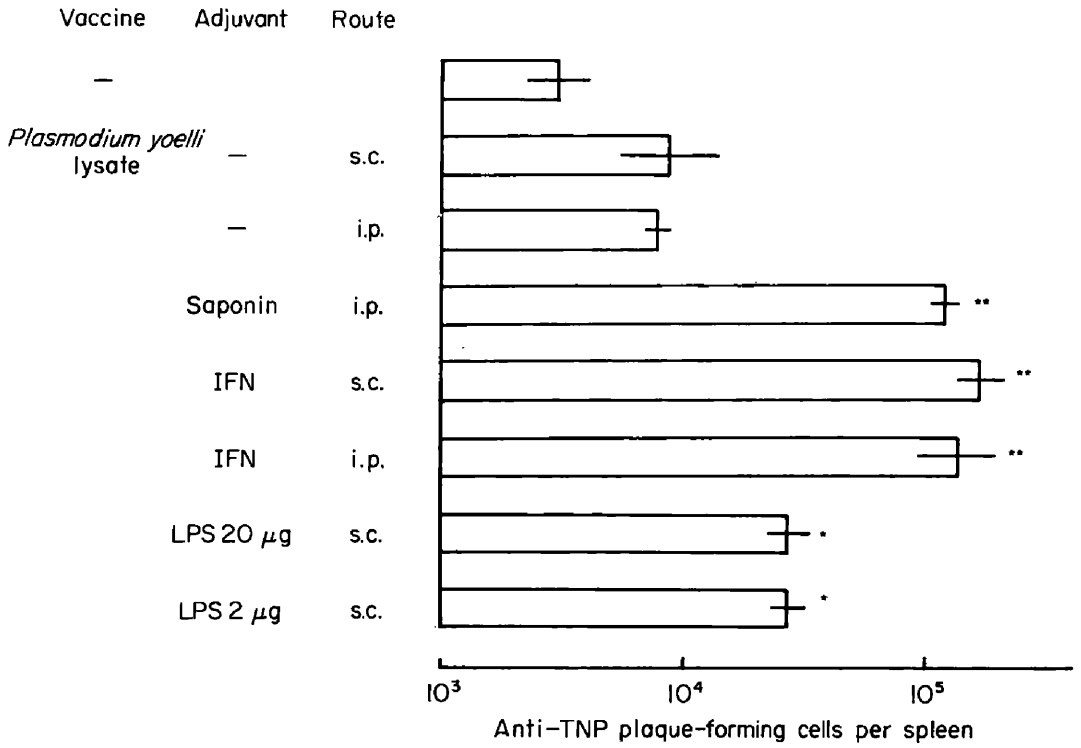

Fig. 3. Effect of adjuvants on the priming of T helper cells to *P. yoelii* antigens in vaccinated mice. Five to ten mice per group. Details and significance values as in Fig. 1.

model because of our previous experience with other adjuvants and the convenience of measuring T helper cell and DTH priming by established assays, and because of the evidence that protection by vaccination in this model is associated with both antibody and cell-mediated immunity (Playfair *et al.*, 1985). However it seems likely that the results would apply to other antigens.

γ-IFN has previously been shown to enhance the primary antibody response to hen-egg lysozyme and dinitrophenylated bovine globulin in mice (Nakamura *et al.*, 1984). Naturally-produced IFN slightly enhanced a DTH (footpad swelling) response to sheep RBC (De Maeyer & De Mayer-Guiguard (1980), and very low doses (8 units) of αβ IFN can enhance antibody and cytotoxic responses in mice (Bastide *et al.*, 1985). However ours appears to be the first demonstration of an effect on protective immunity against a lethal infection. It should of course be borne in mind that *P. yoelii* is not a natural parasite of the mouse, and that our vaccine is a mixture of numerous membrane antigens. Nevertheless, the results seem sufficiently encouraging to justify further study and eventual clinical trial

The mode of action of IFN in these experiments is still unknown, but one obvious possibility is the induction of Class II major histocompatibility complex (MHC) molecules on the antigen presenting cells in the vicinity of the antigen (Nakamura *et al.*, 1984; Zlotnik *et al.*, 1983). Class II MHC antigens are known to be required for efficient triggering of T helper cells (Schwarz, 1985). This idea is supported by preliminary experiments (not shown) in which we found that γ-IFN injected by a different route from the antigen was not effective as an adjuvant. Against it, however, is the finding that γ-IFN given 2 days before antigen was also not effective, although this timing should be ideal for MHC enhancement. We are currently investigating the role of timing and the expression of MHC at the site of injection of IFN.

Taken together with previous demonstrations of adjuvanticity by specific monoclonal IgM antibodies (Harte, Cooke & Playfair, 1983) and by interleukin-1 (Staruch & Wood, 1983), our results suggest that physiological mediators of the immune response, perhaps used in combination, may constitute a safe and effective novel class of adjuvants.

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Vaccination of mice against malaria with soluble antigens. I. The effect of detergent, route of injection, and adjuvant

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Summary Mice were vaccinated against lethal blood stage *P. yoelii* malaria with lysates of parasitised blood prepared in various detergents. Two injections, containing 25 μg of protein, of a Triton X-100 lysate gave optimal protection when injected intraperitoneally with saponin as adjuvant. Such lysates provide a starting point for the isolation of potential antigens for vaccination, selected for their protective activity *in vivo*.

Keywords: malaria, vaccine, adjuvant

Introduction

Mice can be fully protected against a lethal blood-stage *P. yoelii* malaria infection by vaccination with either saponin-lysed formalin-fixed parasitised red cells injected i.v. with *B. pertussis* (Playfair *et al.* 1977) or with a 230 000 mol wt. purified protein injected i.p. with saponin (Holder & Freeman 1981). The latter antigen was fortuitously recognized by a monoclonal antibody which was not itself protective on passive transfer, suggesting that selection by protective antibodies, though frequently useful, may not be the only approach to the identification of potentially protective antigens. In this paper we describe the preparation of an antigenic extract of *P. yoelii* selected uniquely for the ability to protect reliably against infection. In subsequent papers, further purification of protective antigens on the basis of charge and molecular weight will be described, and the immunological properties of protective and non-protective antigens compared.

Materials and methods

MICE

(BALB/c × C57Bl)F1 hybrid mice were bred in our department from parental stocks obtained from the National Institute for Medical Research, Mill Hill, London. Mice of both sexes were used at 10–14 weeks of age.

PARASITES

The virulent YM line of *P. yoelii* was obtained from Dr D. Walliker (University of Edinburgh) as described previously (Freeman & Holder 1983). It was maintained by weekly blood passage. Mice were infected by i.v. injection of 10^4 parasitized red cells. Parasitaemias were counted on Giemsa-stained tail blood films.

PREPARATION OF VACCINES

Donor mice infected i.v. with 10^7 parasitised RBC were bled 4 days later into heparinized PBS, when their parasitaemias were approaching 100%, 90% of the parasites being schizonts. After three washes in PBS the parasitized blood was lysed in 0·01% saponin in PBS at 37°C for 30 min and washed three times in PBS or until the supernatant was free of visible haemoglobin. The pellet was resuspended in extraction buffer (see below) at a ratio of 1 vol pellet to 5 vol buffer. Solubilization was carried out at 4°C for 3 h with vortexing at intervals. Cell debris and insoluble material were removed by microfuging at 8000 ***g*** for 10 min. The supernatant was then dialysed overnight against PBS before being used to immunize mice (see below).

The above method was adopted as a standard for the experiments reported in this paper. In preliminary experiments it was found that for lysis, saponin was superior to distilled water, which caused clumping of parasite material, and that ultra centrifugation at 100 000 ***g*** was no better than microfuging, as judged by the HPLC protein profile of the resulting lysates and their protective effect. The protein content of vaccines was estimated by absorption at 280 nm in an LKB Ultrospec 4050. Typically, one donor yielded 4 mg of protein, which was enough to protect about 200 recipients (see later).

EXTRACTION BUFFERS

The following detergents were used: Triton X-100 (TX-100), Nonidet P40 (NP40), deoxycholate (DOC), sodium dodecyl sulphate (SDS), and *N*-octyl glucoside (OG). The *N*-octyl glucoside was used at 0·7%, and the others at 0·5%; in an initial experiment lysates prepared using TX-100 and NP40 at 1% caused some deaths in the mice. All detergents were made up in 50 mM tris-HCl at pH 8·0, with 5 mM EDTA, 20 mM iodoacetamide, 5 mM PMSF, 1 μg/ml pepstatin (all from Sigma) and 5 μl/ml Trasylol, as described by Deans *et al.* (1982), with the addition of 20 μg/ml of leupeptin (Calbiochem).

In preliminary experiments we found that SDS-PAGE analysis of lysates made with all the detergents showed very similar patterns, with at least 40–50 bands staining clearly with Coomassie Blue.

VACCINATION AND ADJUVANTS

For routine experiments, mice were vaccinated with two intraperitoneal injections of 25 μg of lysate protein at 2 week intervals, with 25 μg of saponin added to each dose as adjuvant (Playfair *et al.* 1985). In some experiments we used *Bordetella pertussis* (10^8 organisms) which had previously been shown to be highly effective with a particulate intravenous vaccine (Playfair *et al.* 1977). We also investigated other routes of injection, namely subcutaneous (flank), intramuscular (thigh) and intradermal (shaved flank).

Results

PATTERNS OF RECOVERY IN VACCINATED MICE

Figure 1 shows the parasitaemias from a pool of four representative experiments. All unvaccinated mice were dead by day 9. Vaccinated mice fell into two clear groups: those which recovered by days 7–10 and those which recovered around days 17–20. The latter group always showed a fall in parasitaemia on day 6 but failed to maintain this. A similar pattern of early and late protection has repeatedly been noted in experiments with the formalin-fixed vaccine. For the purposes of the present study, we have therefore classified protection by vaccines as 'early' if parasitaemia was permanently cleared on or before day 10, and 'late' if clearance occurred subsequently. In fact 'late' recovery in these experiments almost invariably occurred between days 17 and 25.

PROTECTION BY DETERGENT LYSATES

In the first series of experiments we adopted the vaccination regime previously shown to be optimal for the 230 000 mol. wt purified *P. yoelii* antigen—namely two i.p. injections 2 weeks apart of 25 μg of protein with 25 μg saponin, followed by i.v. challenge with 10^4 parasites 3 weeks later (Freeman & Holder 1983). We also tested the effect of one injection of vaccine. Table 1 shows a comparison of lysates made with five different detergents. All the lysates were highly protective but there were some interesting differences: with a single injection OG gave marginally the best protection, but with two injections TX-100 was the best, protecting all the mice in 10 experiments by day 8.

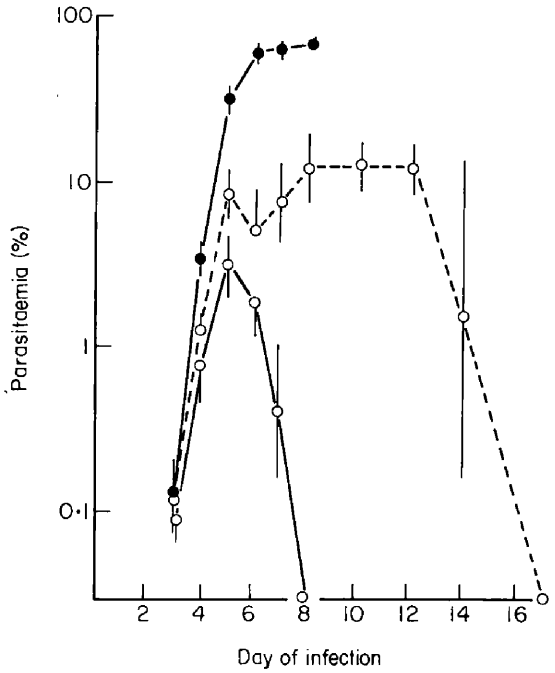

Figure 1. Representative parasitaemias in *P. yoelii* infected normal mice (●) or mice vaccinated with various regimes (○), to illustrate early recovery (———) and late recovery (— — —). Ten mice per group, drawn from the mice represented in Table 1.

Table 1. Protection by *P. yoelii* lysates prepared with different detergents

Detergent	Vaccination	Recovered early	Recovered late	Died	% Protected
TX 100	25 μg × 1 i.p. +saponin	2	3	4	56
NP 40	„	0	1	2	33
DOC	„	0	2	1	67
SDS	„	0	2	1	67
OG	„	0	4	1	80
TX 100	25 μg × 2 i.p. +saponin	36	0	0	100
NP 40	„	17	0	2	89
DOC	„	11	0	1	92
SDS	„	12	2	0	100
OG	„	10	2	1	92

Mice received one or two i.p. injections of 25 μg protein. The values shown are the number of mice recovering early (< 10 days) or late (> 10 days) or dying after infection with 10^4 parasites 2 weeks after the last injection of vaccine. For detergent abbreviations see text.

Various doses of the TX-100 lysate were tested with saponin (Table 2). With two injections, 10 μg or more was consistently protective, and as little as 1 μg protected some mice, but with a single injection, even 250 μg did not give full protection. Two injections of 25 μg were therefore selected as the standard regime for further experiments.

ROUTE OF INJECTION AND THE EFFECT OF ADJUVANT

Table 3 shows that intraperitoneal injection with saponin was the best combination tested. However subcutaneous injection was also protective, and here *B. pertussis* was equally effective. The intramuscular and intradermal routes were also protective. In the absence of adjuvant, the vaccine protected only about 10% of the mice and recovery was invariably delayed until at least 3 weeks, suggesting that neither the Triton X-100 nor the saponin used for the initial lysis had significant adjuvant effects. In the absence of the vaccine, neither of the adjuvants were protective (data not shown).

Discussion

Hopes are currently high that some form of human malaria vaccine will shortly be under test, but it will probably be a considerable time before a particular antigen, or even a single stage, emerges as the first choice (McGregor 1985). Meanwhile, useful data can still be

Table 2. Protection by various doses of a TX-100 *P. yoelii* lysate

TX-100 lysate	Dose (μg)	Recovered early	Recovered late	Died	% Protected
i.p. + saponin × 1					
	250	1	0	1	50
	50	0	2	1	67
	25	2	3	4	56
	10	1	0	2	33
	2·5	0	0	2	0
	0·4	0	0	2	0
i.p. + saponin × 2	250	2	0	0	100
	50	4	0	0	100
	25	36	0	0	100
	10	11	0	0	100
	2·5	5	2	2	78
	1	3	1	0	100
	0·25	1	0	7	12
	0·1	0	0	4	0

Mice received one or two i.p. injections of various doses of a Triton-X 100 lysate plus 25 μg of saponin. Results expressed as in Table 1.

Table 3. Effect of route and adjuvant on protection by a TX-100 *P. yoelii* lysate

Adjuvant	Route	Recovered early	Recovered late	Died	% Protected
Saponin	i.p.	36	0	0	100
25 μg	s.c.	4	2	2	75
B. pertussis 10^8	s.c.	4	4	2	80
organisms	i.m.	4	3	1	88
	i.d.	2	4	4	60
nil	i.p.	0	2	13	13
	s.c.	0	4	28	12
	i.m.	0	2	9	18
	i.d.	0	0	6	0

All mice received two injections of 25 μg of TX-100 lysate mixed with adjuvant or alone. Results expressed as in Table 1.

obtained from the mouse model, which is one of the few in which large-scale protection experiments can be carried out. One approach has been to use immune serum, or protective monoclonal antibodies, to purify antigens from a mixture. This presupposes that the induction of antibody is the prime objective of a vaccine, and ignores the possible role of T cell recognition of antigen which may in some cases be equally important (Mitchison 1984, Playfair *et al.* 1985). Since there is at present no convenient way to purify antigens by virtue of their T cell stimulating properties, we have adopted the alternative strategy of first obtaining a highly protective soluble antigen preparation, and then testing the protective effect of individual components, without reference to their reactivity with antibodies. Once an antigen is isolated, its reactivity with both T and B cells can be evaluated and correlations with protectivity looked for.

In the present paper we describe a simple and reliable soluble vaccine giving effective protection against the highly virulent YM strain of *P. yoelii*. Given intraperitoneally with saponin it is somewhat less effective than a crude formalin-fixed parasite vaccine given intravenously with *B. pertussis*, which requires only one rather than two injections (Playfair *et al.* 1977). However the soluble vaccine is also quite effective when given subcutaneously or intramuscularly, which is of obvious relevance to human vaccination. Moreover, a comparison of the dose-response relationship given in Table 1, with that found for the 230 000 mol. wt purified *P. yoelii* protein (Freeman & Holder 1983), both of which begin to lose full protectivity in the 1–2·5 μg range, suggests that the 230 000 mol. wt protein is not the only protective antigen in our lysate. We are now investigating methods of isolating the protective component or components.

Acknowledgements

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