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*Plasmodium falciparum* merozoite surface and rhoptry proteins as malaria vaccine candidates

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PLASMODIUM FALCIPARUM MEROZOITE SURFACE
AND RHOPTRY PROTEINS AS MALARIA VACCINE CANDIDATES

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# ABSTRACT

*Plasmodium falciparum* merozoite surface proteins were isolated from in

vitro cultured parasites using monoclonal antibody (mAb) 5.2. Serum samples

from rabbits immunized with the native merozoite surface precursor glycoprotein

(gp195) had a mean ELISA titer of 1/560,000 against native gp195 but only 1/890

to denatured, reduced and alkylated (dR/A) gp195, while rabbits immunized with

dR/A gp195 had mean ELISA titers of 1/23,100 and 1/14,650 to native gp195

and dR/A gp195, respectively. Serum samples from rabbits immunized with

native gp195 had a mean parasite growth inhibition of 87% and recognized the 42

and 19 kDa C-terminal processing fragments. Serum samples from rabbits

immunized with dR/A gp195 did not inhibit parasite growth in vitro and poorly

recognized the native C-terminal processing fragment. This study suggests that

recombinant or synthetic peptide gp195 subunit vaccines must contain the native

secondary and/or tertiary protein structures.

Forty-five gp195-specific mAbs were produced in BALB/c, C57/Bl.10, or

Swiss-Webster mice using Freund's complete adjuvant (FCA) or Lipid A-15 PH.

Of 26 mAbs tested for inhibition of parasite growth in vitro, two mAbs (CE2 and

EB2) inhibited parasite growth partially (59% and 52%, respectively) at high

concentrations (500 $\mu$g/ml). These antibodies recognized linear, group specific

epitope(s) on the 83 kDa N-terminal processing fragment. All other mAb,

including mAb 5.2 and two others to the C-terminal 19 kDa processing fragment,

were weakly or non-inhibitory. Combinations of N-terminal or C-terminal mAb

were also not inhibitory. The inability to produce a mAb which inhibits parasite growth at low concentration (1-10 $\mu$g/ml) is discussed.

MAb AC9 was produced and used to isolate the 80, 70 and 40 kDa rhoptry associated protein-1 (RAP-1) complex. The isolated RAP-1 proteins reacted with mAb 30c13, previously used by Perrin to isolate a protective 42 kDa protein and by Braun-Breton to isolate a rhoptry-associated serine protease, as well as mAb 2.13, previously used by Ridley to demonstrate induction of protective immunity by RAP-1. In addition, mAb 219.5 was used to isolate the 140, 130, and 105 kDa RAP-3 complex. Both RAP-1 and RAP-3, along with gp195 as a control, were used to immunize rabbits. Serum samples from rabbits immunized with gp195, RAP-1, and RAP-3 inhibited parasite growth in vitro 87%, 89%, and 89%, respectively. These results suggest that RAP-3 as well as RAP-1, should be investigated as a possible blood-stage malaria vaccine candidates.

Three matrix metalloproteinases (MMP's) having relative molecular weight ($M_r$) 220, 95 and 75K in gelatin zymograms were found to be associated with RAP-1, but not RAP-3 or gp195. The MMP activity was inhibited by EDTA but not PMSF and was restored by addition of calcium. Of eight divalent metal cations tested, 0.1 mM cobalt optimized gelatinolytic activity when combined with 1.0 mM calcium. Gelatinases having the same $M_r$ were also precipitated by antiserums to normal human macrophage and fibroblast MMP's. Therefore, these MMP's are believed to be host enzymes non-covalently associated with RAP-1 proteins.

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CHAPTER I. INTRODUCTION

Malaria is a leading cause of morbidity and mortality in the world.
Annually, more than 100 million inhabitants in 102 developing countries contract
malaria and two million infants die of the most virulent species of human malaria
parasites, *Plasmodium falciparum* (W.H.O. Tropical Disease Report, 1991). The
widespread application of insecticides, such as DDT, and chemotherapeutic
agents, such as chloroquine, has selected for resistant strains of *Anopholine* spp.
mosquitoes and parasites, respectively (W.H.O. Assembly Report, 1969). The
development of a malaria vaccine is considered to be an alternative approach to
supplement current vector and parasite control strategies. Numerous *P.*
*falciparum* proteins are being investigated as vaccine candidates at three stages of
the parasite life cycle.

The pre-erythrocytic or sporozoite vaccine is intended for prevention of the
initial infection and would be useful for non-immune individuals visiting endemic
regions. Experiments have shown that the circumsporozoite protein (CSP) can
prevent infection through the induction of both antibody (Potocnjak *et al.* 1980)
and cell-mediated (Kumar *et al.* 1988) immunity. Cytotoxic T lymphocytes
recognize infected liver cells (Hoffman, *et al.* 1989) and provide protection upon
passive transfer (Romero *et al.* 1989). Although only a few volunteers were
protected from infection in human trials of a sporozoite vaccine, better protection

may have been achieved with the use of a better adjuvant (Ballou *et al.* 1987; Herrington *et al.* 1987).

The sexual, mosquito-stage vaccine is designed to reduce transmission, since mAbs to gametocytes prevent zygote formation (Vermeulen *et al.* 1985), thereby inhibiting parasite development. In addition, transfer of immune T lymphocytes into infected animals can reduce the mosquito transmission of parasites by 95% (Harte *et al.* 1985). Four antigens are currently being investigated as transmission blocking vaccine candidates (reviewed in Oaks *et al.* 1991).

The asexual, blood-stage malaria vaccine is designed to lessen morbidity and mortality after an individual has been infected with the parasite. Antibodies are acquired in natural infections and can provide protection upon passive transfer (Cohen *et al.* 1961). T-cell secretion of cytokines may also facilitate the elimination of parasites from the blood (Ockenhouse *et al.* 1984). At least seventeen different proteins are target antigens for an asexual blood-stage malaria vaccine (reviewed in Oaks *et al.* 1991).

The malaria research group at the University of Hawaii has been working since the early 1970's towards the development of a blood-stage malaria vaccine. Much of the impetus for current work identifying and characterizing potential vaccine candidates grew out of the initial report by Holder and Freeman in 1981 showing that merzoite surface and rhoptry proteins are important vaccine candidates based on the *P. yoelii* mouse malaria model. In 1987, a similar

experiment was reported by Siddiqui and co-workers, using merozoite surface and rhoptry proteins isolated from the human malaria parasite *P. falciparum*. The merozoite surface precursor protein (gp195) induced protective immunity in *Aotus* monkeys; however, the rhoptry proteins were only weakly protective (Siddiqui *et al.* 1987). Overall, the purpose of this study is to further characterize the native, blood-stage merozoite surface coat (gp195) and rhoptry proteins of *P. falciparum* as malaria vaccine candidates.

A malaria vaccine based on these proteins must be a recombinant polypeptide or a synthetic peptide, because it is not practical to develop a native protein vaccine from parasites cultured in vitro for the following reasons: (1) the in vitro culture system is too inefficient to produce antigen for the vaccination of millions of people; (2) the methods for culturing and purifying native antigen are too labor intensive; (3) co-isolation of erythrocyte or serum antigens may lead to autoimmune disease in vaccinees; (4) blood-born viral contamination of the culture system cannot be entirely eliminated, and (5) variability in individual antigen preparations may result in preparations of different potency. Therefore, identification of relevant sequences or structures (e.g. B-cell epitopes) on the native vaccine candidates may assist in the development of an effective recombinant or synthetic peptide blood-stage vaccine candidate.

The precursor to the major merozoite surface coat proteins is a glycosylated protein having a $M_r$ of 195,000. It is processed into 83, 42 and 19 kDa proteins, among others, on the surface of merozoites (Lyon *et al.* 1986;

Holder *et al.* 1987). Although monkeys immunized with the native gp195 together with its processing fragments are protected against lethal malaria infection (Perrin *et al.* 1984; Hall *et al.* 1984; Siddiqui *et al.* 1987; Patarroyo *et al.* 1988; Etlinger *et al.* 1991), it is not known which processing fragment of gp195 is important in the conferring of protective immunity. Thus, the identification of the protective region(s) or processing fragment would facilitate the development of a subunit vaccine based on gp195. The first objective of this dissertation was to isolate the native 83 kDa N-terminal and 42 kDa C-terminal processing fragments of gp195 to determine which processing fragment might be responsible for protection. Currently, there is no concensus as to which processing fragment is important. The 83 kDa N-terminal processing fragment provided partial protection in *Aotus* monkeys (Patarroyo *et al.* 1987b); however, antibodies raised in rabbits did not inhibit parasite growth (Strych *et al.* 1987). Evidence that the 42 kDa C-terminal processing fragment was important in protective immunity came indirectly from two studies using mAbs in in vitro inhibition assays (Pirson and Perkins, 1985; Blackman *et al.* 1990). Therefore, it was important to identify which processing fragment was important in immunity by directly isolating the N- and C-terminal processing fragments and examining their ability to induce growth inhibitory antisera in rabbits. In chapter three, gp195 and its processing fragments were purified by affinity chromatography and SDS-PAGE/electroelution. Monospecific polyclonal antisera were raised in rabbits to the 195 kDa precursor protein, the 83 kDa N-terminal processing fragment and the 42 kDa C-terminal

processing fragment and were evaluated for inhibition of parasite growth in vitro. No conclusion was reached due to problems with co-migrating, contaminating polypeptides. The reasons for the presence of these polypeptides are discussed.

The second objective of this dissertation was to assess the role of native protein conformation in the antigenicity and immunogenicity of native gp195. Recombinant polypeptides produced in bacterial expression systems (Holder *et al.* 1988; Herrera *et al.* 1990) and synthetic peptides (Cheung *et al.* 1986; Patarroyo *et al.* 1987; Ruebush *et al.* 1990) based on gp195 induce low antibody titers compared with native gp195. It has been suggested that the low antibody titers observed may be the result of incorrect protein folding (Holder *et al.* 1988). Consequently, the role of gp195 in antigenicity and immunity was investigated. Chapter four describes experiments in which the disulfide bridges of gp195 were reduced and alkylated and studied for alteration of antigenicity, immunogenicity and the ability to induce growth inhibitory antibodies. New information showing that protein conformation is essential for the induction of high-titered, growth-inhibitory antisera are discussed.

The third objective of this study was to produce mAbs which inhibit parasite growth in vitro at low antibody concentrations. The premise that immunity to blood-stage malaria is antibody-mediated is based partly on the demonstration that passive transfer of immunoglobulin and mAbs is protective in non-immune recipients. Passive transfer of human adult immune globulin reduce *P. falciparum* parasitemias in partially immune children (Cohen *et al.* 1961). With

the development of mAb technology and murine malaria models, it was possible

to define specific parasite molecules and perform passive transfer experiments.

Merozoite-specific mAbs are protective for *P. yoelii* (Freeman *et al.* 1980;

Majarian *et al.* 1984) and *P. chabaudi* (Boyle *et al.* 1982). Although it is not

known if immunity to *P. falciparum* parallels immunity to *P. yoelii* or *P. chabaudi*,

these data support the hypothesis that mAb can confer protection.

To date, no mAb has been shown to passively transfer immunity to *P.*

*falciparum* in humans or in experimental models. Thus, this gap in our knowledge

could begin to be bridged by the production of a mAb which inhibits *P. falciparum*

growth in vitro. Human mAbs specific for gp195 are inhibitory for *P. falciparum*

in vitro at low concentrations (10 $\mu$g/ml) (Brown *et al.* 1986; Schmidt-Ullrich *et al.*

1986). However, this work has not been repeated; hence, the delineation of these

protective epitopes has remained elusive. Although numerous murine mAbs were

produced to gp195, none of these have been demonstrated to inhibit parasite

growth in vitro at low conccentrations. However, two murine mAbs are partially

inhibitory (50%) for *P. falciparum* growth in vitro at very high concentrations (500

$\mu$g/ml) (Pirson and Perkins, 1985; Blackman *et al.* 1990). Since mAb

concentrations of 500 $\mu$g/ml to a single epitope is not likely to occur

physiologically, work was initiated to produce a mAb inhibitory at low

concentration (1-10 $\mu$g/ml). If these mAbs could be produced and the epitope

defined, it would provide the foundation for conducting more definitive passive

transfer experiments. It would also aid in the identification of synthetic peptide

or recombinant polypeptide vaccine candidates. In chapter five, twenty-one mAbs were produced using different adjuvants, mouse strains and lymphocyte sources and evaluated for parasite growth inhibition. Overall, two inhibitory mAbs were identified, however, these were only inhibitory at high concentrations, similar to previous work (Pirson and Perkins, 1985; Blackman *et al.* 1990). Possible reasons for the inability to produce a mAb which inhibit parasite growth at low concentrations are discussed.

The fourth objective of this study was to re-evaluate rhoptry associated proteins as malaria vaccine candidates. Like gp195, rhoptry proteins are potential malaria vaccine candidates based on studies in mouse and monkey experimental models. A 235K rhoptry protein confers protection against *P. yoelii* (Holder and Freeman, 1981), while the rhoptry associated protein-1 (RAP-1) (Ridley *et al.* 1990) and a 41 kDa rhoptry protein (Perrin *et al.* 1985) partially protect *Saimiri* monkeys against *P. falciparum*. The RAP-3 proteins are only weakly protective (Siddiqui *et al.* 1987), although mAbs inhibit parasite growth in vitro to a small, but significant extent (Cooper *et al.* 1988). Because it remains unclear which of the rhoptry proteins is a better vaccine candidate, the RAP-1 and RAP-3 proteins were compared for their immunogenicity and ability to induce growth inhibitory antibodies. In chapter six, RAP-1 and RAP-3 were isolated using mAb affinity purification and used to immunize groups of rabbits. It was found that high-titered antisera to RAP-3 strongly inhibited parasite growth, as did antisera to

RAP-1 and gp195. This new finding re-opened the possibility that RAP-3 may be a potential blood-stage malaria vaccine candidate.

The fifth objective of this dissertation was to assess the proteolytic activity of gp195 and rhoptry proteins. Although specific merozoite proteins have been implicated in erythrocyte (glycophorin) receptor binding (Perkins, 1984; Camus and Hadley, 1985), the role of gp195 and rhoptry proteins in the parasite invasion process remains enigmatic. Some researchers contend that merozoite invasion is mediated in part by rhoptry enzymes (reviewed in Perkins, 1990), since three rhoptry associated proteases have been described: (1) a 68 kDa cysteine protease in the apical complex of *P. berghei* (Bernard and Schrevel, 1987; Schrevel *et al.* 1988); (2) a 76 kDa serine protease (Braun-Breton *et al.* 1988); and (3) a 41 kDa protein sharing significant sequence homology with aldolase (Certa *et al.* 1987; Knapp *et al.* 1990). This hypothesis is also supported by the identification of a lytic substance in the rhoptries of *Toxoplasma gondii* called penetration-enhancing factor, which facilitates host cell invasion (Lycke *et al.* 1975). Hence, gp195, RAP-1 and RAP-3 were assayed for proteolytic activity. In chapter seven, new information showing that three matrix metalloproteinases (MMP) are associated with RAP-1, but not RAP-3 or gp195 is presented. The possibility that these metalloproteinases are derived from the serum is discussed.

In chapter eight, the gelatinase acitivity associated with RAP-1 was further characterized. The metal ion requirements of the gelatinases was evaluated using a variety of metal ions. A new characteristic of the enzyme, that of maximal

activation by 0.1 mM cobalt, is reported. Finally, an overall discussion and conclusion of the results of this study are presented in chapter nine.

# CHAPTER II. MATERIALS AND METHODS

## PARASITES

In vitro cultivation of *Plasmodium falciparum* was performed according to methods previously described (Trager and Jensen, 1976). Each culture consisted of 5% packed type O erythrocytes, 10% serum or plasma and 85% RPMI 1640 (Gibco) supplemented with 0.02 M N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES; Research Organics), 2.0 mM $NaHCO_3$, 10 $\mu$M hypoxanthine and gentamycin sulfate (25 $\mu$g/ml). The gas mixture was 90% $N_2$, 8.0% $CO_2$, and 2.0% $O_2$ (Siddiqui and Palmer, 1981) blended with a Linde FM 4585 automated gas blender (Union Carbide). The primary isolate used in this study is the Falciparum Uganda Palo Alto strain isolated from a patient in 1966 at Stanford Medical Center, Palo Alto, CA (Geiman and Meagher, 1967).

When culture parasitemias rose above 10%, the erythrocytes were lysed with saponin and stored at -70°C. Purified schizonts were prepared using density gradient centrifugation in Percoll[R] (Sigma; Kramer *et al.* 1982). In gelatinase experiments, Percoll[R]-purified schizonts were incubated for one hour at 37⁰C with 5 $\mu$g of phosphatidyl inositol-specific phospholipase C (Sigma) in Phosphate Buffer Saline (PBS).

IN VITRO INHIBITION OF *P. FALCIPARUM*

In vitro inhibition assays were performed according to a procedure previously

described (Hui and Siddiqui, 1988). Synchronous parasites were incubated with

either heat-inactivated rabbit serum adsorbed to human erythrocytes overnight or

with purified mAbs (500 $\mu$g/ml) for 72 hrs. Synchronous parasite cultures had a

1.0% hematocrit and 0.2% parasitemia at the begining of the assay. Thin blood

smears were stained with Leishman's stain and parasitemia determined as the

number of infected erythrocytes per 1000 erythrocytes. Per cent inhibition was

calculated by the following equation:

$$\frac{\text{parasitemia (P - 0 hr) - (T - 0 hr)}}{\text{parasitemia (P - 0 hr)}} \times 100\%$$

P is the parasitemia of the preimmune serum or negative control mAb at 72 hr

and T is the parasitemia containing the immune serum or mAb tested.


ANTIBODIES

MAbs 5.2 and 219.5 were used to purify gp195 and the RAP-3 antigenic

complex, respectively (Siddiqui *et al.* 1987). MAbs CE2, DB8, EB2, AD9 and

BC9 were previously described and characterized (C. Locher Master thesis, 1988).

MAb G13 was produced from splenocytes of B10.PL mice immunized with gp195.

MAb AC9.1 (IgG$_1$) specific for RAP-1 was produced by inoculating Balb/c mice

four times with saponin-lysed parasite antigen in Freund's Complete Adjuvant

(FCA). MAbs 2.13 and 31cl3, previously used to isolate the RAP-1 proteins

(Ridley *et al.* 1990; Perrin *et al.* 1985), were provided by Dr. Robert Ridley and Dr. Luc Perrin, respectively. Rabbit monospecific polyclonal antisera to the 95K human fibroblast gelatinase and the 70K macrophage gelatinase (Vartio and Baumann, 1990) were provided by Dr. Tarpio Vartio. Sheep antiserum to the 70K human fibroblast gelatinase was provided by Dr. Gillian Murphy.

Rabbit monospecific polyclonal antisera designated K22, K41, K42, K43 were raised to mAb 5.2 affinity purified gp195 (Hui and Siddiqui, 1988; Hui *et al.* 1991). K44 was raised to the native 42 kDa C-terminal processing fragment excised from preparative SDS-PAGE gels after immunoaffinity purification with mAb 5.2. K58 was raised to the yeast-expressed 50 kDa N-terminal recombinant polypeptide (195A) and K93 was raised to the yeast-expressed 42 kDa C-terminal recombinant polypeptide (195B) (Chang *et al.* submitted). K15 was raised to the 219.5 affinity purified RAP-3 antigenic complex (Hui and Siddiqui, 1988).

## MONOCLONAL ANTIBODY PRODUCTION

Cell fusions for the production of mAbs followed the protocol previously established (Kohler and Milstein, 1975). Cell cloning was accomplished by limiting dilution in 96 well tissue culture plates previously seeded with peritoneal macrophages in 0.1 ml HT DMEM medium 24 hours in advance. Alternative strategies were evaluated for the production of growth inhibitory mAbs.

One alternative strategy is the use of a different adjuvant (other than FCA). Monophospholipid-A (Lipid A-15 PH) was evaluated for the induction of

inhibitory mAbs in Balb/c mice, since it has been shown to produce high anti-gp195 antibody titers, in mice (Hui *et al.* 1990).

Rabbit/mouse xenhybridomas were produced from rabbit splenocytes after the antisera was shown to inhibit parasite growth in vitro. Rabbit/mouse cell fusions were done in medium containing rabbit serum initially, until the hybridoma cultures were established. The medium was then supplemented with calf serum (Raybould and Takahashi, 1988).

## PURIFICATION OF IgG ANTIBODIES

Purified monoclonal IgG was evaluated for its ability to inhibit parasite growth in vitro. MAb isolated from ascites fluid was delipidated with 0.04 volume of 5.0% heparin with 0.1 volume of 0.25 M $MnCl_2$. The solution was stirred overnight at 4 °C, centrifuged at 12,000 X g for twenty minutes and the supernatant retrieved. The supernatant was mixed slowly with an equal volume of saturated ammonium sulfate solution (pH 7.1). The precipitate was collected by centrifugation at 12,000 X g for twenty minutes. The pellet was suspended in BBS, rocked for two hours at room temperature and the protein concentration was determined using an extinction coefficient at $A_{280}$ of 12.5 for 10 mg/ml.

MAb isolated from culture supernatant was concentrated to approximately 10 mls using Amicon filters with a molecular weight cut off of 50 K. The antibody concentrate was kept at 4 °C until ready for purification using either protein G or the Monoclonal Antibody Purification System II (MAPS II BioRad

Laboratories). One ml of washed Protein G conjugated to sepharose beads (Pharmacia) was incubated with antibody solutions on an end to end rotator overnight at 4°C. The beads were then applied to a glass Econo-column (BioRad) and washed with 50 volumes of BBS. Antibody was eluted with 100mM glycine, pH 2.5 and neutralized with 0.1 volume of Trisaminomethane (TRIS), pH 8.0. Protein concentration was determined using an extinction coefficient at A $_{280}$ of 14 for 10 mgs/ml. Antibody was then dialyzed against four changes of RPMI medium in 50 K cut off minifilters (Pharmacia). The medium with antibody was filter sterilized, adjusted to 1 mg/ml and stored at -20 °C.


ELISA ASSAY

An Enzyme Linked Immuno Sorbent Assay (ELISA) was used to screen hybridoma colonies and titer monospecific polyclonal antisera. MAb 5.2 isolated gp195 antigen (5 ng/well) was adsorbed directly onto polyvinyl 96 well plates (Costar). The plates were washed three times with borate buffer-saline (BBS: 167 mM borate, 134 mM NaCl, pH 8.0). The plates were blocked with 1% bovine serum albumin (BSA) in BBS for one hour. The plates were washed again three times with BBS and stored at -20°C. Incubation with hybridoma supernatant or serial dilutions of sera was carried out for one hour at room temperature. After washing with BBS, the plates were incubated with peroxidase conjugated goat anti-mouse antibody for one hour. The plates were washed three times again with BBS and the enzyme substrate was added (2,2'azino-di-3-ethyl-benzthialzoline

sulfonate, ABTS; Kirkegaard and Perry) for twenty minutes before analysis at an

absorbance of 410 nm with the MR 650 ELISA reader (Dynatech).


IFA ASSAY

The Indirect Immuno Flourescence Assay (IFA) (Voller and Bray, 1962)

was used to identify stage specificity of each mAb produced (ie. schizont and/or

ring-stage) in addition to reactivity with specific parasite isolates from different

geographical locations. The latter was employed to determine whether the mAbs

identified variable, conserved or group specific regions on the gp195 molecule. *P.*

*falciparum*-infected erythrocytes were harvested when the parasitemia reached

approximately 10% and thin blood smears prepared with 22 mm cover slips

(Corning) were fixed in cold acetone and stored at -20 °C for up to six months.

After incubation with MAb supernatant for thirty minutes and three washes in

PBS, pH 7.6, the cover slips were incubated with rabbit anti-mouse IgG, H & L

chain-specific, conjugated to flourescein isothiocyanate (FITC, Miles Laboratories)

for thirty minutes. After three PBS washes, the cover slips were mounted with

50% glycerol and examined using a Zeiss flourescent microscope.


PROTEIN ANALYSIS BY SDS-PAGE AND IMMUNOBLOT

Protein samples were solubolized and electrophoresed according to

methods previously described (Laemmli, 1970). Antigens were mixed with an

equal volume of sample buffer, electrophoresed at 200V for 30 minutes using the

mini-Protean II (BioRad) electrophoretic apparatus and silver stained (BioRad)

or electrophoretically transferred to nitrocellulose using a mini transblot apparatus

(BioRad) at 100V for one hour. The molecular weight standards used were

myosin (heavy chain, 200 kDa), phosphorylase b (92.5 kDa), bovine serum

albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin

inhibitor (21.5 kDa), and lysozyme (14.3 kDa) (Amersham).

Immunoblotting of antigens was performed according to methods

previously described (Towbin *et al.* 1978). Briefly, the SDS-polyacrylamide gel

was equilibrated in Transblot Buffer (0.025 M Tris; 0.192 M Glycine; 20%

Methanol), nitrocellulose was pressed onto the gel and assembled using a BioRad

Mini Transblot apparatus. Electroblotting was carried out at constant voltage

(100 V) for one hour with a BioRad 250/2.5 power supply. The nitocellulose was

blocked with blotto (5% powdered milk in 0.5 M BBS, HSBBS) for thirty minutes.

Strips were cut to approximately 5 mm with and stored frozen. Immunoblots were

probed with various antibodies for one hour at room temperature on a rotator

platform. After three washes in HSBBS containing 0.05% Tween-20 detergent

(Sigma), rabbit anti-mouse or goat anti-rabbit IgG specific antibodies conjugated

to alkaline phosphatase (Kirkegaard and Perry) were incubated with the

nitrocellulose strips for one hour. After washing in the same buffer as above, the

immunoblots were developed using with the enzyme substrate nitro blue

tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (Kirkegaard and Perry).

# PREPARATION OF IMMUNOAFFINITY PURIFICATION COLUMNS

MAbs CE2.1, DB8.2 specific for the N-terminus were used to isolate the 83 kDa N-terminal processing fragment and C-terminal specific mAb 5.2 was used to isolate the 42 and 19 kDa C-terminal processing fragments, in addition to gp195. These were covalently linked to either protein A or G sepharose and used to isolate antigen from crude extract by affinity chromatography.

High-capacity IgG immunosorbent was prepared according to methods previously described (Schneider *et al.* 1978). Approximately 20 mg of IgG were incubated with one ml of protein G-sepharose beads over night at 4°C in BBS. The beads were transferred to an EconoColumn (BioRad) and the column was washed slowly with fifty volumes of 200mM triethanloamine (pH 8.2) in BBS. Fifty volumes of 40 mM dimethyl pimelimidate in 200 mM triethanolamine/BBS buffer (pH 8.2) was added to covalently link the antibody to the protein A or G-sepharose. The column was covalently linked a second time (double linkage) with 40 mM dimethyl pimelimidate in 200 mM triethanolamine in $dH_2O$ and rotated over night at room temperature. The reaction was stopped with fifty volumes of 40 mM ethanolamine (pH 8.2), and the beads were washed with 10 volumes of BBS and stored at 4 °C.

Affinity chromatography was used to isolate gp195 rhoptry antigens from extracted saponin-lysed *P. falciparum* parasites according to methods previously described (Siddiqui *et al.* 1987). Parasites were thawed and sonicated three times for one minute and a crude extract was prepared using HSBBS containing 1 mM

ethylenediaminetetraacetic acid (EDTA), 1 mM ethylenegylcol-bis-N, N, N, N'-

tetraacetic acid (EGTA), 1.0% Non-iden-40 (NP-40) detergent, with 10 mM

iodoacetamide and 1 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma). The

solution was shaken on ice on a rocker platform for 2 hours, centrifuged at 18,000

X g for 30 minutes and the supernatant retrieved. The extract was incubated with

the appropriate IgG immunosorbent overnight on an end to end rotator at 4°C.

The material was then applied to an Econocolumn and the beads and bound

proteins were washed with 50 volumes of HSBBS containing 1 mM EDTA, 1 mM

EGTA, 0.1% NP-40, 0.02% SDS with 10 mM fresh iodoacetamide and 1 mM

fresh PMSF. Bound proteins were eluted with either 50 mM diethylamine (pH

11.5) or 100 mM glycine (pH 2.5) containing 10 mM iodoacetamide and

neutralized with 1/5 volume of 1 M TRIS-HCl (pH 8.0). Triton-X 100 (0.1%)

detergent (Sigma) was included in the elution buffer to help prevent aggregation

of rhoptry polypeptides. Protein determination was made using the Bicinchoninic

Acid (BCA) method (Redinbaugh *et al.* 1986; Pierce Chemicals).


## PURIFICATION OF FRAGMENTS BY SDS-PAGE AND ELECTROELUTION

SDS-PAGE, although generally used as an analytical technique, is also

useful for separating and purifying proteins. Using this method, the desired

processing fragment was retrieved from the gel by cutting protein bands out of the

gel after a section of the gel containing the molecular weight standard and part of

the protein sample was stained. The gel pieces (cut into approximately 1 mm square cubes) were placed into the loading channel of the electroelution cell unit (ECU-040, CBS Scientific) after equilibation in running buffer (50mM Tris acetate, pH 7.8 containing 1 mM EDTA). The sample was electreulted at 70 Volts overnight (15-20 hours). The eluted sample was removed and analyzed by SDS-PAGE with silver stain and immunoblot. Protein determination was performed using the BCA method (see above).

## IMMUNIZATION OF RABBITS

Three New Zealand white rabbits per group were immunized by intramuscular inoculations four times, each with 50 $\mu$g of antigen in Freund's Complete Adjuvant (FCA). Booster injections were given three times at four week intervals in Freund's Incomplete adjuvant (FIA). Serum samples were collected three weeks after each immunization. Preimmune serum was taken prior to immunization and used as a negative control.

## REDUCTION AND ALKYLATION OF gp195

To study the role of disulfide linkage in producing inhibitory antisera, affinity purified gp195 was reduced and alkylated according to methods previously described (Charbonneau, 1989). Briefly, 20 mM dithiothreitol (DTT) in 8M Urea (Sigma) was incubated with purified 750 $\mu$g of gp195 for four hours at 45°C. The protein was alkylated by the addition of iodoacetamide (Sigma) to a final

concentration of 60 mM for thirty minutes at room temperature in a light

protected container. The extent of reduction and alkylation was determined by

immunoblot using antibody reagents recognizing discontinuous (mAb 5.2 and

serum K44) and continuous epitopes (mAb G13 and serum K93).


DETECTION OF GELATINOLYTIC ACTIVITY

Gelatinolytic activity was determined by SDS-PAGE zymography according

to methods previously described (Heussen and Dowdle, 1980). Samples were

diluted with 1 volume of Laemmli's solubilizer and separated on 9.4%

polyacrylamide mini-gels containing 0.1% (w/v) of gelatin (Sigma, nr. G-2500,

Type I). The effect of proteinase inhibitors was determined after electrophoresis.

Gels were washed, sliced into lanes and incubated for 48 hours in incubation

buffer (TRIS buffer containing 2.5% TritonX-100). Activity was determined by

Coomassie stain. Inhibition of activity was determined by supplementing the

incubation buffer with one of the following protease inhibitors: soybean trypsin

inhibitor, antipain, leupeptin, pepstatin A, chymostatin (each at a final

concentration of 50 $\mu$g/ml), EDTA (10mM), pHMB (0.1mM), PMSF (2 mM) and

iodoacetamide (10 mM) (Sigma). Metal ion requirement for enzymatic activity

was determined by the addition of the following ions: $CoSO_4$, $CdSO_4$, $CuSO_4$,

$ZnSO_4$, $MnCl_2$, $MgCl_2$, and $CaCl_2$ at various concentrations. After identification

of calcium and cobalt as the essential metal ion, incubations were performed in

buffers supplemented with 1 mM $CaCl_2$ and 0.1 mM $CoSO_4$.

CHAPTER III.

ISOLATION OF GP195 AND ITS PROCESSING FRAGMENTS

## BACKGROUND AND LITERATURE REVIEW

The native *Plasmodium falciparum* merozoite surface glycoprotein gp195,

isolated by mAb affinity chromatography from in vitro cultured parasites, can

partially or completely protect monkeys against a lethal malaria infection (Hall *et*

*al.* 1984; Perrin *et al.* 1984; Siddiqui *et al.* 1987; Patarroyo *et al.* 1987a; Etlinger, *et*

*al.* 1991). However, neither gp195-based recombinant polypeptides expressed in

*E. coli* (Holder *et al.* 1988; Herrera *et al.* 1990) nor synthetic peptides (Cheung *et*

*al.* 1986; Pattaroyo *et al.* 1987b) have been as successful in affording protection.

Therefore, the study of the native molecule and its processing fragments was

undertaken to identify the protective structures and sequences.

The gp195 precursor molecule is processed into 83 kDa, 30 kDa, 36 kDa,

42 kDa and 19 kDa fragments present on the merozoite surface (Figure 1a;

Holder *et al.* 1987). An alternative processing scheme was proposed by Lyon and

coworkers who noted differences in the processing of the internal region of the

molecule (Figure 1b). The N-terminal sequence for the 83 kDa processing

fragment is the same as the N-terminus of the precursor molecule (Holder *et al.*

1985; Strych *et al.* 1987). N-terminal sequences are also known for the internal 36

kDa processing fragment and the 42 kDa C-terminal processing fragment

**A**



**B**
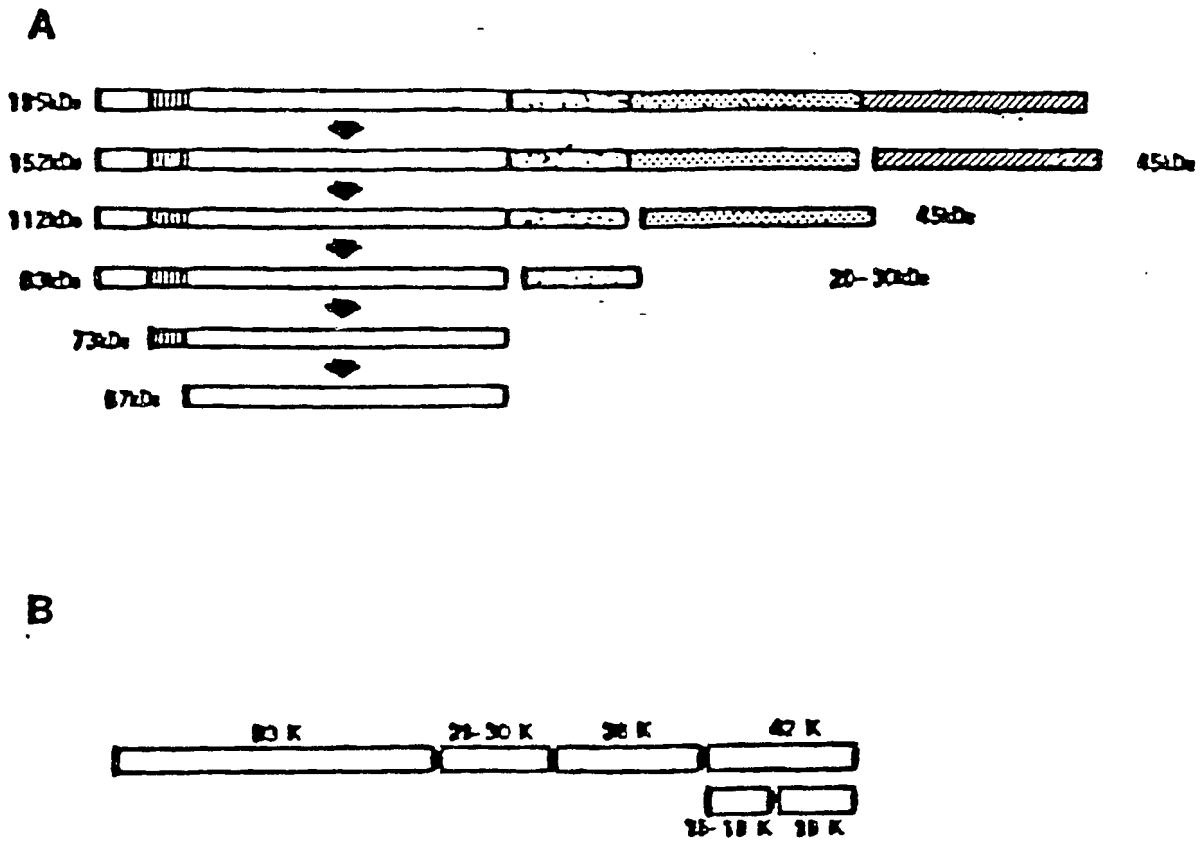


Figure 1.

Processing schemes of gp195 deduced from recombinant expression of the gene using the Wellcome-Lagos isolate (a) by Holder and co-workers and the CAMP isolate (b) by Lyon and co-workers.

(Heidrich *et al.* 1989). These data were in agreement with the processing scheme proposed by Holder and co-workers.

The 42 kDa C-terminal processing fragment is secondarily processed into a 33 kDa polypeptide at the N-terminus and a 19 kDa fragment at the C-terminus. It is a calcium dependent event, mediated by a membrane-associated serine protease (Blackman and Holder, 1992). The 33 kDa fragment is shed upon erythrocyte invasion and is non-covalently associated with the 83 kDa and internal 38 kDa processing fragments (Blackman *et al* 1991a; Blackman and Holder, 1992). The 19 kDa fragment remains with the parasite during ring-stage development, although its function reains unclear (Blackman *et al.* 1990). The N-terminal sequence of the 19 kDa fragment revealed a chymotrypsin-like cleavage site between lysine and asparagine residues (Blackman *et al.* 1991b). The apparent molecular weight of the 19 kDa fragment in SDS-PAGE is greater than would be expected, possibly due to the addition of a glycosylphosphatidylinositol anchor (Haldar *et al.* 1985) since it could not be accounted for by N-glycosylation.

The C-terminal region of gp195 contains thirteen cysteine residues clustered on the 42 kDa processing fragment and its 19 kDa subfragment (Holder *et al.* 1985; Chang *et al.* 1988). The arrangement of intrachain disulfide bridges may resemble epidermal growth factor (EGF) since the cysteine residues of the 19 kDa fragment appear to align with those of EGF (Blackman *et al.* 1991b). This arrangement also occurs in analogous merozoite surface antigen sequences of *P. yoelii* and *P. chabaudi* (Blackman *et al.* 1991b) and the 25 kDa ookinete surface

antigen of *P. falciparum* (Kaslow *et al.* 1988). These structural motifs may be involved in recognition of a receptor and adhesion (Appella *et al.* 1988).

The 83 kDa N-terminal processing fragment was previously purified from extracted merozoite surface proteins using gel sieve chromatography followed by anion exchange chromatography on FPLC Mono Q resin (Strych *et al.* 1987). Purified rabbit IgG antibodies raised to this antigen were not inhibitory. It was concluded that the 83 kDa processing fragment did not play a critical role in merozoite invasion. It was postulated to serve a protector function for the other processing fragments, where the 83 kDa fragment hides the critical epitopes on another fragment (ie. the C-terminus). This seems to be a reasonable hypothesis, since a baculovirus-expressed recombinant polypeptide based on the C-terminal sequence of gp195 was shown to produce growth inhibitory antibodies (Chang *et al.*, submitted). In addition, a mAb specific for the 19 kDa fragment was shown to inhibit parasite growth in vitro (Blackman *et al.* 1990). These data are supporting evidence that the C-terminal portion of the molecule is important in protective immunity based on gp195. The fact that the C-terminus remains with the parasite during the erythrocyte invasion process also indicates it plays an essential role in parasite development (Blackman *et al.* 1990).

In an independent study, *Aotus* monkeys immunized with the native 83 kDa processing fragment showed partial protection against a lethal malaria infection (Patarroyo *et al.* 1987b). This antigen had been electroeluted from merozoites separated on preparative SDS-PAGE. The possiblity of some denaturation and

loss of immunogenicity cannot be ruled out in the previous study (Strych *et al.* 1987). It is possible that the electroeluted 83 kDa fragment (Patarroyo *et al.* 1987b) served as a better immunogen than the 83 kDa fragment separated on the anion exchange column (Strych *et al.* 1987). Taken together, there is evidence that both fragments contain inhibitory epitopes.

The overall objective of this phase of the study was to isolate the N- and C-terminal native processing fragments in order to determine which of these fragments may be important in immunity. The specific objectives were to (1) isolate native gp195 from parasite cultures, (2) purify the 195 kDa precursor, the 83 kDa N-terminal and 42 kDa C-terminal processing fragments, and (3) raise monospecific polyclonal antisera to each of these purified polypeptides.

RESULTS

**Isolation of Polypeptides by mAb 5.2.** Native gp195 was isolated by immunoaffinity chromatography using the C-terminal specific mAb 5.2. Table 1 summarizes a series of extractions used to purify gp195. A total of 19.8 mgs of native gp195 antigen was purified from 306 vials of saponin-lysed parasites in 23 separate extractions using mAb 5.2. Each extraction was evaluated by silver stained SDS-PAGE gel and immunoblot assays. The antigen preparations were enriched in the C-terminal processing fragments (Figure 2). When antigen was electrophoresed under non-reducing conditions (lane 1), the 42 kDa C-terminal processing fragment migrated as a 38 kDa protein. However, under reducing
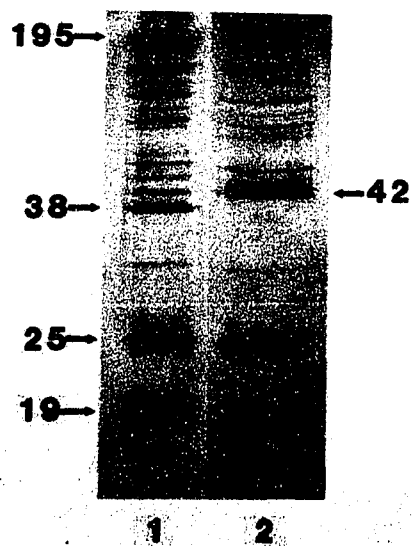
Figure 2.

Silver stain of a 12.5% SDS-PAGE gel mAb 5.2 immunoaffinity purified

antigen separated under non-reduced (lane 1) or reduced (lane 2)

conditions. Molecular weights of C-terminal processing fragments are

noted at 38 kDa, 25 kDa and 19 kDa under non-reducing conditions.

conditions (Fig. 2, lane 2), it migrated as a 42 kDa fragment, indicating the presence of intramolecular disulfide linkage. Although the majority of the cysteine residues were also contained within 19 kDa fragment, its migration in SDS-PAGE did not change upon reduction. There was a second prominent C-terminal processing fragment of 25 kDa.

Immunoblots probed with different antibody reagents distinguished the various processing fragments in the antigen preparation (Fig. 3). An antiserum to the native 42 kDa fragment K44 (lane 2) recognized the C-terminal fragments, while an antiserum to the recombinant N-terminal polypeptide K58 (lane 3) recognized only N-terminal fragments. Similar results were obtained with mAbs to the 83 kDa fragment (lanes 4 and 5) and the 42 kDa fragment (lane 6 and 7). The 83 kDa N-terminal processing fragment remained associated with the C-terminal processing fragments since it was isolated with C-terminal specific mAb 5.2. These data are consistent with previous observations using radiolabeled antigens, where mAbs immunoprecipitated an antigenic complex of gp195 processing fragments with N- and C-terminal specific mAbs (McBride and Heidrich, 1987).

**Isolation of the 83 kDa N-terminal Fragment Using an N-terminal Specific Monoclonal Antibody DB8.** A specific goal of this research was to isolate and characterize the 83 kDa processing fragment. It was decided that the initial
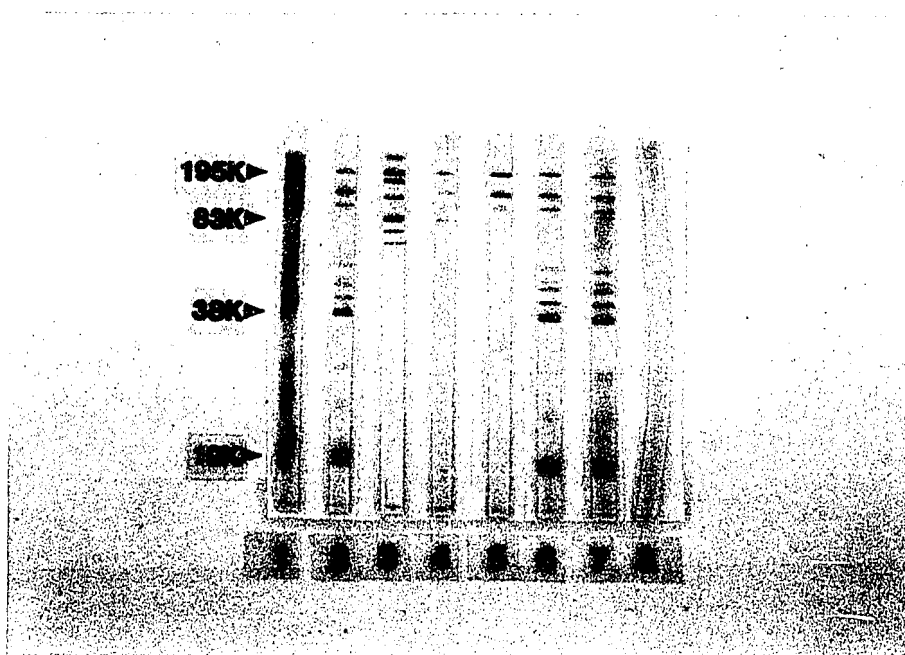
Figure 3.

Immunoblots of mAb purified gp195 separated by 10% SDS-PAGE.

Immunoblots were probed with a monospecific polyclonal serum against

gp195 (K41, 42 and 43) (lane 1), a monospecific polyclonal antiserum

against the 42 kDa processing fragment (K44) (lane 2), a monospecific

polyclonal antiserum against the gp195 N-terminal recombinant polypeptide

(K58) (lane 3), mAb CE2 (lane 4), mAb DB8 (lane 5), mAb AD9 (lane 6),

mAb 5.2 (lane 7) and a monospecific polyclonal antiserum against RAP-3

(K15) (lane 8).

isolation should be made using the C-terminal specific mAb 5.2, since this was the

standard protocol used for isolating the polypeptide shown to be protective in this

laboratory (Siddiqui *et al.* 1987). Therefore, we wished to characterize the

fragments isolated with an N-terminal specific mAb.

The experiments above demonstrated that the 83 kDa fragment could be

isolated using the C-terminal specific mAb 5.2. This was possible because the N-

and C-terminal fragments appear to be non-covalently associated. While it was

therefore possible to isolate the 83 kDa fragment along with the 42 kDa fragment

using the C-terminal mAb, we wished to determine if additional 83 kDa

processing fragment could be isolated from the remaining parasite extracts

depleted of gp195 using the 42 kDa specific C-terminus mAb 5.2. Immunoaffinity

columns were made with the 83 kDa N-terminal mAb DB8. Crude parasite

extracts were repeatedly applied to the mAb 5.2 column until parasite proteins

were completely removed, then applied to a mAb DB8 affinity column. In 23

such extractions, 19.8 mg of gp195 was recovered with the C-terminal mAb 5.2

(Table I). An additional 1.6 mg of gp195 was recovered using the N-terminal

specific mAb DB8, an average of less than 10% residual gp195.

To determine if greater amounts of the 83 kDa fragment was recoverable

using the N-terminal specific mAb DB8 relative to the C-terminal specific mAb

5.2, equal amounts of protein were analyzed by SDS-PAGE with silver-stain and

immunoblot analysis. Silver-staining of proteins resolved in 6.25% polyacrylamide

TABLE 1

mAb Affinity Purification of gp195 from *Plasmodium falciparum*

| Extraction | Flasks | Vials | 5.2* | DB8 |
|---|---|---|---|---|
| 61-65 | 500 | 100 | 7.10 | 0.95 |
| 66-70 | 360 | 73 | 3.50 | 0.21 |
| 71-77 | 480 | 93 | 4.10 | 0.39 |
| 78-84 | 300 | 60 | 5.10 | - |
| **Total** | 1640 | 306 | 19.80 | 1.56 |

* Total protein isolated by mAbs in milligrams

Extractions refers to the number of individual experiments where membrane proteins were extracted from saponin-lysed parasites. Flasks refers to the number of 2.8 litre erlynmeyer flasks used to culture parasites in vitro. Approximately five of these flasks produced one vial of saponin-lysed parasites. 12 vials were used in each extraction experiment. mAb 5.2 is specific for the C-terminus of gp195, while mAb DB8 is specific for the N-terminus.
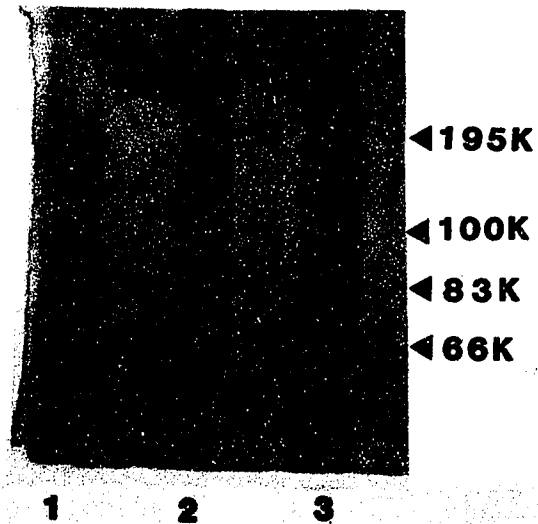
Figure 4.

Silver-stained 6.25% SDS-PAGE gel of mAbs 5.2 and DB8 immunoaffinity

purified antigens. Lane 1 is the molecular weights standard of myosin (heavy

chain, 200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa),

ovalbumin (46 kDa). Lane 2 is the antigen purified with mAb 5.2 specific for

the C-terminus of gp195. Lane 3 is the antigen purified with mAb DB8 specific

for the N-terminus of gp195.

gels suggested that the N-terminal specific mAb DB8 was more efficient at recovering the 83 kDa fragment (Fig. 4). In addition a doublet at the MW 195,000 region was isolated with mAb DB8 (lane 3); whereas, only a single band at this MW was isolated using mAb 5.2. Also, bands in the region of 110 and 66 kDa (lane 3), probably corresponding to N-terminal processing fragments reported by Lyon and co-workers (1986) were detected. These polypeptides were not isolated using mAb 5.2 (lane 2).

To further characterize the protein isolated by mAb DB8, proteins resolved on 6.25% (Fig. 5) and 10% (Fig. 6) polyacrylamide gels were probed with various antibodies specific for N- and C-terminal processing fragments in immunoblots. This is a more sensitive method than silver staining for detecting minor quantities of proteins. The gp195 preparation obtained with mAb DB8 contained an "intermediate processing fragment" of about 100 kDa detected by the N-terminal specific antiserum (Fig. 5, lanes 2). A co-migrating C-terminal processing fragment was recognized by C-terminal specific antiserum (lane 3).

Since low MW proteins are not resolved on 6.25% polyacrylamide gels, the same experiment was performed using 10% polyacrylamide gels (Fig. 6). Immunoblots probed with the C-terminal specific antiserum K44 (lane 3) indicated that the gp195 isolated with the N-terminal specific mAb did contain the C-terminal processing fragments. Thus, C-terminal fragments can be isolated with N-terminal specific mAbs, presumably because the fragments are non-covalently associated.
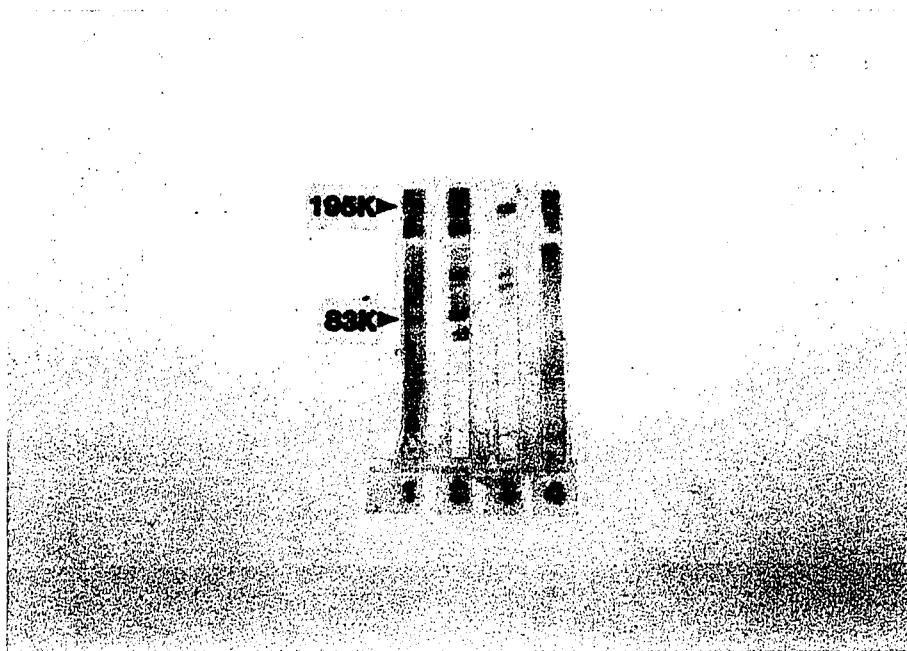
Figure 5.

Immunoblot of antigen isolated by mAb DB8 separated by 6.25% SDS-PAGE.

Immunoblots were probed with a pool of monospecific polyclonal antiserum

(K41, K42, and K43) (lane 1), a monospecific polyclonal antisera against the

gp195 N-terminal recombinant polypeptide (K58) (lane 2), a monospecific

polyclonal antiserum against the 42 kDa processing fragment (K44) (lane 3)

and a monospecific polyclonal antiserum against RAP-3 (K15) (lane 4).
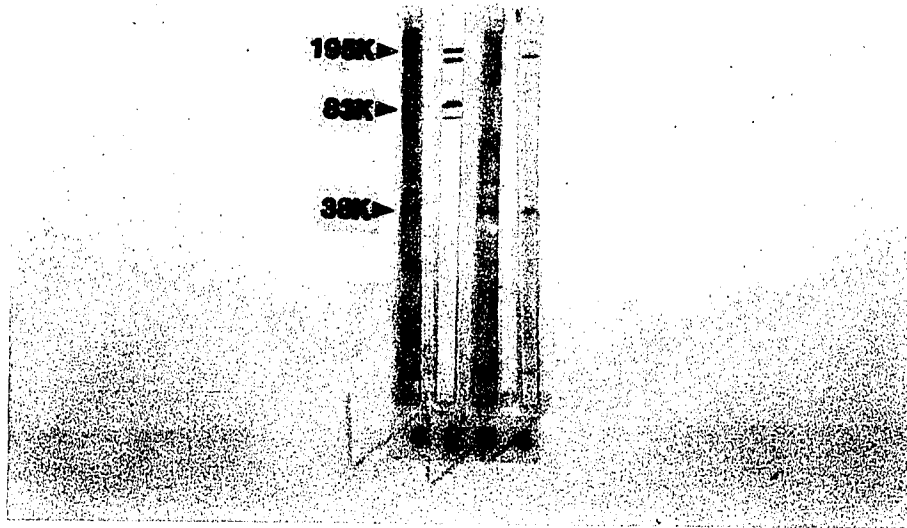
Figure 6.

Immunoblot of antigen isolated with mAb DB8 separated by 10% SDS-PAGE gel. Immunoblots were probed with a pool of monospecific polyclonal serum to native gp195 (K41, K42, K43, lane 1), a monospecific polyclonal serum to the yeast-expressed N-terminal region of gp195 (K58, lane 2), a monospecific polyclonal serum to the native 42 kDa C-terminal processing fragment (K44, lane 3) and a yeast-expressed C-terminal region of gp195 (K93, lane 4).
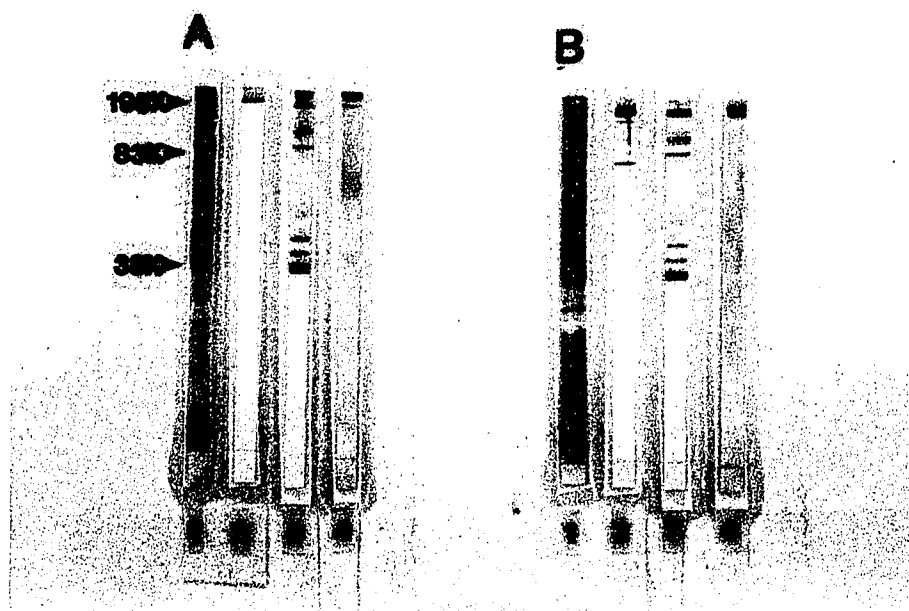
Figure 7.

Immunoblots of antigens isolated with mAb 5.2 (A) and mAb DB8 (B)

separated by 10% SDS-PAGE. Immunoblots were probed with a pool of

monospecific polyclonal serum to native gp195 (K41, K42, K43, lane 1), a

monospecific polyclonal serum to the yeast-expressed N-terminal region of

gp195 (K58, lane 2), a monospecific polyclonal serum to the native 42 kDa C-

terminal processing fragment (K44, lane 3) and a monospecific polyclonal

antiserum to RAP-3 (K15, lane 4).

The overall results are summarized in Fig. 7A and 7B, where immunoblots of both mAb 5.2 (C-terminal) and mAb DB8 (N-terminal) affinity purified gp195 preparations are compared on the same gel. Whether a C-terminal (Fig. 7A) or N-terminal (Fig. 7B) mAb was used for isolation, the C-terminal processing fragments were isolated as shown by the bands at 38K (lanes 3 in both Figs.) There was an enrichment of the N-terminal 83 kDa processing fragment in the antigen preparation isolated with N-terminal mAb DB8 (Fig. 7A, lane 2 vs. Fig. 7B, lane 2). Some of the 83 kDa processing fragment was associated with C-terminal fragments isolated with mAb 5.2, but a small amount was also detected with mAb DB8. However, this too was associated with C-terminal processing fragments. In summary, although affinity purification with mAb DB8 resulted in increased yields of the N-terminal 83 kDa fragment, it was not free of C-terminal polypeptides. Presumably, this was due to non-covalent association of N-terminal and C-terminal polypeptides.

**Attempts to Disrupt the Non-covalent Association of N-terminal and C-terminal Polypeptides.** A number of experiments were performed to determine if the noncovalent association of the C-terminal processing fragment with the N-terminal processing fragments could be dissociated. First, the standard non-ionic detergent used in extractions (NP-40) was supplemented with the ionic detergent sodium dodecyl sulfate (SDS). Parasites were extracted in detergent consisting of a mixture of 0.2% SDS and 1.0% NP-40. Use of this detergent combination

resulted in very low yields of antigen (less than 100 μg) isolated by both mAb 5.2 and mAb DB8 (less than 100 ug each). In addition, immunoblots of the antigen purified using this detergent showed that the 42 kDa and 19 kDa C-terminal processing fragments were still present in the polypeptides isolated with the N-terminal mAb DB8 (Fig. 8).

Secondly, the ionic strength of the buffer was increased in attempts to dissociate the antigenic complex formed by the N- and C-terminal processing fragments. Parasites were extracted in buffer containing 750mM NaCl, 0.2% SDS and 1.0% NP-40. It was necessary to perform isolations in the cold, since this extraction buffer became opaque at room temperature. Again, the yield was much lower than usual (less than 100 ug) and the C-terminal processing fragments were co-isolated with mAb DB8.

A third attempt to dissociate the antigenic complex formed by the N- and C-terminal processing fragments was tried by differential elution with high and low pH buffers. Previous analysis of affinity purified antigens differentially eluted from the mAb affinity columns with 50 mM diethylamine (pH 11.5) followed by 100 mM glycine (pH 2.5) showed that some of the 83 kDa fragment eluted from mAb 5.2 columns with the glycine buffer. This experiment was repeated using the mAb DB8 and an extraction buffer containing 750 mM NaCl, 0.2% SDS and 1.0% NP-40. Analysis of eluted antigens by immunoblot assay again showed that the N- and C-terminal fragments were not completely disrupted (data not shown).
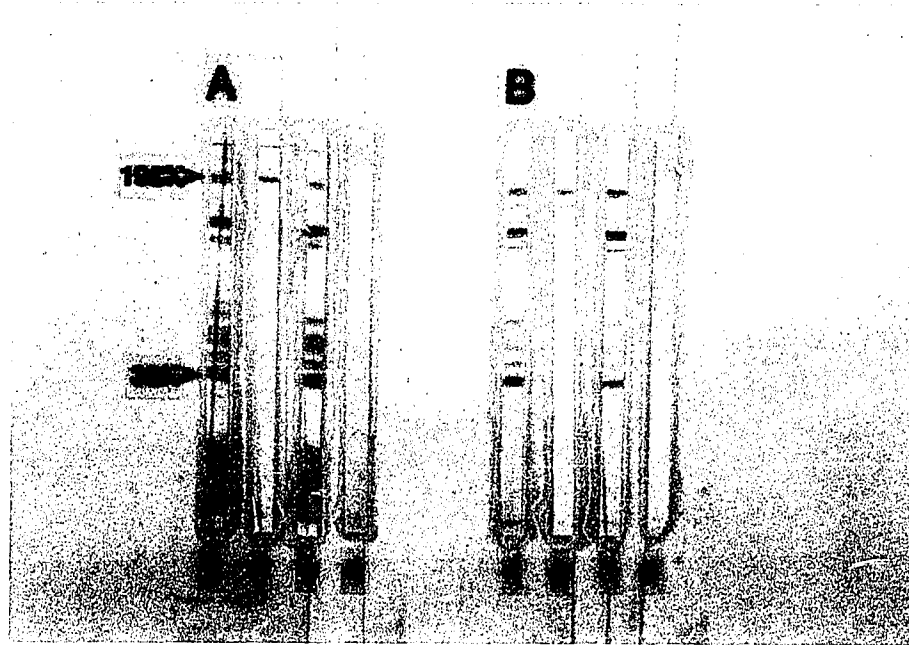
Figure 8.

Immunoblots comparing the antigens isolated with C-terminal-specific mAb 5.2

(A) and N-terminal-specific mAb DB8 (B) using ionic detergent. Proteins were

separated on a 10% SDS-PAGE gel. A pool of rabbit monospecific polyclonal

antierum to native gp195 (K41, K42, K43 pool, lane 1), a rabbit monospecific

polyclonal antserum to the yeast-expressed N-terminal polypeptide (K58, lane

2), a rabbit monospecific polyclonal antiserum to the native 42 kDa C-terminal

polypeptide (K44, lane 3) and a rabbit monospecific polyclonal antiserum to

RAP-3 (K15, lane 4) were the antibody reagents used to probe the

immunoblot.

**Electroelution.** An alternative strategy of preparative SDS-PAGE and electroelution of mAb 5.2 affinity purified antigen was implemented to isolate the 195 kDa precursor and the 83 kDa N-terminal and the 42 kDa C-terminal processing fragments. After three electroelution experiments, it was found that the recovery of the 83 kDa fragment from antigen preparations isolated by mAb DB8 was not enough for the immunization of rabbits. Preparations of gp195 isolated with the C-terminal mAb 5.2 were used to isolate the 83 kDa fragment. A total of sixteen preparative SDS-PAGE and electroelution experiments were conducted for the purification of the 195 kDa precursor and its 83 kDa N-terminal and 42 kDa C-terminal fragments (Table II). The recovery of these polypeptides was 51% and is close to the expected values using this electroelution apparatus (Hunkapillar, 1984). As expected, the 83 kDa polypeptide had the lowest yield.

A silver stained SDS-PAGE gel of the electroeluted proteins suggested that some proteolytic breakdown had occurred, especially with the 195 kDa fragment (Fig. 9). In addition, some aggregation had occurred, as the 195 kDa polypeptides appeared as a heterogenous band. An immunoblot of the electroeluted 195 kDa polypeptide probed with various N- and C-terminal antibodies showed clearly that the 195 precursor had been degraded into numerous polypeptides (Fig. 10). The C-terminal polypeptides recognized by K44 and mAb 5.2 were clearly evident. The presence of bands with MW's greater than 195,000 suggested that some of the antigens had aggregated.

TABLE 2


Isolation of gp195 Processing Fragments by Electroelution


| Antigen | Yield ($\mu$g) | Recovery |
| --- | --- | --- |
| 195 kDa | 200 | 17% |
| 83 kDa | 215 | 11% |
| 42 kDa | 365 | 23% |

Total: 51%
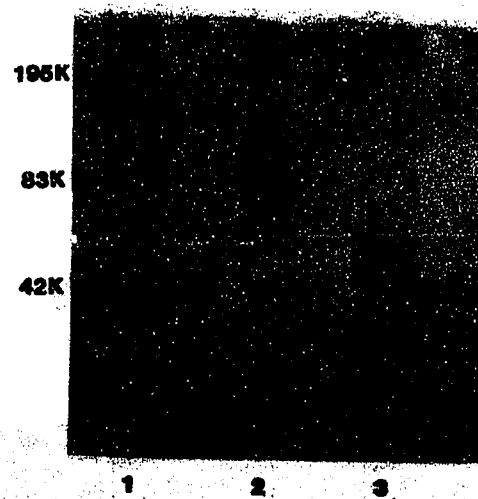
Figure 9.

A 10% SDS-PAGE gel of antigens purified by electrelution and visualized by silver staining. The 195 kDa precursor protein (lane 1), the 83 kDa N-terminal processing fragment (lane 2), and the 42 kDa C-terminal processing fragment were purified from gel slices electroeluted from SDS-PAGE.
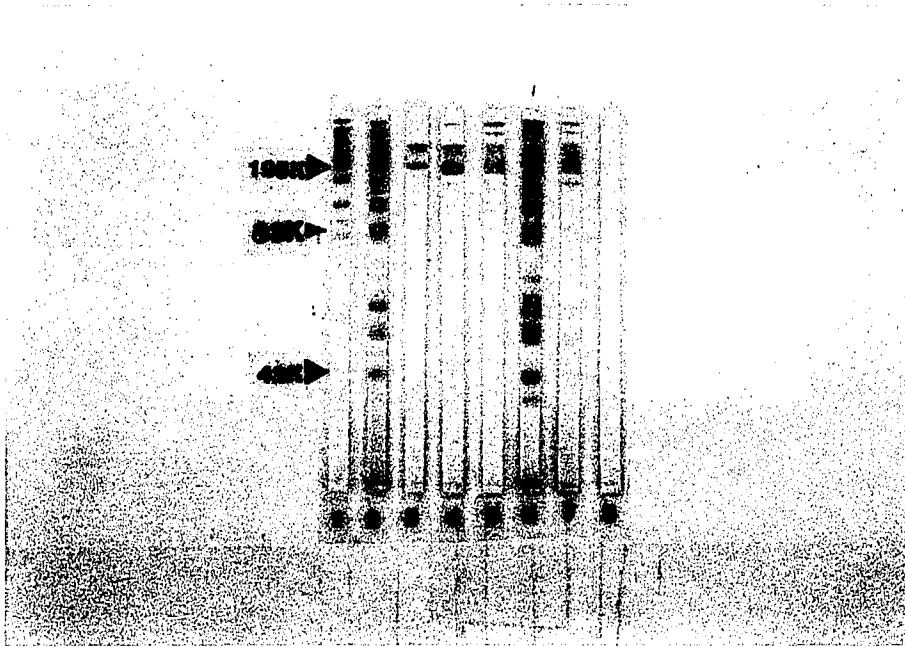
Figure 10.

Immunoblot of electroeluted 195 kDa precursor protein probed with a

monospecific polyclonal antiserum to the N-terminal yeast-expressed

polypeptide (K58, lane 1), a monospecific polyclonal antiserum to the native 42

kDa polypeptide (K44, lane 2), mAbs specific for the N-terminus (CE2 and

DB8, lanes 3 and 4, respectively), a mAb specific for an internal region of

gp195 (S1.7, lane 5), mAbs specific for the C-terminus of gp195 (5.2 and BC9,

lanes 6 and 7, respectively) and a monospecific polyclonal antiserum to RAP-3

(K15, lane 8).

An immunoblot of the electroeluted 83 kDa processing fragment probed with the N-terminal specific antibodies K58 (lane 1), mAb CE2 (lane 2) and mAb DB8 all identified the electroeluted 83 kDa processing fragment, while the C-terminal antibodies did not (Fig. 11). The weakness of the bands is probably due to the low initial yield with this protein and the inability to load higher protein amounts into the electrophoresis apparatus, since the initial antigen concentration was low.

The 42 kDa electroeluted processing fragment reacted with polyclonal rabbit serum K44 (lane 1), raised to the native 42 kDa processing fragment (Fig. 12). In addition, each of four C-terminal mAb tested also showed reactivity to the 42 kDa processing fragment (lanes 2 to 5). However, N-terminal specific polyclonal rabbit antiserum K58 (lane 6) and mAb CE2.1 did not react, indicating that no co-migrating N-terminal processing fragments were present. Electroelutions of the 19 kDa C-terminal processing fragment were also free from contamination from N-terminal polypeptides, since there was no reaction detectable with N-terminal specific antiserum K58 (Fig. 13).

Rabbits were immunized with the electroeluted processing fragments and their reciprocal ELISA antibody titers were determined (Table III). Rabbits K73, K75 and K76 immunized with the total polypeptides purified by mAb 5.2 had high titers (1/12,000, 1/80,000 and 1/25,000) to native gp195. In general, rabbits immunized with electroeluted antigens had slightly lower titers. For example, rabbits receiving the 195 kDa precursor had ELISA titers of (1/10,000, 1/30,000 and 1/17,000). Rabbits receiving the 83 kDa fragment had the lowest titers
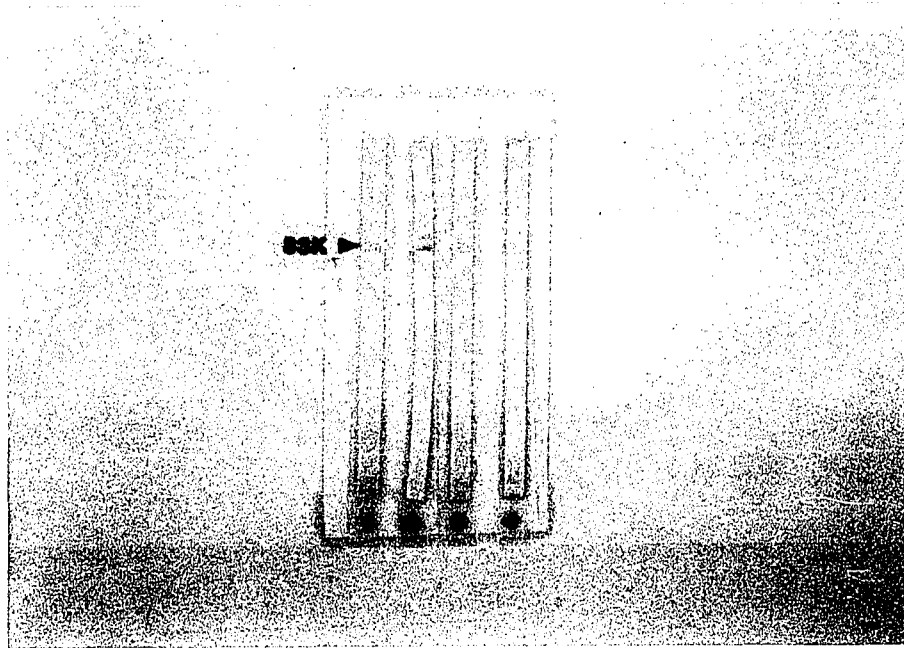
Figure 11.

Immunoblot of the electroeluted 83 kDa fragment probed with a monospecific

polyclonal antiserum raised to the yeast-expressed N-terminal polypeptide

based on the gp195 sequence (K58, lane 1), mAbs specific for the N-terminus

of gp195 (CE2 and DB8, lanes 2 and 3, respectively) and a monospecific

polyclonal serum raised to the C-terminal 42 kDa fragment of gp195 (K44, lane

4).
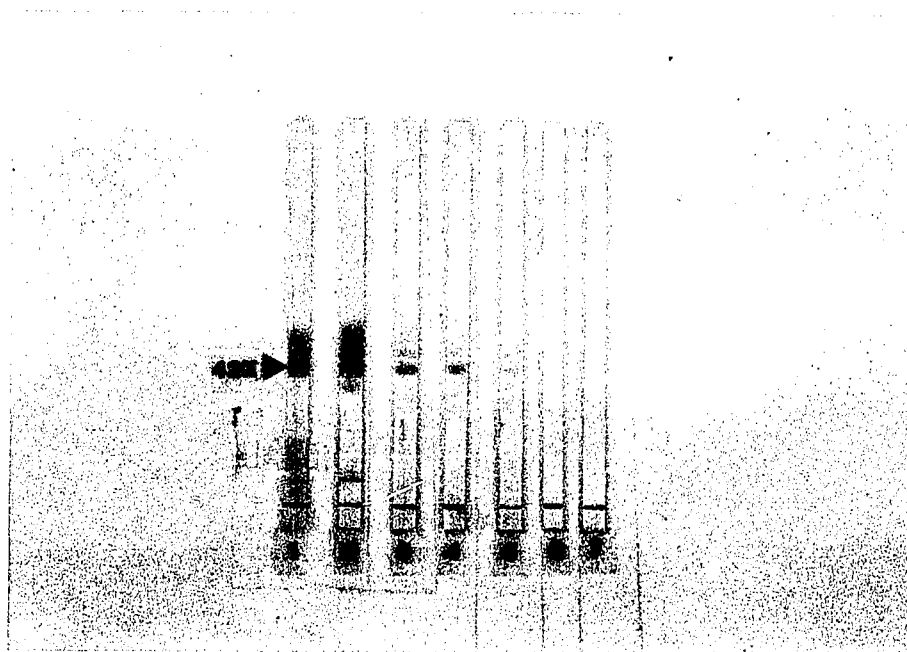
Figure 12.

Immunoblot of the electroeluted 42 kDa fragment probed with a monospecific

polyclonal antiserum raised to the C-terminal 42 kDa fragment of gp195 (K44,

lane 1), a mAb specific for the C-terminus of gp195 (5.2, lane 2), a mAb

specific for a internal region of gp195 (S1.7, lane 3), mAbs specific for the C-

terminus of gp195 (AD9 and BC9, lanes 4 and 5, respectively), a monospecific

polyclonal antiserum raised to the yeast-expressed N-terminal polypeptide

based on the gp195 sequence (K58, lane 6) and a mAb specific for the N-

terminus of gp195 (CE2, lane 7).

46
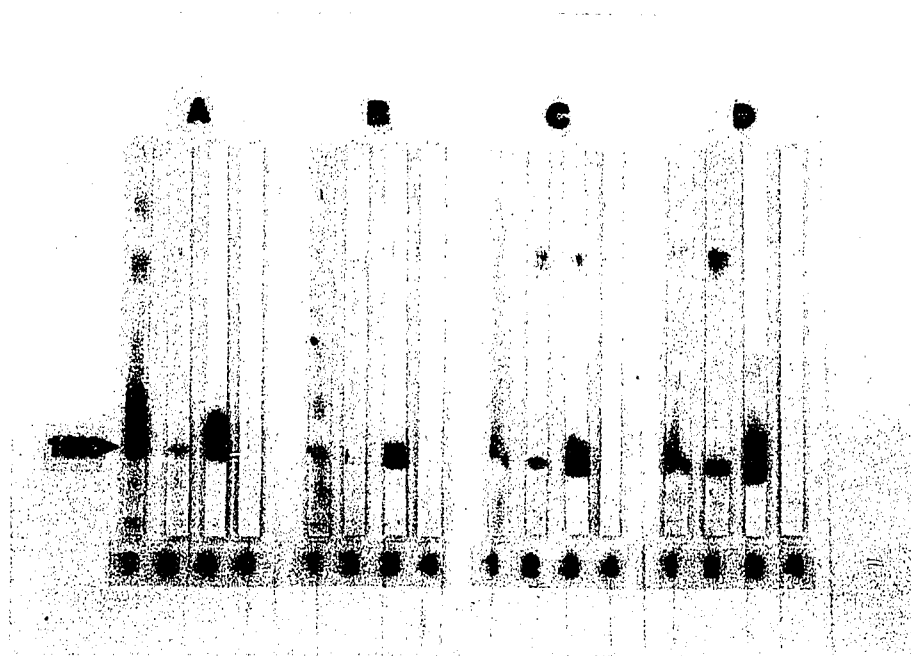


Figure 13.

Immunoblot of the electroeluted 19 kDa fragment from four individual
experiments probed with A pool of rabbit monospecific polyclonal antierum to
native gp195 (K41, K42, K43 pool, lane 1), a rabbit monospecific polyclonal
antiserum to the native 42 kDa C-terminal polypeptide (K44, lane 2) and a
rabbit monospecific polyclonal antserum to the yeast-expressed C-terminal
polypeptide (K93, lane 3), a rabbit monospecific polyclonal antserum to the
yeast-expressed N-terminal polypeptide (K58, lane 4)

## TABLE 3

### ELISA Titers of Serum Samples from Rabbits Immunized with gp195 and its Processing Fragments

| Rabbit No. | Immunogen[a] | Reciprocal ELISA titer[b] |
| --- | --- | --- |
| 73 | Native gp195 | 12,000 |
| 75 | " | 80,000 |
| 76 | " | 25,000 |
| 120 | Electroeluted 195 kDa | 10,000 |
| 121 | " | 30,000 |
| 126 | " | 17,000 |
| 122 | Electroeluted 83 kDa | 10,000 |
| 123 | " | 9,500 |
| 127 | " | 1,800 |
| 124 | Electroeluted 42 kDa | 19,000 |
| 125 | " | 17,500 |
| 128 | " | 12,000 |

[a]Rabbits were immunized with 50 $\mu$g native gp195 or SDS-PAGE electroeluted polypeptides four times intramuscularly in multilamellar liposomes containing B30-MDP/LA-15-PH synthetic adjuvants.

[b]Serum samples were taken 21 days after the secondary immunization (21d $2^0$)

(1/10,000, 1/9,500 and 1/1,800), which may reflect the enrichment of C-terminal fragments isolated by mAb 5.2 in the antigen used in the preparation of the ELISA.

The above antisera were used to probe immunoblots of total gp195 polypeptides and to determine their specificity. Antisera from rabbits K120, K121 and K126 immunized with the electroeluted 195 kDa precursor protein recognized N- and C-terminal processing fragments. Antisera from rabbits K122, K123, and K127 immunized with the electroeluted 83 kDa N-terminal processing fragment recognized the 83 kDa fragment in addition to the 42 and 19 kDa C-terminal fragments, indicating that the electroeluted preparation contained C-terminal contaminants (not shown). Serum samples from rabbits K124, K125 and K128 immunized with the electroeluted 42 kDa preparation recognized the gp195 precursor as well as the 38 kDa (42 kDa under reducing conditions) and 19 kDa C-terminal processing fragments. In addition, these antisera also identified the 83 kDa N-terminal fragment (not shown), indicating that this preparation contained N-terminal fragments. Therefore, the experiments using the electroeluted processing fragments were discontinued.

**In vitro Parasite Growth Inhibition by Antisera to the Electroeluted 195 kDa Precursor.** Antisera from rabbits immunized with electroeluted 195 kDa precursor and rabbits immunized with native gp195 were compared by ELISA, IFA and the in vitro growth inhibition assay. Rabbits immunized with native

gp195 had a mean ELISA titer of 1/560,000 to native gp195 and its processing

fragments. However, these antisera had a mean ELISA titer of only 1/4,400 to

the electroeluted 195 kDa precursor (Table IV). Rabbits immunized with the

electroeluted 195 kDa precursor had a mean ELISA titer of 1/152,000 to native

gp195 and a mean ELISA titer of only 1/9,400 to the electroeluted antigen.

Nevertheless, the antibody titers induced by electroeluted gp195 to native gp195

by ELISA and IFA were lower than the antibody titers to native gp195. These

data indicated that the SDS-PAGE electroeluted antigen was less antigenic and

less immunogenic than the native antigen. Finally, antisera from 2 of the 3

rabbits immunized with native gp195 efficiently inhibited parasite growth (Table

IV), while antisera from rabbits immunized with the electroeluted 195 kDa

precursor were generally less efficient at inhibiting parasite growth in vitro. This

suggests that the electroeluted antigen had lost its antigenicity, immunogenicity

and ability to induce biologically active antibodies.


DISCUSSION

**Non-covalent association of the 83 kDa processing fragment with C-terminal**

**processing fragments.** The method for isolation of protective polypeptides using

mAb 5.2 (Siddiqui et al. 1987) produces, in addition to the precursor gp195

molecule, a preparation enriched in the 42 kDa, 25 kDa and 19 kDa C-terminal

fragments (Fig. 2). This is not surprising, since mAb 5.2 recognizes an epitope in

the C-terminal 19 kDa fragment and all its larger, parent fragments including the

TABLE 4

ELISA titer and Parasite Growth Inhibition by Serum
Samples from Rabbits Immunized with Native or SDS-PAGE Electroeluted gp195

| Rabbit No. | Immunogen[a] | Reciprocal ELISA titer[b] Native | Electro. | IFA titer | %Inhibition Exp.1 | Exp.2 |
|---|---|---|---|---|---|---|
| 108 | Native | 580,000 | 4,500 | 20,000 | 93 | 100 |
| 109 | " | 760,000 | 5,000 | 20,000 | 95 | 93 |
| 110 | " | 340,000 | 3,700 | 10,000 | 52 | 72 |
| 120 | Electro. | 220,000 | 12,000 | 2,500 | 10 | 40 |
| 121 | " | 85,000 | 6,250 | 5,000 | 53 | 36 |
| 126 | " | 150,000 | 10,000 | 10,000 | 44 | 10 |

[a]Rabbits were immunized with 50 $\mu$g native gp195 or SDS-PAGE electroeluted polypeptides four times intramuscularly in multilamellar liposomes containing B30-MDP/LA-15-PH synthetic adjuvants.

[b]Serum samples were taken 21 days after the fourth immunization (21d, $4^0$).

42 kDa fragment and its 195 kDa precursor. However, the 83 kDa N-terminal processing fragment remains associated with these C-terminal proteins during the purification procedure. These data are similar to those reported for radiolabeled merozoite surface antigens immunoprecipitated with various mAbs to the 19 kDa C-terminal gp195 fragment (McBride and Heidrich, 1987). The 83 kDa and 42 kDa processing fragments can form an antigenic complex with each other and two additional proteins of 36 kDa and 22 kDa. These are dissociated when isolated in the presence of ionic detergent (zwitterions), or are frozen and thawed after extraction, indicating that the associations are non-covalent ionic interactions (McBride and Heidrich, 1987). Apparently, the inclusion of 0.2% SDS ionic detergent in the extraction buffer was insufficient to dissociate the antigenic complex in these experiments. In addition, the significantly lower yield of antigen indicated that this was not an efficient method of purifying processing fragments.

**Low yield of 83 kDa fragment in parasite extracts.** The major question we wished to answer was whether additional N-terminal 83 kDa fragment remained in the crude parasite protein extracts after depletion using the C-terminal mAb 5.2. In order to answer this question, the mAb DB8 against the 83 kDa polypeptide was used in a second, sequential adsorption following depletion with mAb 5.2. Less than 10% of the 83 kDa associated gp195 polypeptides remained in such preparations (Table I). The lower yield of the 83 kDa N-terminal fragment may be due to the shedding of this antigen into the culture medium (Holder *et al.* 1985; Camus *et al.* 1987). The 19 kDa processing fragment remains

with the parasite during ring-stage development (Blackman *et al*, 1990). Although more of the 83 kDa fragment was recovered with mAb DB8 (Figs. 4 & 5), the total amount of 83 kDa polypeptide was still very low, when compared to the C-terminal processing fragments. Thus, use of the N-terminal mAb DB8 did not increase the yield of the 83 kDa fragment, which appears to be inherently related to its being shed from the parasite surface following merozoite invasion.

**Inability to Break Non-covalent Associations.** In spite of the presence of added ionic detergent (0.2% SDS) and the inclusion of 750 mM NaCl, the 83 kDa processing fragment remained associated with the C-terminal processing fragments, and it was not possible to disrupt the noncovalent interaction using these methods. In retrospect, the zwitterion and freeze-thaw methods of McBride and Heidrich (1987) could have been investigated. However, we have observed that significant antigen aggregation occurs following repeated freezing and thawing of preparations. This procedure may be useful in qualitative studies with small quantities of metabolically labeled parasite proteins, but it was not thought to be a reasonable method for purification of processing fragments. Therefore, the decision was made to attempt electroelution of fragments from mAb 5.2 isolated gp195 polypeptide preparations.

**Degradation of Electroeluted Proteins.** During the electroelution process, some protein degradation occurred. Many of the degradation sites on the precursor molecule lay in the C-terminus, as determined by immunoblots probed with C-terminal specific antibody. Although the running buffer should be free of

protease activity due to the presence of SDS and the proteolytic inhibitor EDTA, protease activity may have been present and subjected the precursor to proteolytic degradation. Degradation of antigen preparations has been noted in frozen gp195 preparations stored for long periods, even at -70°C.

**Presence of Minor N-terminal Contaminants in the Electroeluted C-terminal Preparation.** The fact that serum samples from rabbits immunized with the electroeluted 42 kDa polypeptide preparation contained antibodies that reacted in immunoblots with the 83 kDa N-terminal fragment indicates that there was a small amount of contamination with N-terminal fragments. This N-terminal contaminant probably was about the same molecular size and co-migrated with the C-terminal fragment in the 42K region of the preparative SDS gel. This was probably a minor contaminant, and could not be detected in immunoblots of the electroeluted polypeptide preparation. Thus, while the electroeluted antigen preparations appeared uncontaminated by silver-staining and immunoblot assays, antisera produced in rabbits showed that minor, contaminating polypeptides were present. These data suggest that electroelution is not capable of providing antigens which can induce N-terminal or C-terminal specific antisera.

**Diminished Antigenicity and Immunogenicity of Electroeluted Polypeptides.** Electroeluted 195 kDa precursor had reduced antigenicity when compared with native gp195 in ELISA (Table IV). This was not due to disruption of disulfide bonds, since the separation of polypeptides was performed in the absence of reducing agent (this work will be described in Chapter IV). One possible

explanation for the diminished antigenicity is that putative epitopes formed by the quaternary association of individual processing fragments were destroyed by the presence of SDS in the non-reduced Laemmli's electrophoresis buffer. Another explanation for the reduced antigenicity is that it is a technical artifact. It is possible that detergent SDS molecules remained associated with the electroeluted 195 kDa polypeptide, causing some denaturation of the molecule, which resulted in decreased antigenicity.

The electroeluted 195 kDa precursor also appeared to have diminished immunogenicity in rabbits. The lower antibody titer to native gp195 by ELISA and IFA (Table IV) might be explained by the presence of detergent SDS molecules with the molecule, which interfere with liposome formation, interaction with the B30-MDP/LA-15-PH adjuvant, or antigen processing by antigen presenting cells during antibody formation. Denaturation by SDS detergent may have also decreased the immunogenicity of the electroeluted antigen. However, the decrease in immunogenicity was not as dramatic as that due to reduction and alkylation (Chapter IV).

**Diminished In Vitro Inhibition by Electroeluted 195 kDa Polypeptide.**
Antisera from rabbits immunized with electroeluted 195 kDa precursor also had low parasite growth inhibitory activity in vitro when compared to antisera from rabbits immunized with antive protein. The low parasite growth inhibition may be due to the low antibody titer. In an attempt to correct for this, rabbits were given a fourth booster injection, but this failed to increase antibody titers to levels

comparable with the levels obtained with native, unelectroeluted gp195 preparations.

Recently, Chang and co-workers reported that the baculovirus-expressed 42 kDa processing fragment (Chang *et al.* submitted, 1992) induces antibodies which completely inhibit parasite growth. It is possible that these epitopes are absent from the 195 kDa precursor molecule and form only after proteolytic cleavage of this processing fragment. That is, the protective epitopes may be present only within the processing fragment itself. It is not likely that these epitopes are formed by the quaternary structure of the association of processing fragments, since the 42 kDa baculovirus expressed C-terminal fragment could induce growth inhibitory antibodies by itself.

**Conclusion:** Attempts to devise methods for the isolation of antigenic and immunogenic N- and C-terminal processing fragments were undertaken in order to determine which of the fragments contained in the gp195 polypeptide pool through non-covalent association, were responsible for protective immunity. The problems of non-covalent association of the polypeptides could not be resolved, and the plan for using differential adsorption with N-terminal followed by C-terminal specific mAbs, and vice versa, was not pursued. In addition, the alternative electroelution approach yielded preparations with low, but undetectable levels of contaminating polypeptides, probably due to uncontrolled proteolysis. Thus, the method of electroelution, in our hands, gave unequivocal answers. In the end, the ultimate goal of identifying a relevant protective

processing fragment was resolved by the ongoing parallel track employing

recombinant polypeptides, rather than by isolation of native, parasite derived

polypeptides (Chang *et al.* 1992; submitted). It is still not clear if the N-terminal

83 kDa processing fragment is important in inducing inhibitory antibodies, since

the results of yeast-expressed polypeptides have not inhibited parasite growth in

vitro (Chang, unpublished). However, the finding that the baculovirus expressed

C-terminal 42 kDa polypeptide induces antibodies which completely inhibit

parasite growth in vitro (Chang *et al*, 1992; submitted) indicates that this region of

gp195 is important in parasite invasion.

CHAPTER IV. IMMUNOGENICITY OF REDUCED/ALKYLATED GP195

BACKGROUND AND LITERATURE REVIEW

The C-terminal 42 kDa processing fragment of gp195 is further processed into a 33 kDa fragment that is shed upon erythrocyte invasion (Blackman *et al.* 1991a) and a membrane bound C-terminal 19 kDa fragment that remains with the parasite during ring-stage development (Hall *et al.* 1983). Cysteine residues in the 19 kDa fragment (Holder *et al.* 1985) undergo intrachain disulfide bridging (Holder *et al.* 1987) and form two domains which resemble epidermal growth factor (EGF) (Blackman *et al.* 1991b). However, it is unclear if the EGF-like domains are involved in surface recognition.

While immunization with native gp195 is strongly protective, recombinant gp195 polypeptides produced in bacterial expression systems (Holder *et al.* 1988; Herrera *et al.* 1990; Etlinger *et al.* 1991) and some synthetic peptides (Cheung *et al.* 1987; Patarroyo *et al.* 1987) have been less effective. It has been suggested that the low antibody titers may be the result of incorrect protein folding (Holder *et al.* 1988). Recently, baculovirus expression systems have been shown to produce recombinant proteins containing conformation-dependent epitopes which closely resemble the native protein (Murphy *et al.* 1990; Chang *et al.* 1992; submitted). In these studies, we found that disulfide bridges were critical in the antigenicity and immunogenicity of gp195. In addition, they are important for the induction of growth inhibitory antibodies.

RESULTS

Native gp195 and its processing fragments were isolated by mAb affinity

chromatography and resolved by SDS-PAGE and silver staining. Under reducing

conditions, the 195 kDa precursor and the 42 kDa, 25 kDa and 19 kDa C-terminal

processing fragments were the prominent polypeptides (Fig. 2, lane 2). Under

non-reducing conditions (lane 1) the 42 kDa fragment migrated as a 38 kDa

protein, indicating that intramolecular disufide linkages were present, as

previously described (Holder *et al.* 1987; McBride and Heidrich, 1987). Although

the majority of the cysteine residues are contained within the 19 kDa fragment, its

molecular weight did not increase under reducing conditions. The prominent 25

kDa processing fragment is also a C-terminal processing fragment, since it was

recognized in immunoblots using C-terminal specific polyclonal and monoclonal

antibodies (see below).

In order to characterize the denatured, reduced and alkylated (dR/A)

gp195, immunoblots were probed with mAbs against conformational and linear

determinants. MAb 5.2 specific for a conformational epitope reacted with the

non-reduced antigen (Fig. 14a, lane 1) but did not react with the dR/A gp195

(Fig. 14b, lane 1). On the other hand, mAb G13, specific for a linear epitope on

the 42 kDa fragment, reacted with both the non-reduced and reduced

preparations (Figs. 14a & 14b, lane 2). Similar results were seen with other mAbs

specific for the 19 kDa processing fragment (not shown). A monospecific

polyclonal rabbit antiserum
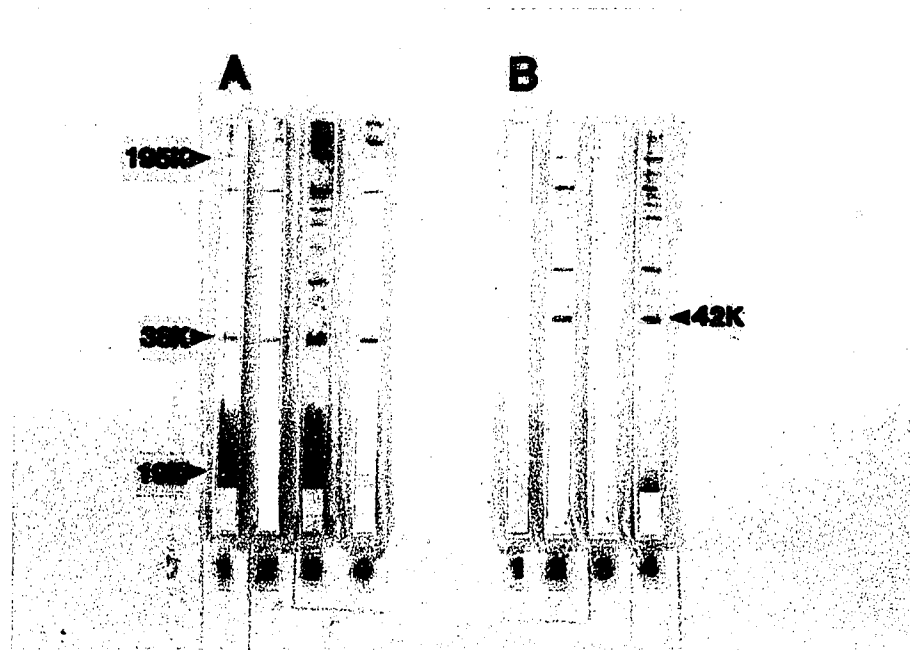
Figure 14.

Immunoblots of gp195 under non-reduced (A) and reduced (B) conditions.

Monospecific polyclonal serum to native C-terminal 42 kDa (lane 1), yeast

recombinant C-terminal 42 kDa (lane 2), mAb G13, specific for a linear C-

terminal epitope (lane 3) and mAb 5.2, specific for a conformational C-

terminal epitope (lane 4) were used to probe the purified antigen.

(K44) raised against the native 42 kDa fragment reacted strongly with non-reduced gp195 (Figure 14a, lane 3) but poorly with dR/A gp195 (Fig. 14b, lane 3). Immunoblots probed with a monospecific polyclonal antiserum (K93) raised to the yeast C-terminal recombinant polypeptide (Yp42) reacted better with reduced than with non-reduced gp195 preparations (Fig 14b, lane 4).

To determine the effect of denaturation, reduction and alkylation on the antigenicity and immunogenicity of gp195, rabbits were immunized with either native gp195 or dR/A gp195 and the antibody levels were evaluated by ELISA and IFA. After four inoculations, serum samples from rabbits receiving native, non-reduced gp195 had a mean ELISA titer of 1/560,000 against the native antigen, but only 1/890 to the dR/A gp195 (Table V), indicating that reduction and alkylation had destroyed much of the antigenicity of the molecule. In addition, rabbits immunized with dR/A gp195 had a mean ELISA titer of only 1/14,650 to the homologous antigen and 1/23,100 to the native gp195, indicating that the immunogenicity of dR/A gp195 was abrogated as well. Rabbits immunized with the native antigen had approximately a 25-fold greater ELISA titer than rabbits immunized with the reduced and alkylated antigen. Similar, but less striking, results were observed when the antibody titers were determined by IFA. Rabbits immunized with native gp195 had a mean IFA titer of 1/17,000 against native gp195, while rabbits immunized with dR/A gp195 had a mean IFA titer of 1/2800 (Table V), again indicating that dR/A gp195 was less immunogenic.

TABLE 5

ELISA Titer and Parasite Growth Inhibition by Serum Samples
from Rabbits Immunized with Native or Reduced/Alkylated gp195.

| Rabbit No. | Immunogen[a] | Reciprocal ELISA titer[b] gp195 | dR/A gp195 | IFA | %Inhibition Exp. 1 | Exp. 2 |
|---|---|---|---|---|---|---|
| K108 | Native | 580,000 | 1,350 | 20,000 | 93 | 100 |
| K109 | " | 760,000 | 520 | 20,000 | 95 | 93 |
| K110 | " | 340,000 | 810 | 10,000 | 52 | 91 |
| K111 | dR/A | 25,000 | 25,000 | 2,500 | 0 | 0 |
| K112 | " | 44,000 | 23,000 | 5,000 | 20 | 0 |
| K113 | " | 18,000 | 7,600 | 1,250 | 0 | 1 |
| K114 | " | 5,300 | 3,000 | 2,500 | 0 | 0 |

[a]Rabbits were immunized by four intramuscular inoculations with 50 $\mu$g native
or reduced/alkylated gp195 in multilamellar liposomes containing B30-MDP/LA-
15-PH synthetic adjuvants.

[b]21d,4° serum samples.

The ability of dR/A gp195 to induce antibodies capable of inhibiting parasite growth in vitro was also evaluated. After the fourth inoculation, serum samples from rabbits immunized with native gp195 had a mean growth inhibition of 83%, while serum samples from rabbits immunized with dR/A gp195 failed to inhibit parasite growth (Table V). This indicated that the reduced and alkylated antigen was incapable of inducing growth inhibitory antibodies.

The specificity of the antibodies was evaluated by immunoblots of SDS-PAGE resolved gp195 polypeptides separated under non-reducing and reducing conditions. Serum from rabbits immunized with native gp195 (Figure 15a, lanes 1-3) recognized the native but not the reduced (Figure 15b, lanes 1-3) 42 kDa and 19 kDa C-terminal processing fragments, indicating that the immunodominant epitopes on these fragments are conformational rather than linear. These data are consistent with those in Fig. 2, in which the antiserum (K44) raised to the native 42 kDa processing fragment identified primarily conformation-dependent epitopes. Serum from rabbits immunized with dR/A gp195 recognized reduced (Fig. 15b, lanes 4-7) but did not react well with native, non-reduced C-terminal processing fragments (Fig. 15a, lanes 4-7). These data are consistent to those obtained with antiserum K93 raised to the yeast expressed polypeptide Pv42 (Fig. 16). Taken together, these data indicated that the native structure(s) formed by disulfide bridge(s) at the C-terminus are essential for sustaining the antigenicity, immunogenicity and the induction of biologically active, growth-inhibitory antibodies.
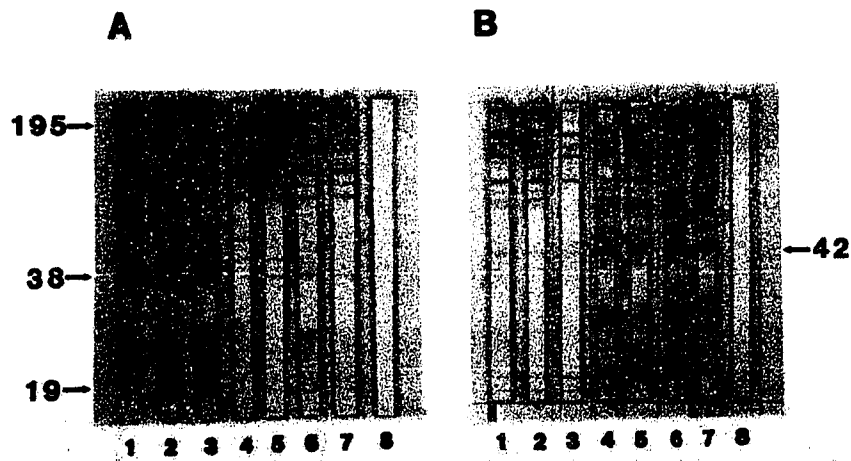
Figure 15.

Immunoblots of native (A) and reduced/alkylated gp195 (B) probed with

rabbit sera immunized with native gp195 (K108, K109, and K110, lanes 1-3,

respectively) or reduced and alkylated gp195 (K111, K112, K113, and K114,

lanes 4-7, respectively).

DISCUSSION

Reduction and alkylation of native gp195 significantly decreased the antigenicity of the native molecule, as determined by the lack of binding by conformational-specific mAb and decreased monospecific polyclonal antisera binding to dR/A gp195 in ELISA. Endpoint ELISA titers of rabbit antisera raised to the native molecule decreased almost one thousand fold (Table V), indicating that the majority of the epitopes recognized by these antibodies are conformational. In addition, the native 42 kDa antigen elicited antibodies reactive with mostly conformational determinants by immunoblot analysis. In contrast, the yeast expressed polypeptide induced antibodies which were reactive with primarily denatured determinants. Both rabbit serum K93 raised against Yp42 and mAb G13, which appear to recognize continuous gp195 determinants, reacted better with dR/A gp195 than the native gp195, indicating that more epitopes were available for binding in the unfolded state with these antibodies.

The dR/A gp195 was also much less immunogenic than the native antigen. Rabbits immunized with the reduced and alkylated antigen had lower antibody titers by ELISA and IFA (Table V). The ELISA results were consistent whether the capture antigen was native or reduced and alkylated. The reduced immunogenicity of dR/A gp195 indicates that native structures formed by the disulfide bridges at the C-terminus play an essential role in determining the magnitude of the antibody response.

Serum from rabbits immunized with reduced and alkylated antigen failed to inhibit parasite growth in vitro. It may be that the lack of inhibition was due both to the decreased immunogenicity of the dR/A antigen and an alteration of the specificity of the antibody response. Inhibition of parasite growth by gp195 antisera is correlated with high ELISA antibody titer, and the threshold titer was significantly greater than that obtained with the dR/A gp195 (Hui *et al.* 1991). This indicates that gp195-specific antibody inhibition of parasite growth could be related to its immunogenicity. In addition, a mAb specific for the 19 kDa processing fragment inhibited 50% of parasite growth in a similar inhibition assay (Blackman *et al.* 1990) and was previously shown to be a reduction-sensitive epitope (McBride and Heidrich, 1987). These data indicate that discontinuous epitope of gp195 may be a target for inhibitory antibodies.

An unusual structural feature of gp195 is the presence of thirteen cysteine residues clustered in the C-terminal 42 kDa processing fragment and its 19 kDa subfragment. This subfragment is carried into the ring-stage of newly infected erythrocytes, while other processing fragments, e.g. the 83 kDa N-terminal fragment and the 33 kDa C-terminal subfragment, are shed following parasite invasion, indicating that the 19 kDa fragment is essential for parasite development. The thirteen cysteine residues appear to produce a compact, highly folded C-terminus, as judged by the apparent increase in $M_r$ of this fragment from 38 kDa to 42 kDa under reducing conditions. These data suggest that the highly folded structure provided by the cysteine-rich cluster may be immunodominant

and play a critical role in the induction of growth inhibitory antibodies. Other regions of the molecule may participate in disulfide bonding, but the primary protein structure has eight possible cysteine residue which might form disulfide bonds (Holder *et al.* 1985). In addition, a recombinant baculovirus-expressed 42 kDa protein induces inhibitory antibodies in rabbits (Chang *et al.* 1992; submitted). Taken together, these data suggest that disulfide-dependent folding is critical in the design of an effective gp195-based malaria vaccine.

Immunization of monkeys with recombinant polypeptides based on both N-terminal and C-terminal sequences resulted in only partial protection (Holder *et al.* 1988; Herrera *et al.* 1990; Etlinger *et al.* 1991). It was suggested that the conformation of the C-terminal recombinant polypeptide was not correct in the *E. coli* expression system (Holder *et al.* 1988). Similarly, immunization with synthetic peptides based on gp195 N-terminal sequences resulted in incomplete protection (Cheung *et al.* 1987; Patarroyo *et al.* 1987). However, immunization of monkeys with native gp195 provided complete protection (Siddiqui *et al.* 1987; Etlinger *et al.* 1991), indicating that native conformation is necessary for protection. Therefore, the production of a gp195-based vaccine will require a eukaryote expression vector providing native conformation in order for a malaria vaccine based on gp195 to be effective.

CHAPTER V. MONOCLONAL ANTIBODIES TO GP195

BACKGROUND AND LITERATURE REVIEW

The precursor to the major merozoite surface coat proteins (MSA-1 or PMMSA) induces protective immunity to several species of *Plasmodium* (Freeman *et al.* 1980; Perrin *et al.* 1984; Siddiqui *et al.* 1987; Etlinger *et al.* 1990). Protection achieved with these antigens was also shown to be largely antibody-mediated since mice can be protected with passive transfer of immune serum or mAbs in rodent malaria models (Holder *et al.* 1980; Boyle *et al.* 1984; Majarian *et al.* 1984). In *P. falciparum* malaria, inhibition of parasite growth by immune serum and mAbs indicates that immunity to gp195 is also primarily antibody-mediated (Pirson and Perkins, 1985; Schmidt-Ullrich *et al.* 1986; Brown *et al.* 1986; Hui and Siddiqui, 1988; Blackman *et al.* 1990). Although the specific mechanism remains to be determined, it has been suggested that antibodies may inhibit erythrocyte attachment or cause an agglutination of merozoites in vitro (Epstein *et al.* 1981). In addition, antibody combined with cellular and complement components may facilitate parasite clearance in vivo.

Passive transfer of a murine mAb to a gp195 analogue (230 kDa merozoite surface antigen) protects mice against lethal challenge with *Plasmodium yoelii* (Majarian *et al.* 1984). This epitope was localized to the C-terminal portion of the 230 kDa antigen using recombinant polypeptides (Burns, *et al.* 1988). Since there is a 50-70% sequence homology between gp195 and the 230 kDa *P. yoelii*

merozoite surface antigen, it has been suggested that this region may be important in the development of a *P. falciparum* blood-stage vaccine (Burns *et al.* 1988).

A mouse mAb (5B1.A8), specific for a merozoite surface epitope on a 200 kDa protein and a 50 kDa fragment partially inhibits parasite growth in vitro (Pirson and Perkins, 1985). It was thought that this 50 kDa antigen may be the C-terminal fragment of gp195 since it was immunoprecipitated from $^3$H-glucosamine metabolically-labeled parasites. In a similar study, the mouse mAb 12.10 is partially inhibitory (50% inhibition at 250-500 $\mu$g/ml) for two parasite clones (Blackman *et al.* 1990). MAb 12.10 identifies a conserved, disulfide-dependent epitope on the C-terminal 19K processing fragment (McBride and Heidrich, 1987). However, inhibition of parasite growth required high antibody concentrations of approximately 500 $\mu$g/ml. Moreover, inhibition was concentration dependent; lower antibody concentrations were not inhibitory. The physiological significance of such inhibitory antibodies is unknown, since a high antibody concentration to a single epitope is unlikely to occur in vivo.

Human mAbs specific for gp195 inhibit *P. falciparum* growth in vitro at low concentrations (approximately 10 $\mu$g/ml); however, the processing fragment or epitope was not identified (Schmidt-Ullrich *et al.* 1986). In a similar study, pooled transformed human B-cell culture supernatants specific for gp195 were 85% inhibitory although the purified Ig fractions were not inhibitory (Brown *et al.* 1986).

The purpose of this study was to produce mAbs to gp195 that inhibit parasite growth at low concentrations. The project involved the use of different adjuvants, alternative screening assays, the outbred Swiss-Webster mouse strain, and an attempt to produce rabbit-mouse xenohybridomas.

## RESULTS

**Monoclonal Antibodies Produced in Balb/c mice with Freund's Complete Adjuvant.** Initially, six mAbs were produced to gp195 and characterized using IFA, immunoblot, and immunoprecipitation (C. Locher Master's thesis). These were subsequently purified from delipidated ascitic fluid and evaluated for their ability to inhibit parasite growth in vitro (Table VI). Monoclonal antibody CE2 reproducibly showed partial inhibition at high antibody concentrations, while inhibition by mAb EB2 was partial and variable (Table VI and Table VIII). These mAbs recognize linear, group specific epitope(s) on the 83 kDa N-terminal processing fragment. None of the inhibitory mAbs were specific for conserved, conformational C-terminal epitopes.

To determine if mAbs could demonstrate additive or synergistic inhibition of parasite growth, these mAbs were mixed at the same concentration and evaluated for growth inhibition. No additive or synergistic effects were observed (Table VII). However, mAbs CE2 and EB2 still inhibited parasite growth when used together, but not when mixed with mAb DB8. These data suggest that inhibition of parasite growth is concentration dependent.

Table 6

Inhibition of *P. falciparum* Growth in vitro
by N-terminal-specific mAbs

| mAb[a] | Isotype[b] | Processing fragment[c] | %Inhibition | |
| --- | --- | --- | --- | --- |
| | | | Exp. 1 | Exp.2 |
| CE2 | $IgG_1$ | 83 kDa N-terminal | 53 | 55 |
| DB8 | $IgG_{2b}$ | " | 0 | 11 |
| EB2 | $IgG_1$ | " | 48 | 57 |
| 24A1.7 | $IgG_1$ | Internal | 28 | 29 |
| AD9 | $IgG_1$ | 19 kDa C-terminal | 32 | 42 |
| BC9 | $IgG_1$ | " | 28 | 17 |
| 5.2 | $IgG_{2a}$ | " | 4 | 23 |

[a]mAbs were purified from ascitic fluid using protein G-sepharose and adjusted to a final concentration of 500 $\mu$g/ml in RPMI culture medium.

[b]Isotypes were determined using ELISA.

[c]Immunoblot was used to determined processing fragment specificity.

Table 7

mAb Inhibition of *P. falciparum* used in Combination

| mAb[a] | Processing fragment[b] | % Inhibition[c] |
| --- | --- | --- |
| CE2/EB2 | 83 kDa N-terminal | 51 |
| EB2/DB8 | " | 15 |
| CE2/DB8 | " | 21 |
| CE2/DB8/EB2 | " | 0 |
| AD9/BC9 | 19 kDa C-terminal | 35 |
| AD9/5.2 | " | 24 |
| BC9/5.2 | " | 14 |
| AD9/BC9/5.2 | " | 26 |

[a]Final mAb concentration was 500 μg/ml.

[b]Specificity was determined by immunoblot and immunoprecipitation.

[c]Per cent inhibition is a representitive experiment.

Table 8.

Growth Inhibition by mAbs is Concentration Dependent

Per cent growth inhibition

| mAb | 500 $\mu$g/ml | 250 $\mu$g/ml | 125 $\mu$g/ml |
| --- | --- | --- | --- |
| CE2 | 60 | 16 | 0 |
| EB2 | 24 | 6 | 0 |
| 5.2 | 20 | 0 | 0 |

Per cent inhibition data is a representitive experiment.

Indeed, when mAbs CE2 and EB2 were even diluted two-fold, inhibition of parasite growth was no longer significant (Table VIII).

Additional mAbs were produced to gp195 in Balb/c mice using Freund's complete adjuvant (FCA). Seven mAb were detected by ELISA (Table IX). An unusual characteristic of mAbs 97.2, 97.3, 97.4 and 97.5 was the lack of reactivity in immunoblots, even when the blotted gp195 had been electrophoresed under unreducing conditions. This suggested that the epitopes identified by these mAb might be sensitive to denaturation by SDS-PAGE. To determine if these were specific for gp195, IFA's were performed to observe merozoite surface staining. These four mAbs were also negative by IFA, indicating that the epitopes were absent on parasites fixed in acetone. To determine if these undefined epitopes were dependent on disulfide-linkage of the molecule, reactivity to reduced and alkylated gp195 was evaluated using ELISA (Table IX). The ELISA revealed that mAb 97.5 showed reactivity, indicating that this mAb identified a linear epitope which was present on both non-reduced and reduced gp195. Since ELISA plates are blocked with bovine serum albumin (BSA) to prevent non-specific proteins from attaching to plastic, it was thought that the remaining three mAb might be directed against BSA or human serum. Therefore, ELISA assays were performed with plates coated with these antigens. One mAb (97.4) was found to crossreact with both BSA and human serum, indicating this mAb was not specific for gp195. Finally, these seven mAb were purified from mAb culture supernatants grown in serum-free medium.

Table 9.

Monoclonal Antibodies Produced from Mice Immunized with FCA

| mAb | Isotype[b] | Relative ELISA reactivity[a] | | | | IFA[c] | Fragment[d] |
| | | gp195 | R/A | BSA | Serum | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| 97.1 | $G_{2a}$ | 2 | - | - | - | 1 | 42 |
| 97.2 | $G_1$ | 2 | - | - | - | - | - |
| 97.3 | M | 4 | - | - | - | - | - |
| 97.4 | $G_1$ | 2 | - | 3 | 3 | - | - |
| 97.5 | $G_1$ | 3 | 3 | - | - | - | - |
| 97.6 | $G_1$ | 3 | - | - | - | 4 | 83 |
| 97.7 | $G_1$ | 3 | - | - | - | 3 | 19 |

[a]Relative ELISA reactivity was scored from one through four, where one showed a weak positive reaction was scored (1) and a strong positive reaction was scored (4).
Negative reactions were scored (-).

[b]Isotypes were determined using ELISA with gp195 as capture antigen.

[c]IFA was used to observe merozoite surface-staining by mAbs, characteristic of mAbs to gp195.

[d]Specificity was determined by immunoblots of purified gp195.

Three antibodies (mAb 97.1,mAb 97.6, and mAb 97.7) identified undefined

determinants, none inhibited parasite growth (Table X).

**Monoclonal Antibodies Produced in Balb/c Mice Using Lipid A-15-PH.**

Recently, work in this laboratory has revealed that adjuvants affect the specificity

of the immune response to gp195 (Hui *et al*, 1992). Because we had failed to

produce inhibitory mAb using FCA, we investigated the use of an alternative

adjuvant LA-15-PH, which induces a high antibody titer to gp195 in mice (Hui *et*

*al.* 1990). Moreover, serum samples from B10 mice immunized with gp195 in

Lipid A-15-PH inhibited parasite growth in vitro (Hui *et al*.1992; submitted),

indicating that these animals were ideal for producing growth inhibitory mAbs.

To determine the ability of Lipid A-15-PH to produce growth inhibitory

antibodies, four cell fusions were performed using spleen cells from Balb/c mice.

Supernatants from 32 hybridomas were positive by ELISA, with intensities ranging

from $1^+$ (weak) to $4^+$ (strong) (Table XI). All were negative by immunoblot

assay and radioimmunoprecipitation (RIP) of metabolically labeled parasite

antigens, therefore, no processing fragment specificity could be identified. Five

mAb were positive for the merozoite surface staining pattern characteristic of

antibodies to gp195, but the fluorescence was weak. Eighteen of 32 hybridomas

generated were of the IgM isotype. Twenty-six antibodies recognized undefined

epitopes absent in ELISA's with R/A gp195. Despite the lack of reactivity by

IFA and immunoblot, it was thought that these hybridomas might actually

produce growth inhibitory antibodies effective at low concentrations.

Table 10

Purification of mAbs from Mice Immunized with FCA

| mAb | Isotype[a] | Yield[b] | % Inhibition[c] |
|------|------|------|------|
| 97.1 | $G_{2a}$ | 1,780 | 16 |
| 97.2 | $G_1$ | 2,020 | 19 |
| 97.3 | M | 780 | 0 |
| 97.4 | $G_1$ | 810 | 23 |
| 97.5 | $G_1$ | 540 | 23 |
| 97.7 | $G_1$ | 1,200 | 0 |

[a]Isotype was determined using ELISA with purified gp195 as capture antigen.

[b]Yield is total protein in $\mu$g isolated with protein G-sepharose from 500 mls. of hybridoma culture supernatant grown in serum-free medium.

[c]Final protein concentration in inhibition assay was 500 $\mu$g/ml. Lack of parasite growth inhibition (< 50%) is considered negative. Data is from one experiment.

Table 11

Monoclonal Antibodies Produced from Mice Immunized with Lipid A

| mAb | Isotype | Relative ELISA Reactivity* to: | | | | |
| | | gp195 | R/A | BSA | Serum | IFA |
| --- | --- | --- | --- | --- | --- | --- |
| 99.1 | $G_1$ | 1 | - | - | - | 1 |
| 99.2 | M | 2 | 2 | - | - | 2 |
| 99.3 | M | 4 | - | - | - | - |
| 99.4 | M | 3 | - | 3 | 3 | - |
| 99.5 | M | 1 | 3 | - | - | - |
| 99.6 | M | 4 | - | - | - | - |
| | | | | | | |
| 100.1 | $G_1$ | 1 | - | - | - | - |
| 100.2 | $G_{2a}$ | 4 | 3 | 3 | 3 | - |
| 100.3 | $G_1$ | 1 | - | - | - | - |
| 100.4 | M | 1 | - | - | - | - |
| 100.5 | $G_1$ | 1 | - | - | - | - |
| 100.6 | M | 2 | - | - | - | - |
| | | | | | | |
| 101.1 | M | 3 | 3 | - | - | - |
| 101.2 | $G_1$ | 1 | - | - | - | - |
| 101.3 | $G_1$ | 3 | - | - | - | - |
| 101.4 | M | 3 | - | - | - | - |
| 101.5 | M | 3 | - | - | - | - |
| 101.6 | $G_1$ | 2 | - | - | - | - |
| 101.8 | $G_1$ | 1 | - | - | - | 1 |
| 101.9 | M | 4 | - | - | - | - |
| 101.10 | $G_1$ | 2 | - | - | - | - |
| 101.11 | M | 4 | - | - | - | - |
| 101.13 | M | 3 | - | - | - | - |
| 101.16 | $G_1$ | 3 | - | - | - | 2 |
| 101.17 | M | 4 | 3 | - | - | - |
| 101.19 | $G_1$ | 3 | - | - | - | - |
| 101.23 | $G_1$ | 2 | - | - | - | - |
| | | | | | | |
| 102.1 | M | 4 | - | - | - | 2 |
| 102.2 | $G_1$ | 3 | - | - | - | - |
| 102.3 | M | 4 | - | 3 | - | - |
| 102.6 | M | 4 | 3 | - | - | - |
| 102.7 | M | 4 | - | - | - | - |

*see Table IX for table legend

Therefore, thirteen randomly selected hybridomas were grown in serum-free medium and Ig was purified on protein G sepharose columns. None of these mAbs were inhibitory for parasite growth (Table XII).

**Production of mAb S1.7 and Others in Swiss-Webster Mice.** Mice of different H-2 haplotypes were shown to differentially recognize specific processing fragments (Chang *et al.* 1989). To determine if growth inhibitory mAbs could be produced from Swiss-Webster (an outbred strain) mice, hybridomas were made from splenocytes of hyperimmunized Swiss mice. Seventeen mAbs were generated using the ELISA and IFA screening assays; however, only ten remained stable after subsequent growth and expansion (Table XIII). The majority of mAbs were found to be of the $IgG_1$ isotype and all were reactive with the different parasite isolates, as determined by IFA. This indicates that the epitopes recognized by these mAbs were conserved between strains. In addition, these were primarily specific for the 83 kDa N-terminal processing fragment, as determined by immunoblotting (Fig. 18) and all identified linear epitopes, since these were reactive with antigen separated under reducing conditions (not shown). One mAb (S1.7) identified an epitope in an internal gp195 fragment, since it did not recognize either the N or C-terminal fragments. Instead, two fragments were identified at the 50 and 55 kDa regions (Fig. 16). Although this mAb recognized determinants on gp195 purified from the FVO isolate, the bands at 50 kDa and 55 kDa were not identified (Fig. 17). Immunoprecipitation of $^{35}$S-methionine

Table 12

Purification of mAbs from Mice Immunized with Lipid A

| mAb | Isotype[a] | Yield[b] | % Inhibition[c] |
|---|---|---|---|
| 99.1 | $G_1$ | 430 | 26 |
| 99.2 | M | 400 | 23 |
| 99.4 | M | 480 | 25 |
| 99.5 | M | 460 | 21 |
| 100.1 | $G_1$ | 1,760 | 17 |
| 100.3 | $G_1$ | 660 | 6 |
| 100.4 | M | 520 | 19 |
| 100.5 | $G_1$ | 500 | 23 |
| 101.2 | $G_{2a}$ | 360 | 33 |
| 101.13 | M | 260 | 0 |
| 101.19 | $G_1$ | 180 | 22 |
| 101.23 | 1 | 210 | 23 |
| 102.6 | M | 300 | 15 |

[a]Isotype was determined using ELISA with purified gp195 as capture antigen.

[b]Yield is total protein in $\mu$g isolated with protein G-sepharose from 500 mls of hybridoma culture supernatant grown in serum-free medium.

[c]Lack of parasite growth inhibition (< 50%) is considered negative. Data is from one experiment.

## TABLE 13

### mAbs to gp195 Produced from Swiss Mice

| mAb | Isotype[a] | ELISA[b] | IFA Reactivity[c] | |
| --- | --- | --- | --- | --- |
| | | | FUP | FVO |
| S1.1 | $IgG_1$ | 2 | 3 | 3 |
| S1.2 | $IgG_{2a}$ | 2 | 4 | 3 |
| S1.3 | $IgG_{2a}$ | 2 | 4 | 4 |
| S1.4 | $IgG_1$ | 2 | 3 | 3 |
| S1.5 | $IgG_1$ | 2 | 3 | 3 |
| S1.6 | $IgG_1$ | 3 | 4 | 4 |
| S1.7 | $IgG_1$ | 3 | 4 | 4 |
| S1.8 | $IgG_1$ | 2 | 4 | 3 |
| S1.9 | $IgG_1$ | 2 | 3 | 3 |
| S1.10 | $IgG_1$ | 2 | 3 | 3 |

[a]Isotype was determined using ELISA with purified gp195 as capture antigen.

[b]Relative ELISA reactivity was scored from one through four, where one showed a weak positive reaction and four showed a strong positive reaction.

[c]Relative IFA reactivity was scored from one through four, where one showed a weak positive reaction and four showed a strong positive reaction.

metabolically-labeled parasite proteins did not reveal the antigens at 50 kDa and 55 kDa (not shown). Cultures of five hybridomas were grown expanded, the IgG was purified and evaluated for parasite growth inhibition (Table XIV). None of these mAbs inhibited parasite growth at relatively high concentrations (500 μg/ml).

**Production of Monoclonal Antibodies from Rabbit Splenocytes.** Since attempts to produce strongly inhibitory mAb from mice had failed and it was known that rabbits hyperimmunized (four inoculations with FCA) produced serum antibodies which inhibited parasite growth by greater than 90%, attempts were made to produce rabbit-mouse xenohybridomas secreting rabbit mAbs. Spleen cells from rabbit K22, known to produce growth inhibitory serum antibodies, were fused with P3 and SP 2/0 fusion partners (Table XV). The SP 2/0 fusion partner provided more hybridomas (56 vs. 39) and more mAb cell lines (11 vs. 6) compared with the P3 fusion partner. Although seventeen mAb cell lines were selected initially based on screening by ELISA and expanded, these were not positive in subsequent ELISA, IFA and immunoblot screenings.

DISCUSSION

**Inhibitory Monoclonal Antibodies to gp195 Identify Linear Determinant(s) on the N-terminal 83 kDa Fragment.** An overall objective of this study was to determine the structural features of gp195 responsible for protective immunity.
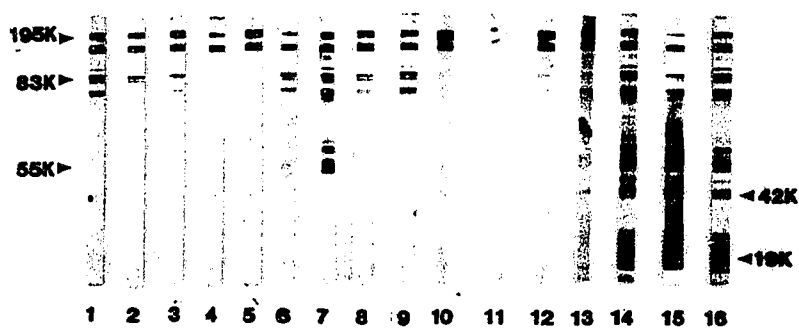
Figure 16.

Immunoblot of purified gp195 antigen separated on a 7.5% SDS-PAGE gel

under non-reducing conditions. mAbs produced from Swiss mice (S1.1-

S1.10) were used to probe both antigen preparations (lanes 1-10,

respectively). N-terminal mAbs (CE2, DB8, lanes 11 and 12, respectively)

and C-terminal mAbs (5.2, AD9 and BC9, lanes 13-16, respectively) were

used as controls.
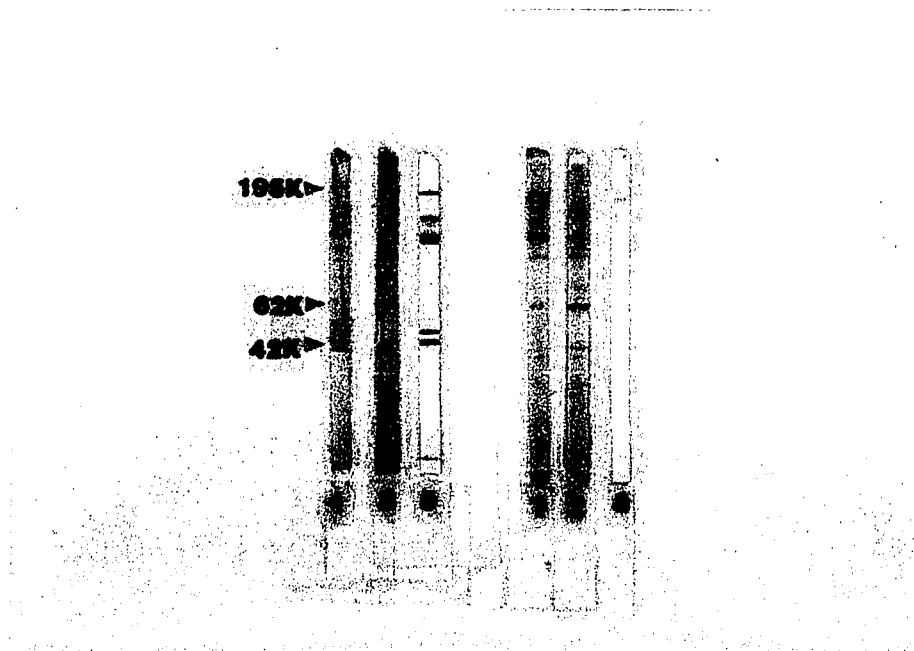
Figure 17.

Immunoblot of FVO antigen separated on a 8.75% SDS-PAGE gel under
non-reduced (A) and reduced (B) conditions.  mAbs S1.7 (lane 1), DB8
(lane 2) and 5.2 (lane 3) were used to probe the immunoblot.

TABLE 14

Parasite Growth Inhibition by Swiss mAbs

| mAb | Isotype[a] | Yield[b] | % Growth Inhibition[c] | |
|-----|-----------|----------|---------|---------|
| | | | Exp.1 | Exp.2 |
| S1.2 | $IgG_{2a}$ | 1.50 | 30 | 28 |
| S1.3 | $IgG_{2a}$ | 0.80 | 26 | 14 |
| S1.4 | $IgG_1$ | 1.43 | 16 | 0 |
| S1.7 | $IgG_1$ | 1.70 | 38 | 0 |
| S1.10 | $IgG_1$ | 1.45 | 0 | 0 |

[a]Isotype was determined using ELISA with purified gp195 as capture antigen.

[b]Yield is total protein in mg isolated with protein G-sepharose from 500 mls. of hybridoma culture supernatant.

[c]Lack of parasite growth inhibition (< 50%) is indicated as negative (-). Final Ig concentration in inhibition assay was 500 μg/ml.

TABLE 15

Comparison of Fusion Partners for the Development of

Rabbit/Mouse Xenohybridomas

| Cell Line | Number of positives in ELISA[a] | |
| | Primary Hybridomas | Primary Clones |
| --- | --- | --- |
| SP2/0 | 22/56 (39%) | 11/22 (50%) |
| P3 | 17/39 (48%) | 6/17 (35%) |

[a]Total number of hybridomas positive by the ELISA using purified gp195 as capture antigen.

We had learned that the induction of growth inhibitory antibodies was dependent on the presence of disulfide bonds in gp195 (Chapter IV), suggesting that inhibitory monoclonal antibodies would identify conformational determinants. In addition, the work of Chang and co-workers indicate that the C-terminal 42 kDa fragment is an important protective processing fragment. Therefore, it was surprising that the inhibitory mAbs CE2 and EB2 recognized non-conformational, linear epitope(s). Moreover, the epitope(s) resided on the 83 kDa N-terminal fragment, rather than the C-terminal 42 kDa fragment. Since this fragment is shed from the merozoite surface prior to erythrocyte invasion (Holder *et al.* 1985), we suspected that this fragment does not participate in the merozoite invasion process and is not a potential gp195 vaccine fragment. Yet, the published data are inconsistent. A monospecific polyclonal antiserum raised to the 83 kDa processing fragment is not inhibitory (Strych *et al.* 1987); however, an electroeluted 83 kDa antigen provides partial protection in *Aotus* vaccination studies (Patarroyo *et al.* 1988). In addition, the inhibitory mAb 12.10 (Blackman *et al.*, 1990) is specific for a conformational epitope on the 19 kDa fragment on the C-terminal 19 kDa processing fragment. Thus, the discovery that mAbs CE2 and EB2 are specific for linear epitopes on the 83 kDa N-terminal fragment is surprising and adds to the confusion.

It is possible that the inhibition demonstrated by mAb CE2 and EB2 may be a phenomenon peculiar to the in vitro assay. High concentrations of antibodies to linear gp195 epitopes may result in inhibition of parasite growth,

and may not be relevant to the observation that R/A gp195 is poorly

immunogenic and unable to induce growth inhibitory antibodies. Inhibition was

observed only at very high antibody concentrations (500 ug/ml), and at

concentrations of 250 ug/ml the inhibition dropped off rapidly. Hui and Siddiqui

(1987) have shown that antibodies purified from inhibitory rabbit antisera inhibit

parasite growth strongly (90% or greater) only when antibody concentrations

approximating 800 ug/ml are used in the in vitro assay. While the distribution of

antibodies to gp195 epitopes in hyperimmune rabbit antisera is unknown,

antibodies are likely to be directed to epitopes spread throughout the gp195

molecule. High mAb concentrations such as 500 $\mu$g/ml, in which each antibody

recognizes the same epitope, are not likely to occur in nature. Although

reproducible, the inhibition may be viewed as an in vitro phenomenon, which may

have little physiological significance. Similarly, mAb 12.10 (Blackman *et al* 1990)

also inhibits parasite growth by approximately 50% at concentrations of about 500

$\mu$g/ml, and this mAb may not identify a epitope which plays an important role in

inhibition of merozoite invasion.

There are only two reports of inhibitory mAb to gp195 at low

concentrations. Human monoclonal antibodies at concentrations of about 10

$\mu$g/ml produce 90% inhibition (Brown *et al*; 1986; Schmidt-Ulrich *et al.* 1986).

However, these mAbs were not characterized. It is not known if they identify

linear or conformational epitopes and whether the epitopes reside on N- or C-

terminal processing fragments.

**Other Attempts to Produce Growth Inhibitory Monoclonal Antibodies.**

We hypothesized that the epitopes defined by putative growth inhibitory monoclonal antibodies may not be detected by immunoblots and IFA. That is, our strategy for producing growth inhibitory mAb had been to screen hybridomas by ELISA, IFA and immunoblot. Yet, there was no evidence indicating that an inhibitory mAb needed be positive by IFA and immunoblot. It was possible that an inappropriate screening assay had been selected and we had actually been discarding the inhibitory hybridomas. For example, an inhibitory mAb may detect an epitope formed by the quaternary association of both N- and C-terminal processing fragments through non-covalent interactions. Such an epitope would certainly be lost upon resolution of processing fragments by SDS-PAGE. Seven additional mAbs produced using FCA as adjuvant (Table X) and thirteen other mAb produced using Lipid A-15-PH as adjuvant (Table XII) and five mAb produced in Swiss-Webster mice (Table XIV) screened by ELISA, immunoblot and IFA had variable characteristics, but none inhibited parasite growth in vitro. Recently, the ability of the C-terminal 42 kDa fragment to produce growth inhibitory antibodies in rabbits (Chang *et al.* 1992; submitted) indicates that quaternary structure formed by different fragments is not required for the induction of growth inhibitory antibodies. Thus, this hypothesis of inhibitory antibodies specific for quaternary structure was rejected.

Immunization of Balb/c mice with gp195 in liposomes containing LA 15-PH generated 36 mAbs that were negative by immunoblot and

radioimmunoprecipitation assays. In addition, these antibodies had variable reactivities by IFA and ELISA using R/A gp195. Four mAbs with these characteristics were also produced in fusion 97 (Table IX); FCA had been used as the adjuvant in the generation of antibody producing spleen cells. Three of the mAbs reacted with BSA and were therefore not gp195-specific. The specificity of the remaining mAbs for gp195 remains putative, since most of these mAbs were negative by IFA and all were negative by immunoblot and radioimmunoprecipitation.

Since these mAbs were only positive by ELISA, specificity for gp195 could be further characterized using this assay. For example, several additional ELISA assays containing different antigens could be used to determine the extent of cross-reactivity: (1) recombinant polypeptides based on the gp195 sequence; (2) purified parasite proteins other than gp195 as capture antigens, ie. rhoptry antigens; (3) competitive ELISA assay using whole parasite antigen as capture antigen and purified gp195 as the competitor protein. If the undefined mAbs were unreactive with recombinant polypeptides, but cross-reactive with other parasite antigens or whole parasite antigen, it could be that these mAbs are specific for erythrocyte proteins present in the antigen preparations (although these mAbs were negative for erythrocytes by IFA). An ELISA assay using erythrocyte ghosts as capture antigen could be used to detect erythrocyte specificity since these epitopes maybe absent on erythrocytes fixed in acetone. ELISA experiments using the above antigens would enable more conclusive

statements to be made about the specificity of these undefined, putative gp-195 specific mAbs as well as the extent of their cross-reactivity with other antigens.

Why the Lipid A-15 PH mAbs were negative by immunoblot and radioimmunoprecipitation assays remains unclear. Since the epitopes are present only in ELISA, it appears that the epitopes are sensitive to mild denaturation. Alternatively, it may be that the epitopes are formed after gp195 has been bound to the polyvinyl plastic. This could be the result of transformed protein conformation, resulting in the unique presentation of epitopes absent in the other assays.

It seems unlikely that the undefined mAbs were induced solely by the adjuvant Lipid A-15 PH, since three mAbs with these characteristics were produced using FCA as adjuvant. It is possible that the use of the SP-2/0 fusion partner facilitated the selection of undefined hybridomas, although further experimentation comparing the two fusion partners would need to be conducted before this conclusion is substantiated.

Immunization of mice with gp195 in liposomes containing LA 15-PH as adjuvant results in high titers of gp195-specific antibodies in mice (Hui *et al.* 1991). However, this was done in the B10 congenic mouse strain. One possible explanation for the failure to produce inhibitory mAb using Lipid A-15-PH in the present study, is that this adjuvant enhances the production of high titered anti-gp195 antibodies in the B10 strain, but does not enhance the production of inhibitory antibodies in the BALB/c strain. This might reflect the fact that, while

generalized recognition of gp195 is not restricted genetically, production of antibodies to protective epitopes might be restricted to certain MHC haplotypes, as suggested by Chang *et al.* (1989). Moreover, potentiation of these gp195 antibody responses might be adjuvant restricted, and Lipid A-15-PH may be unsuitable for use in the BALB/c strain commonly used for hybridoma production. Eighteen of the 36 hybridomas were of the IgM isotype. The predominance of these seen in the development of mAbs may be attributed in part to LA 15-PH being a polyclonal B-cell mitogen (Kotani *et al.* 1986).

The majority of mAbs generated with Swiss-Webster mice were specific for the N-terminal 83 kDa processing fragment. This is an unusual finding, since the Balb/c mouse strain has produced more C-terminal mAbs. Again, this may reflect a strain-specific response reflecting the animals response to gp195, the adjuvant used, or both. The 55 kDa and 50 kDa fragments identified by mAb S1.7 have been recognized by serum samples from B10.D2 and B10.WB congenic mice immunized with gp195 (Chang *et al.* 1989). Processing fragments of a similar size have been described for the analogous merozoite surface coat protein of *P. knowlesi* (David *et al.* 1984). However, antigens of 22 kDa and 36 kDa have been shown to be the central processing fragments of gp195 by immunoprecipitation and direct N-terminal sequencing of the 36 kDa protein (Holder *et al.* 1987; Heidrich *et al.* 1988).

The specificity of rabbit/mouse xenohybridomas for gp195 were found to be negative by ELISA, IFA and immunoblot after being cloned and expanded.

This instability may have been avoided by the use of a rabbit/mouse xenohybridoma fusion partner, since the stability of antibody secreting rabbit/mouse xenohybridomas is proportional to the number and type of rabbit chromosomes present (Raybould and Takahashi, 1989). The SP 2/0 fusion partner produced more hybridoma colonies and twice the positive clones as the P3 myeloma. These results may not accurately reflect the inherent characteristics of the two cell lines since repeated experimentation would be required for a conclusive analysis.

**Conclusion.** An overall objective of this study was to determine the structural features of gp195 responsible for protective immunity. In particular, we sought to define protective epitopes using monoclonal antibodies and the in vitro growth inhibition assay, as has been done with many potential vaccines. Initially, use of the Balb/c mouse strain produced five antibodies (mAb5.2, mAb AD9, mAb BC9, 97.1 and 97.7) to the C-terminal 42 kDa processing fragment which did not inhibit parasite growth greater than 50%. Similarly, eight antibodies (CE2, DB8, EB2, 97.6, S1.2, S1.3, S1.4 and S1.10) to the 83 kDa N-terminal fragment were produced; mAb S1.7 appeared to recognize an internal 55 kDa fragment. In addition, 13 immunoblot and IFA negative mAbs were produced using Lipid A-15-PH. In all, 26 mAb were produced and evaluated for growth inhibition. With the exception of mAbs CE2 and EB2, no inhibition was detected. The inhibition was observed with mAbs CE2 and EB2, but antibody concentrations approaching 500 $\mu$g/ml were required. Growth inhibition at high antibody concentrations may

represent an in vitro phenomenon, and none of the above antibodies may represent biologically important epitopes. Overall, it is possible that strongly protective epitopes exist on gp195; however, they have not yet been identified.

# CHAPTER VI. RHOPTRY PROTEINS AS MALARIA

# VACCINE CANDIDATES

## BACKGROUND AND LITERATURE REVIEW

The apical region of the *Plasmodium* merozoite contains two pear-shape organelles called rhoptries. There are four major groups of rhoptry proteins: (1) a complex of 80 kDa and 65 kDa called RAP-1 (Ridley *et al.* 1990) or QF3 (Bushell *et al.* 1988), which is non-covalently associated with a 42 kDa protein (RAP-2) and an uncharacterized 40 kDa protein (Ridley *et al.* 1991); (2) the RAP-3 complex containing 140, 130 and 105 kDa proteins (Holder *et al.* 1985), later shown to be unrelated polypeptides (Cooper *et al.* 1988); (3) a 240 kDa protein and its 225 kDa derivative localized to the neck (peduncle) of the rhoptry (Roger *et al.* 1988); and (4) the 80 kDa apical membrane antigen-1 (AMA-1), unrelated to RAP-1, which is also localized to the peduncle region (Crewther *et al.* 1990). The complete gene sequence of the 105 kDa polypeptide in the RAP-3 complex has recently been reported (Brown and Coppel, 1991).

The role of the rhoptries in erythrocyte invasion has been recently reviewed (Perkins, 1989). Rhoptry proteins may be involved in the formation of the parasitophorous vacuole membrane through the insertion of membraneous elements (Bannister *et al.* 1986) and alteration of the erythrocyte cytoskeleton as they are emptied onto the cell surface (Sam-Yellowe *et al.* 1988). Evidence suggests that the RAP-3 complex forms large aggregates and may be membrane

bound since these antigens are not entirely digested with trypsin and are released

by phospholipases (Etzion *et al.* 1991). These could be directly introduced into

the erythrocyte lipid membrane to facilitate parasite invasion, since they are

inserted into liposomes (Sam-Yellowe and Perkins, 1991). In addition, the

invasion process is thought to be mediated in part by enzyme activity associated

with rhoptry proteins. This is the subject of Chapter VII.

Rhoptry proteins are important malaria vaccine candidates, based on the

demonstration that a 235 kDa rhoptry protein confers protection against *P. yoelii*

malaria in mice (Holder and Freeman, 1981). In addition, an 80 kDa rhoptry

protein (Ridley *et al.* 1990) and a 41 kDa rhoptry associated protein (Perrin *et al.*

1985) protects *Saimiri* monkeys against *P. falciparum* malaria. Moreover, a mAb

to the 80 kDa protein inhibits *P. falciparum* growth in vitro (Schofield *et al.* 1986).

However, work from this laboratory showed that the RAP-3 proteins were only

weakly protective when gp195 and the RAP-3 complex were used to immunize

groups of *Aotus* monkeys. Upon challenge with a lethal *P. falciparum* infection,

monkeys immunized with gp195 were completely protected; whereas, only one

monkey of three immunized with the RAP-3 complex showed partial protection

and the remaining two monkeys required drug treatment (Siddiqui *et al.* 1987). In

this study, however, the antibody titers to RAP-3 were lower than the anti-

antibody titers to gp195. In addition, serum samples from monkeys immunized

with RAP-3 did not inhibit parasite growth in vitro, while serum samples from

monkeys immunized with gp195 did (Hui and Siddiqui, 1987). Recently, inhibiton

of parasite growth in vitro by anti-gp195 antibodies has been shown to be dependent upon high antibody titers in serum samples (Hui *et al.* 1990). If this is also true for the RAP-3 complex, high titered antisera would be necessary for inhibition to occur. Therefore, it was hypothesized that RAP-3 could induce growth inhibitory antibodies provided high titered antisera were raised. In this study, mAb 219.5 (Siddiqui *et al.* 1986) was used to isolate the RAP-3 complex, while mAb AC9 was produced and used to isolate the RAP-1 complex. High-titered antisera from rabbits immunized with these complexes inhibited parasite growth.

## RESULTS

The RAP-1 and RAP-3 complexes were isolated by affinity chromatography with mAb AC9 and mAb 219.5, respectively. Approximately 7-8 mgs were isolated in eighteen extractions (Table XV). The yields were slightly lower than the yields for gp195. A silver stained SDS-PAGE gel of the RAP-1 polypeptides (Fig. 18) revealed 85 kDa, 77 kDa and 42 kDa polypeptides, similar to the RAP-1 polypeptides isolated by mAb 2.13 (Ridley *et al.* 1990). When separated under reducing conditions on SDS-PAGE, RAP-1 had greater relative migration (not shown). In addition, the mAb 219.5 isolated 140 kDa, 130 kDa and 105 kDa polypeptides, similar to the RAP-3 polypeptides isolated by mAb 61.3 (Holder *et al.* 1985).

RAP-1, isolated with mAb AC9, was also recognized by other RAP-1

specific mAbs 2.13 (Ridley *et al.* 1990) and 30cl3 (Perrin *et al.* 1985) (Fig. 19). In

immunoblots, mAb 2.13 identified a 2-ME insensitive (linear) determinant, while

mAbs AC9 and 30cl3 identified 2-ME labile (conformational) determinants. An

epitope on the 130 kDa polypeptide was identified by mAb 219.5 by immunoblot

(Fig. 20). In addition, two other mAb produced in this laboratory were

characterized. Mab 8.311 and mAb 25D2.1 identified epitopes on the 130 kDa

and the 105 kDa polypeptide, respectively (Fig. 20). RAP-3-specific rabbit serum

K15 recognized the 140 kDa, 130 kDa and the 105 kDa antigens in addition to

several other bands on these immunoblots; however, its reactivity was diminished

with antigen separated under reducing conditions.

Groups of rabbits were immunized with RAP-1, RAP-3 and gp195,

respectively, in FCA. Serum samples were taken 21 days after the third booster

injection and evaluated by ELISA, IFA and the in vitro parasite growth inhibition

assay (Table XVI). IFA antibody titers of serum samples from rabbits immunized

RAP-1 and RAP-3 complexes were comparable to the those of sera from rabbits

immunized with gp195. However, serum samples from rabbits immunized with

the RAP-3 complex had a slightly lower antibody titers by ELISA than serum

samples from rabbits immunized with RAP-3 complex. Sera from rabbits

immunized with gp195 had a mean parasite growth inhibition of 87%, while sera

from rabbits immunized with RAP-1 and RAP-3 both had mean parasite growth

inhibition of 89%.

TABLE 16

Isolation of gp195 and Rhoptry Polypeptides

| Extraction[a] | Flasks[b] | Vials | gp195[c] | RAP-1 | RAP-3 |
|---|---|---|---|---|---|
| 66-70 | 360 | 73 | 3.5[d] | 1.7 | 0.6 |
| 71-78 | 540 | 105 | 5.2 | 4.7 | 4.0 |
| 80-84 | 890 | 178 | 3.3 | 1.4 | 2.1 |
| Total | | | 12.0 | 7.8 | 6.7 |

[a]Each extraction of membrane proteins from saponin-lysed parasites was given a lot number.

[b]Flasks refers to the number of 2.8 liter erylenmeyer flasks used to culture parasites in vitro. Approximately five of these flasks produced one vial of saponin-lysed parasites. 12 vials were used in each extraction.

[c]Gp195, RAP-1 and RAP-3 were purified by mAbs 5.2, AC9 and 219.5, respectively.
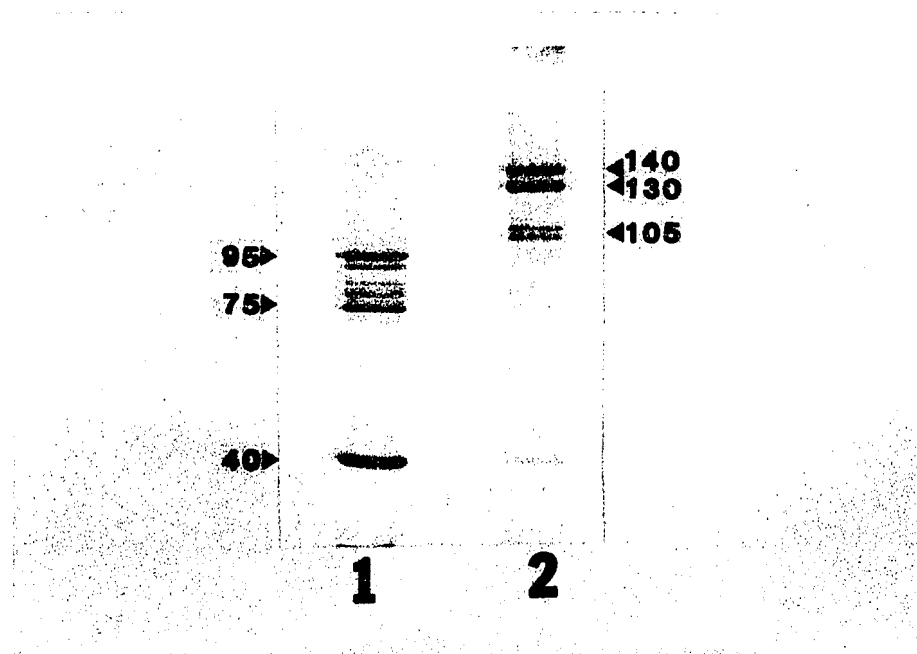
[d]Protein yield in milligrams.

Figure 18.

A silver stained SDS-PAGE gel of rhoptry antigen preparations separated under non-reducing conditions. The RAP-1 complex of was isolated by mAb AC9 (lane 1) and the RAP-3 complex was isolated by mAb 219.5 (lane 2).
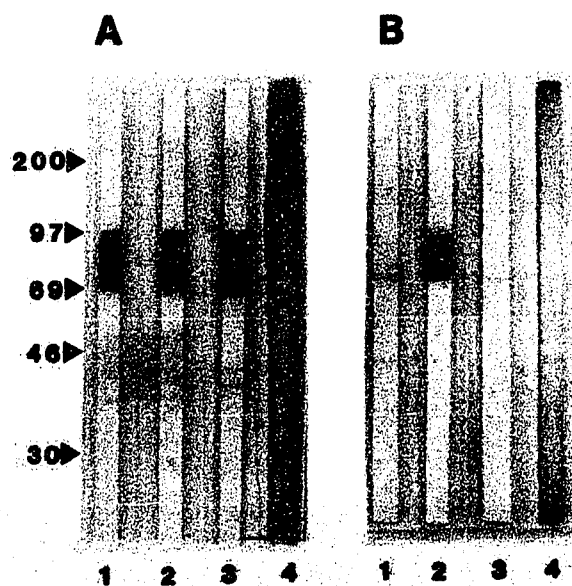
Figure 19.

Immunoblot of polypeptides of the RAP-1 antigen complex separated by SDS-PAGE under non-reducing (A) and reducing conditions (B). MAbs AC9, 2.13, 30cl3 and 5.2 (lanes 1-4, respectively) were used to probe the antigen preparation. The MW markers are indicated.
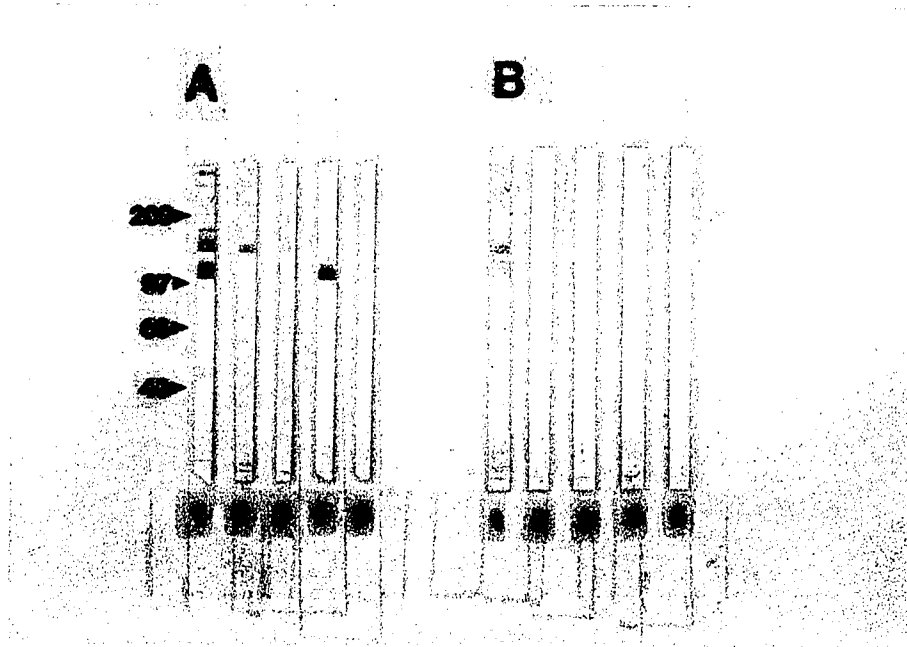
Figure 20.

Immunoblot of the RAP-3 antigen complex separated under non-reducing

(A) and reducing (B) conditions. Polyclonal rabbit antiserum K15 (raised

to RAP-3) (lane 1) and RAP-3 specific mAbs 219.5, 8.311 and 25D2.1

(lanes 2-4, respectively) and anti-gp195 specific mAb 5.2 (lane 5) were used

to probe the antigen preparation.

TABLE 17

ELISA Titer and Parasite Growth Inhibition by Serum Samples
from Rabbits Immunized with gp195
and Rhoptry Polypeptides

| Rabbit | Immunogen[a] | Reciprocal ELISA titer[b] | Reciprocal IFA titer | %Inhibition Exp. 1 | Exp.2 |
|--------|-----------|-----------|-----------|--------|--------|
| K108 | gp195 | 580,000 | 20,000 | 93 | 100 |
| K109 | " | 760,000 | 20,000 | 95 | 93 |
| K110 | " | 340,000 | 10,000 | 52 | 91 |
| K138 | RAP-1 | 320,000 | 10,000 | 88 | 96 |
| K139 | " | 900,000 | 10,000 | 68 | 100 |
| K140 | " | 950,000 | 20,000 | 91 | 96 |
| K130 | RAP-3 | 220,000 | 20,000 | 95 | 96 |
| K133 | " | 270,000 | 10,000 | 68 | 100 |
| K137 | " | 120,000 | 10,000 | 77 | 96 |

[a]Rabbits were inoculated intramuscularly with 50 $\mu$g of native RAP-1 or RAP-3 four times in FCA adjuvant.

[b]21d,$4^0$ serum samples.

Antibody titers of rabbits K130, K133 and K137 immunized with the RAP-3

complex had higher ELISA and IFA antibody titers than rabbit antiserum K15

raised to RAP-3 previously in this laboratory. In addition, these sera inhibited

parasite growth in vitro; whereas, antisera from rabbit K15 did not.

Sera from rabbits immunized with RAP-3 complex did not strongly react

with the 140 kDa antigen in immunoblots of saponin-lysed parasite extracts (Fig.

21). Similar results were obtained with RAP-3 specific antiserum K-15 (Hui and

Siddiqui, 1987) (Fig. 20). In addition, a 72 kDa antigen was recognized by each of

the RAP-3 antisera, but did not react with RAP-1 antisera in immunoblots (not

shown). Since a similar band was identified by antisera against RAP-3, the 72

kDa antigen may be a fragment of one of the RAP-3 proteins.

Immunoblots probed with sera from rabbits K138, K139 and K140 immunized

with the RAP-1 complex did not react strongly with the 40 and 42 kDa proteins

(Fig. 21); however, better reactions were seen with the 85 kDa, 77 kDa and the 65

kDa antigens. Diminished reactivity of both RAP-1 and RAP-3-specific rabbit

antisera was observed in immunoblots of antigen separated under reducing

conditions (not shown). In addition, sera from rabbits immunized with RAP-1

and RAP-3 were also negative for reactivity to gp195 in an immunoblot assay (not

shown).

Figure 21.

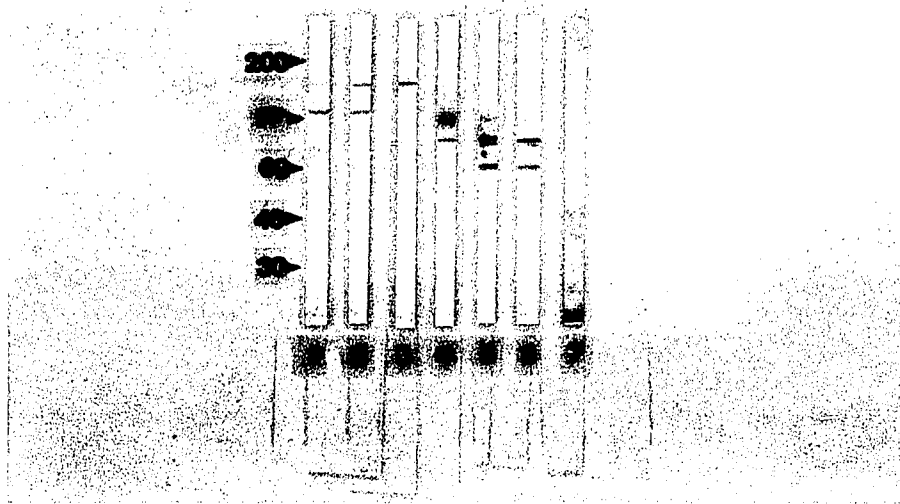Immunoblots of saponin-lysed parasites separated on a 7.5% SDS-PAGE

gel and probed with 21d,4° serum samples diluted 1/1000 from rabbits

K130, K133 and K137 immunized with the RAP-3 complex (lanes 1, 2 and

3, respectively) and rabbits K138, K139 and K140 immunized with the

RAP-1 complex (lanes 4, 5 and 6, respectively). A rabbit serum to human

serum carnosinase was used as a negative control (lane 7).

DISCUSSION

The RAP-1 antigenic complex isolated with mAb AC9 is the same complex described by Perrin and co-workers (1985) and Ridley and co-workers (1990) since mAbs (30cl3 and 2.13, respectively) obtained from these laboratories reacted with AC9 purified antigens in immunoblots. Other mAbs have been produced to RAP-1 (Stanley et al. 1984; Schofield et al. 1986).

Eight cysteine residues are present within RAP-1 (Ridley et al. 1990). MAbs AC9 and 31cl3 recognized conformational epitopes on RAP-1, indicating that intramolecular disulfide linkages are present. Some RAP-1-specific mAbs inhibit parasite growth in vitro (Perrin et al. 1981; Schofield et al. 1986); however, the relationship between native disulfide linkages and growth inhibition remains unclear. The enhanced mobility of the RAP-1 polypeptides under reducing conditions may be attributed to an asymmetrical distribution of amino acids resulting in a higher mass to charge ratio of SDS binding under reducing conditions (Howard and Reese, 1990).

Some of the mAb epitopes of RAP-3 antigenic complex were also conformation-dependent, since mAbs 219.5 and 8.311 did not react with reduced antigen in immunoblots. Reduction-sensitive epitopes have been described for the 130 and 105/110 kDa antigens (Cooper et al. 1988; Sam-Yellowe et al. 1988). The decreased reactivity of both the RAP-1 and RAP-3-specific rabbit antisera to reduced antigens in immunoblots indicates that much of the antibody response is towards conformational epitopes.

Antibodies to the RAP-1 antigenic complex with diminished reactivity to

the 42 kDa RAP-2 antigen in immunoblots has also been reported (Ridley *et al.*

1990). It is possible that the antigen is less immunogenic than RAP-1. Similarly,

antibodies to the RAP-3 antigenic complex reacted poorly to the 140 kDa antigen.

It remains unclear if the 42 kDa RAP-2 antigen and the 140 kDa RAP-3 antigen

are less immunogenic than their non-covalently associated counterparts.

The fact that rabbits immunized with both rhoptry antigenic complexes

produced growth inhibitory antibody suggests that each complex is important in

parasite invasion. The lack of parasite growth inhibition by K15 rabbit serum

suggests that a high antibody titer to the RAP-3 antigenic complex is essential for

biological activity. Similarly, sera from *Aotus* monkeys immunized with RAP-3

were only partially inhibitory and the antibody titers, as shown by IFA, were low

when compared to gp195 (Hui and Siddiqui, 1987). Since high-titered

monospecific polyclonal antiserum to RAP-3 inhibits parasite growth in vitro, it

may be that immunity to these antigens is antibody-mediated.

Gp195 is a promising malaria vaccine candidate based on several

vaccination experiments in monkeys in which complete protection has been

demonstrated. Serum samples from such monkeys (Hui and Siddiqui, 1987) and

from rabbits immunized with native gp195 (Hui *et al.* 1990) strongly inhibited

parasite growth in vitro (90%). In these studies, serum samples from rabbits

immunized with native gp195 also inhibited parasite growth strongly (89%).

However, serum samples from rabbits immunized with RAP-3 as well as RAP-1

inhibited parasite growth (89%). This finding suggests that RAP-3 may be a future blood-stage malaria vaccine candidate, the vaccine potential of which should be evaluated in monkey vaccination experiments.

# CHAPTER VII. ENZYME ACTIVITY ASSOCIATED WITH RAP-1

## BACKGROUND AND LITERATURE REVIEW

Invasion of the malaria merozoite into the erythrocyte is mediated in part by the rhoptry organelle (Perkins, 1990). Two theories of rhoptry mediated erythrocyte invasion have emerged. First, rhoptry proteins may disrupt the erythrocyte membrane integrity by insertion of hydrophobic domains of the proteins. In support of this theory, the 105 kDa RAP-3 protein is inserted into the erythrocyte membrane (Sam-Yellowe and Perkins, 1989) and binding to inside-out erythrocyte vesicles is competitively inhibited by liposomes (Sam-Yellowe and Perkins, 1991). Alternatively, rhoptry enzymes may disrupt the erythrocyte cytoskeleton (ie. by the cleavage of spectrin) to facilitate merozoite invasion since several rhoptry enzymes have been described. These include a 68 kDa cysteine protease in the apical complex of *P. berghei* (Bernard and Schrevel, 1987; Schrevel *et al.* 1988), a 76 kDa serine protease (Braun-Breton *et al.* 1988), and a 41 kDa protein sharing significant sequence homology with aldolase (Certa *et al.* 1987; Knapp *et al.* 1990).

Since antisera to gp195, RAP-1 and RAP-3 antigen complexes is inhibitory, it is possible that the growth inhibition was due to inhibition of enzyme activity. Therefore, it was hypothesized that gp195, RAP-1 and RAP-3 possess proteolytic activity. To determine if gelatin could serve as a substrate for these parasite proteins, gelatin zymograms were performed with the isolated proteins. It was

found that three metalloproteinases having $M_r$ 220 kDa, 95 kDa and 70 kDa were noncovalently associated and co-isolated with the RAP-1 complex, but not RAP-3 or gp195.

RESULTS

Gelatinase activity was initially observed in a NP-40 detergent extract of *Plasmodium falciparum* cultured in vitro. Three predominant bands at 220K, 95K and 70K regions were detected (Fig. 23a, lane 1). Heat treatment at 100°C for one minute (lane 2) as well as reduction of disulfide bonds with 2-mercaptoethanol (lane 3) abolished activity.

The 220K gelatinase and to a lesser degree the 95K and 70K gelatinases were also seen in preparations of RAP-1 isolated with mAb AC9 (Fig. 23b, lane 1) . However, preparations of RAP-3 isolated with mAb 219.5 (lane 2) and gp195 isolated with mAb 5.2 (lane 3) had no gelatinase activity. Extracts depleted of RAP-1 by affinity purification had strong residual gelatinase activity (lane 4). Supernatants from phosphatidyl inositol-specific phospholipase C treated schizonts were analyzed in the zymogram assay, but no activity was observed (not shown). Zymograms using BSA as substrate also displayed no activity (not shown).

To determine whether the gelatinase activity was of parasite or host origin, a titration of washed uninfected erythrocytes and *P. falciparum* infected erythrocytes from the same donor were analyzed by zymogram. Both uninfected as well as infected erythrocytes had gelatinase activity (Fig. 23a). Titrations of *P.*
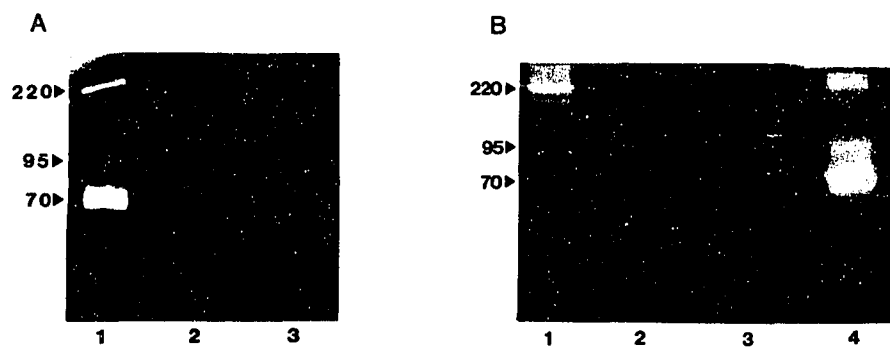
Figure 22.

A. Zymogram of a saponin-lysed *P. falciparum* crude extract. An untreated

sample (lane 1), heat treated (lane 2) and reduced with 2-mercaptoethanol

(lane 3). B. Zymogram of affinity purified antigens from parasite extracts:

RAP-1/2 (lane 1), RAP-3 (lane 2), gp195 (lane 3) and the same extract

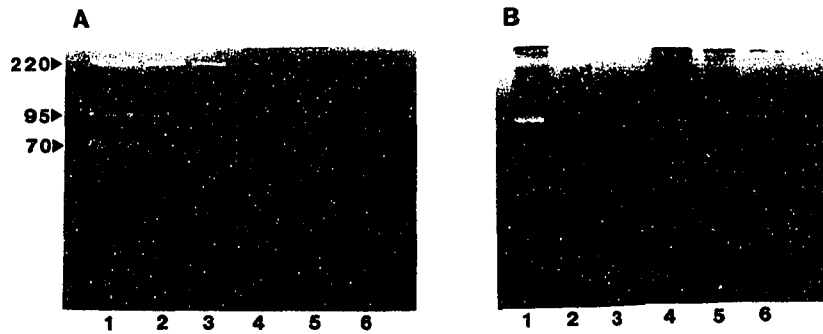after affinity purification of each antigen (lane 4).

**Figure 23.**

A. Zymogram of uninfected erythrocytes (lanes 1-3) and *P. falciparum* infected erythrocytes (lanes 4-6) titrated at 5.0 µl (lanes 1 and 4), 1 µl (lanes 2 and 5) and 0.1 µl (lanes 3 and 6). B. Zymogram of control medium (lanes 1-3) and *P. falciparum* culture medium (lanes 4-6) titrated with the same volumes as above.

*falciparum* culture supernatant and culture medium (supplemented with 10% human plasma) also revealed gelatinase activity (Fig. 23b). To determine if the gelatinases were the same human enzymes described by others, a parasite extract was reacted with specific antisera against the 95K human macrophage gelatinase and a 70K human fibroblast gelatinase (Vartio and Baumann, 1987). Both antisera precipitated gelatinases from parasite extracts (Fig. 24). In order to detect intracellular localization of the gelatinases, IFA's were performed with anti-MMP rabbit sera, however, the results were negative (not shown).

The enzymes were shown to be metalloproteinases since gelatinolytic activity was inhibited by EDTA but other protease inhibitors tested (soybean trypsin inhibitor, antipain, leupeptin, pepstatin A, pHMB, PMSF, chymostatin and iodoacetamide; not shown). After incubation with EDTA, only calcium was able to restore the gelatinolytic activity of the 220K, 95K and 70K enzymes. Of all other metal ions tested, 0.1 mM cobalt optimized gelatinolytic activity when it was combined with 1.0 mM calcium. Under these assay conditions, optimal activity was achieved at pH 7.5 and a temperature of 37°C.

## DISCUSSION

The characteristics of the 220K, 95K and 70K gelatinases non-covalently associated with RAP-1 are identical to those described for MMP also known as type IV collagenase (Woessner, 1991; Emonard and Grimand, 1990). Gelatin
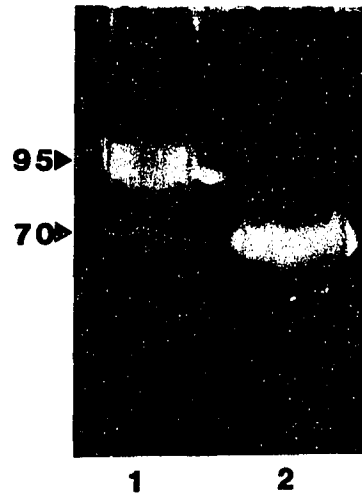
Figure 24.

Zymogram of immunoprecipitated gelatinases from saponin-lysed *P.*

*falciparum* crude extract. Monospecific polyclonal rabbit serum to the 70K

human fibroblast gelatinase (lane 1) and the 95K human macrophage

gelatinase (lane 2).

specificity, relative molecular weight, sensitivity to disulfide reduction, inhibition by EDTA, and calcium dependence are in agreement with previous studies. No differences were detectable when parasites and culture supernatants were titrated on zymograms in parallel with control samples. Moreover, each of these gelatinases reacted with specific antisera raised to the human enzymes. The monspecific rabbit serum to macrophage and fibroblast MMP did not stain the infected erythrocytes by IFA, indicating that the gelatinases may have associated with RAP-1 during the extraction process. Matrix metalloproteinases have evolved since amphibians and have not been identified in lower life forms (Woessner, 1991). These data suggest that human plasma is the source of the gelatinases seen in parasite extracts.

Matrix metalloproteinases (MMPs) or matrixins are a family of nine groups of enzymes which degrade different forms of the extracellular matrix (Woessner, 1991; Emonard and Grimand, 1990). Although matrixins are synthesized by many different cells, they share common structural and functional features. One group (MMP-2 or type IV collagenase) is a human plasma component (Vartio and Baumann, 1990) secreted by macrophages, neutrophils and fibroblasts in culture (Vartio *et al.* 1982; Hibbs *et al.* 1985; Murphy *et al.* 1989). Macrophage culture supernatants contain two polypeptides of 225 kDa and 92 kDa (Vartio and Hovi, 1987). It is thought that the high molecular weight enzyme is formed by the disulfide-linkages of the 92 kDa gelatinase.

The RAP-1 complex isolated with mAb AC9 is the same rhoptry complex isolated from *P. falciparum* extracts by other workers (Ridley *et al.* 1990; Perrin *et al.* 1985). In addition to reacting with RAP-1, mAb 31cl3 has been reported to isolate a novel 76K serine protease and minor 90K and 68K gelatinases from phosphatidyl inositol-specific phospholipase C treated percoll purified schizonts (Braun-Breton *et al.*, 1988). The 76K serine protease was inhibited by PMSF and chymostatin but not by EDTA indicating that it is a serine protease and not a metalloproteinase. We found that the 220K, 95K and 70K metalloproteinases co-isolated with the RAP-1 complex were completely inhibited by EDTA but not PMSF or chymostatin. Thus, the metalloproteinases appear to be distinct from the novel 76K serine proteinase. However, we could not detect 75K gelatinase activity in schizonts treated with phosphatidyl inositol-specific phospholipase C. In addition, the isolated RAP-1 complex itself did not have gelatinase activity in the 75K region, even in the presence of EDTA, which we expected would enhance detection of non-metalloproteinases by inhibiting activity of the 220K, 95K and 70K metalloproteinases. The novel 76K serine gelatinase appears not to be related to the RAP-1, since the gene encoding RAP-1 has been sequenced and has no homology with known serine proteases (Ridley *et al.* 1990). Therefore, it appears that RAP-1 may be noncovalently associated with a number of proteins, including the 42K RAP-2 and an uncharacterized 40K protein (Ridley *et al.* 1991), the novel 76K serine protease (Braun-Breton *et al.* 1991), and the metallo-

proteinases described here. The role of these associated proteins and function of RAP-1 remains unclear.

The specificity of mAb 30cl3 is clearly RAP-1 as seen by immunoblot (Fig. 19); however, there may be cross-reacting epitopes on the two molecules that accounts for the co-isolation of the novel 72 kDa serine gelatinase with RAP-1. In addition, it remains unclear if this 72 kDa serine gelatinase is a parasite enzyme. Although antigens having these $M_r$ were immunoprecipitated with mAb 30cl3 from metabolically-labeled parasites (Braun-Breton *et al.* 1988), closer examination reveals that they may in fact be RAP-1 and not the serine gelatinase.

The fact that not all of the gelatinase activity is immunoprecipitated by mAb AC9 indicates that only a fraction of the enzyme is associated with RAP-1. Why serum gelatinase associates specifically with RAP-1 but not with RAP-3 or gp195 is unclear. In serum, gelatinases are often bound to inhibitors such as α-2 macroglobulin. It is possible that a structure of the RAP-1 complex is similar to that of an inhibitor (ie. α-2 macroglobulin or tissue inhibitor of metalloproteinases) (TIMP), resulting in the association with RAP-1 and the metalloproteinases.

Further characterization is necessary to conclusively determine if the malaria parasite utilizes host metalloproteinases or serine gelatinases to facilitate erythrocyte invasion. Recent evidence may support this hypothesis. Parasite invasion of erythrocytes is calcium-dependent from an extracellular source and is not supplemented by other metal ions (McCallum-Deighton and Holder, 1992).

In addition, secondary processing of the gp195-derived 42 kDa C-terminal fragment is mediated by a calcium-dependent serine protease, which may be membrane-bound to the parasite (Blackman and Holder, 1992). It is possible that both the metalloproteinases described here and the 72 kDa serine protease co-isolated with RAP-1 play a role in either erythrocyte invasion or in secondary processing of merozoite surface proteins or both.

Although the metalloproteinase-specific antisera did not react in IFA and immunoblot assays, it may be that the metalloproteinases are present in concentrations too low to be detected by these assays. Serum molecules are transported into the malaria parasite through a duct that transverses the erythrocyte and parasitophorous vacuole membrane (Pouvelle *et al.* 1991). It possible that the metalloproteinases are transported into the parasite through these ducts and thereby associate with RAP-1, although in amounts too low to be detected by IFA. Thus, if the metalloproteinases associate with RAP-1 prior to membrane extraction remains undetermined.

# CHAPTER VIII.

## ACTIVATION OF SERUM METALLOPROTEINASES WITH COBALT

Three gelatinases of 225, 95 and 70 kDa were a identified in *Plasmodium falciparum* cultures and were also non-covalently associated with RAP-1 (Chapter 8). Based on several characteristics, it was concluded to be the human serum enzyme described by other workers (Vartio and Baumann, 1990), which is secreted by macrophages, neutrophils and fibroblasts in culture (Vartio *et al.* 1982; Hibbs *et al.* 1985; Murphy *et al.* 1989). The 95K and 70K proteins are separate gene products, and the 225K polypeptide is believed to be formed from the 95K species through intermolecular disulfide bond (Vartio and Hovi, 1987).

Since it was initially thought to be a novel parasite enzyme, the parameters of activation were investigated: substrate specificity, class of protease, temperature and pH optimum. Protease inhibitors that are known to prevent the activity specific classes of enzymes were tested for their ability to abrogate gelatinase activity in zymograms. Only EDTA inhibited gelatinase activity, indicating that the gelatinases were metalloproteinases. Of eight divalent metal ions tested, only calcium was able to restore activity, suggesting that calcium was essential for activity. In this study, a new characterisitic of the gelatinases was identified. The metal ion requirement of the gelatinases associated with RAP-1 was evaluated using several metal ions in a concentration dependent manner.

Although calcium alone was previously reported to be sufficient for serum MMP activity in gelatin zymograms (Vartio and Baumann, 1990), only 25% of relative activity was achieved (Fig. 25). A concentration of 1.0 mM calcium was used in additional zymogram experiments.

To study the additive or synergistic effects of other metal ions, dose dependency curves were determined in the presence of calcium. Maximal gelatinase activity was achieved when 0.1 mM cobalt was used in conjunction with 1.0 mM calcium (Fig. 26). Magnesium could not be evaluated since its presence affected the Coomassie stain of the gel. The dose dependency curves were similar for each of the serum MMPs. Under our assay conditions, optimal activity was achieved at pH 7.5 and 37°C.

It is interesting to note that calcium was necessary for activation of the RAP-1 associated metalloproteinases and cobalt synergistically enhanced their activity. Previous studies showed that 1 $\mu$M zinc combined with 5 mM calcium is optimal for activating collagenase in a different assay system (Seltzer *et al.* 1977). Whether the addition of extracellular calcium and cobalt could enhance parasite invasion by heightened activation of the metalloproteinases remains speculative. The testing of these hypotheses may establish a role for these enzymes in the host/plasmodia relationship. Since the matrix metalloproteinases are implicated in the pathology of cancer and arthritis (Moscatelli and Rifkin, 1988), optimal conditions for performing zymogram assays are advisable. In vivo, manganese or zinc rather than cobalt are possibly the metal ions required for optimal activation.
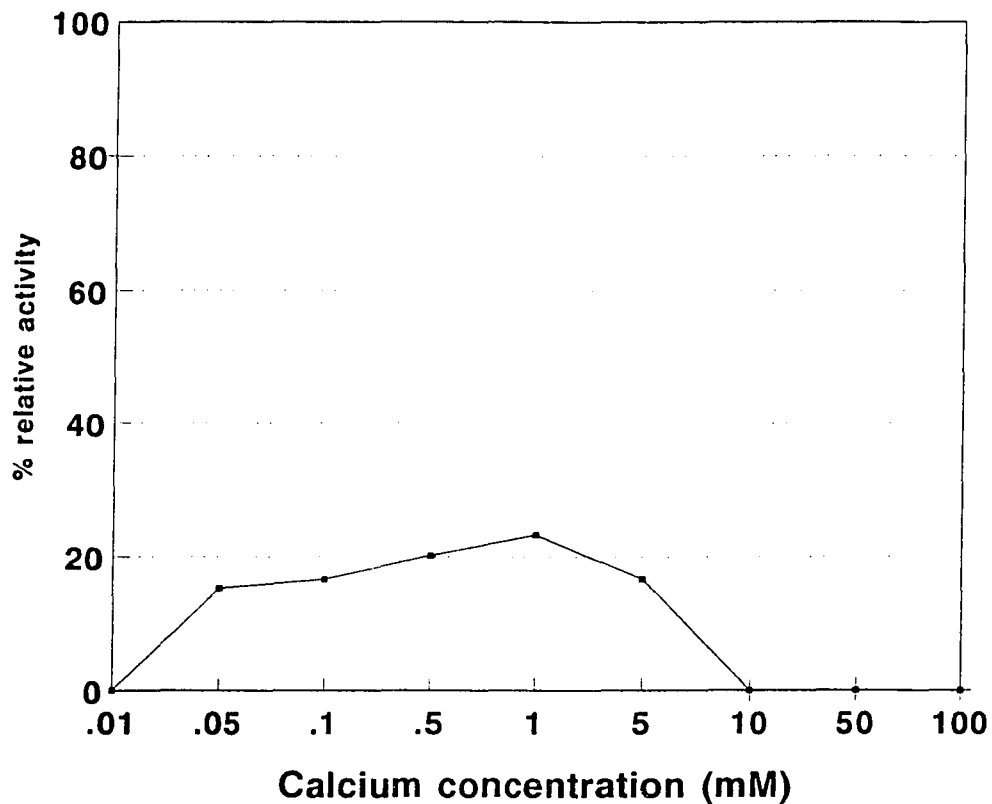
Figure 25.

Dose dependency curve of calcium for serum matrix metalloproteinases. Normal human serum (1.0 $\mu$l) was separated on a SDS-PAGE gelatin zymogram and relative gelatinase activity was determined. Per cent relative activity was evaluated on the zymogram assay using values from one to ten. Maximum activity was achieved in combination with 0.1 mM cobalt (see below).
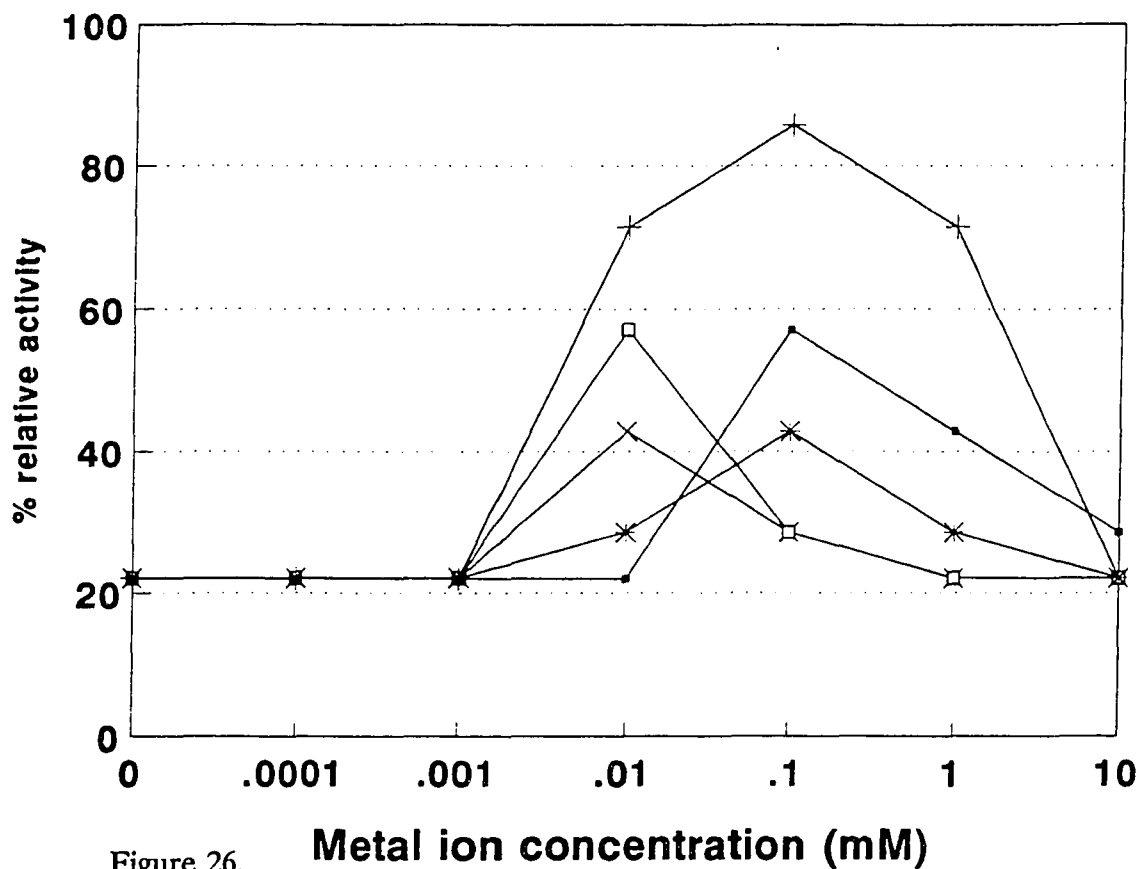
Figure 26.   **Metal ion concentration (mM)**

Dose dependency curve of various metal ions in the presence of 1.0 mM calcium. The metals used were (-+-) manganese, (-+-) cobalt, (-*-) copper, (-□-) zinc, and (-*-) cadmium.   Per cent relative activity was evaluated on the zymogram assay using values from one to ten.

# CHAPTER IX. CONCLUSION

*Plasmodium falciparum* is the most virulent species of parasite causing human malaria. In nature, immunity develops slowly and is only acquired after repeated infection, usually not until adulthood. Antibodies are important, since passive transfer of adult immune serum can confer protection to non-immune children (Cohen *et al.* 1961). Although the constituents of protective immunity have not been clearly defined, some blood-stage proteins have induced protective immunity in experimental models.

The merozoite surface (gp195) and rhoptry proteins are promising malaria vaccine candidates, since the native antigens are protective (Perrin *et al.* 1984; Hall *et al.* 1984; Siddiqui *et al.* 1987; Patarroyo *et al.*1987; Ridley *et al.* 1990; Etlinger *et al.* 1991). Based on new findings reported in this dissertation, it was concluded that the magnitude of the antibody response to gp195 was dependent upon native protein conformation. Reduction and alkylation of gp195 altered its antigenicity, immunogenicity and ability to induce growth inhibitory antibodies in rabbits.

These results may provide some insight as to why some recombinant gp195 polypeptides produced in bacterial expression systems (Holder *et al.* 1988; Herrera *et al.* 1990) and synthetic peptides (Cheung *et al.* 1986; Patarroyo *et al.* 1987; Ruebush *et al.* 1990) have met with limited success. On the other hand, a recombinant C-terminal polypeptide expressing conformational epitopes induced

antibodies in rabbits which completely inhibited parasite growth in vitro (Chang *et al.* 1992; submitted).

The results in this study suggest that gp195 resembles hepatitis B surface antigen (Dreesman *et al.* 1973), human immunodeficiency virus-1 gp120 (La Rosa *et al.* 1990) and the Japanese encephalitis virus envelope glycoprotein (Mason *et al.* 1989), in that neutralizing antibodies are dependent upon the presence of disulfide bridges. Thus, the native secondary and tertiary structure maintain immunological integrity of gp195, and the production of growth inhibitory antibodies is dependent upon protein conformation. Therefore, a recombinant polypeptide vaccine based on gp195 must be possess the native protein conformation in order to be efficacious.

The failure to identify a single mAb which inhibits parasite growth at low concentrations leaves this research problem without resolution. It may be that a single protective epitope exists, however, it was not identified in this study. Alternatively, since none of the mAbs evaluated in this study inhibited parasite growth at physiological concentrations, the hypothesis that a single inhibitory epitope exists on gp195 must be re-evaluated. A number of possibilities exist. (1) It may be that antibodies with several specificities are required for this inhibition to occur. That is, the requirement that antibodies be formed to multiple epitopes on gp195 may be a parasite strategy for immune evasion. If this is the case, a mAb to gp195 that inhibits parasite growth at low concentrations may never be defined. (2) It is possible that the in vitro growth inhibition assay requires high

antibody concentrations in order for inhibition to occur. That is, the requirement for high antibody concentrations is simply an artifact of the in vitro assay system. This possibility could be resolved by comparing concentrations of mAb required for growth inhibition in vitro with concentrations of mAb required for passive transfer of protection.

The immunogenicity of native rhoptry proteins was studied using rabbit monospecific polyclonal antisera. Like gp195, these sera has high antibody-titers and were effective in parasite growth inhibition. The lower antibody titer seen in monkeys immunized with RAP-3 (Siddiqui *et al.* 1987) may explain why previous experiments from this laboratory suggested that RAP-3 was less promising as a blood-stage vaccine candidate compared to gp195. The new evidence showing that RAP-1 can induce antibodies in rabbits which strongly inhibit parasite growth at levels comparable to gp195 re-opens the possibility that these proteins may be vaccine candidates as well. Based on this finding, vaccination experiment using the native rhoptry antigens, both RAP-3 and RAP-1, are appropriate. If such experiments are successful, the possibility of a polyvalent asexual blood-stage vaccine involving both merozoite surface and rhoptry proteins must be re-evaluated.

The use of a strong adjuvant is essential for a vigorous antibody response to gp195 and rhoptry proteins (Siddiqui *et al.* 1986), and growth inhibition in vitro is correlated with high antibody titers (Hui *et al.* 1990). In this study, inhibition by anti-gp195 and anti-rhoptry antisera was dependent upon high antibody levels as

well. No single mAb was capable of inhibiting parasite growth in vitro greater than 50%; whereas, high-titered, polyclonal antisera presumably directed against multiple epitopes were capable of inhibiting parasite growth by 90% or more. The picture emerging from these studies is that the overall antibody response to native gp195 and rhoptry antigens may be important in maintaining the fine balance between parasite survival and host immunity. In nature, parasites co-exist with circulating antibody and are necessary for the induction of protective immunity, a concept sometimes termed "premunition." Since it is not to the parasite's advantage to terminate its host, perhaps it is the parasite strategy to require a vigorous antibody response to multiple epitopes and proteins before solid immunity can be achieved. Only in the immune adult does a symbiotic relationship exist between man and *Plasmodium falciparum*. Thus, it is possible that a recombinant blood-stage malaria vaccine at its best will not alter the basic host-parasite relationship; that is, some parasites will ultimately survive in the presence of circulating antibodies.

BIBLIOGRAPHY

Apella, E., Weber, I.T., and Blasi, F. (1988) Structure and function of epidermal growth factor-like regions in proteins. FEBS Lett. 231, 1-4.

Ballou, W.R., S.L. Hoffman, J.A. Sherwood, M.R. Hollingdale, F.A. Neva, W.T. Hockmeyer, D.M. Gordon, I. Schneider, R.A. Wirtz, J.F. Young, G.F. Wasserman, P. Reeve, C.L. Diggs, and J. D. Chulay. (1987) Safety and efficacy of a recombinant DNA Plasmodium flaciparum sporozoite vaccine. Lancet 1, 1281.

Bannister, L.H., Mitchell, G.H., Butcher, G.E., and Dennis, E.D. (1986) Lamellar membranes associated with rhoptries inerythrocytic merozoites of Plasmodium knowlesi: a clue to mechanism of invasion. Parasitology. 92, 291.

Bernard, F., and Schrevel, J. (1987) Purification of a Plasmodium berghei neutral endopeptidase and its localization in merozoite. Mol. Biochem. Parasit. 26, 167.

Blackman, M.J., Heidrich, H.J., Donachie, S., McBride, J.S., and Holder A.A. (1990) A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. 172, 379.

Blackman, M.J., H. Whittle and A.A. Holder. (1991a) Processing of the Plasmodium falciparum major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocyte invasion. Mol. Biochem. Paras. 49, 35.

Blackman, M.J., I.T. Ling, S.C. Nicholls and A.A. Holder (1991b) Proteolytic processing of the Plasmodium falciparum merozoite surface protein-1 produces a membrane fragment containing two epidermal growth factor-like domains. Mol. Biochem. Paras. 49, 29.

Blackman, M.J. and A.A. Holder (1992) Secondary processing of the Plasmodium falciparum merozoite surface protein-1 (MSP-1) by a calcium-dependent membrane-bound serine protease: shedding of $MSP_{33}$ as a noncovalently associated complex with other fragments of the MSP1. Mol. Biochem. Paras. 50, 307.

Boyle, D., Newbold, C., Smith, C., and Brown, K. (1982) Monoclonal antibodies that protect in vivo against Plasmodium chabaudi recognize a 250,000-dalton parasite polypeptide. Infect. Immun. 38, 94.

Braun-Breton, C., Rosenberry, T.L. and Pereira da Silva, L. (1988) Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. Nature. 332, 457.

Brown, H.J. and R.L. Coppel (1991) Primary structure of a *Plasmodium falciparum* rhoptry antigen. Mol. Biochem. Paras. 49, 99.

Brown, J., H.C. Whittle, K. Berzins, R.J. Howard, K. Marsh, and K. Sjoberg (1986) Inhibition of *Plasmodium falciparum* growth by IgG antibody produced by human lymphocytes transformed with Epstein-Barr virus. Exp. Immunol. 63, 357.

Burns, J.M., T.M. Daly, A.B. Vaidya, and C.A. Long (1988) The 3' portion of the gene for a *Plasmodium yoelii* merozoite surface antigen encodes the epitope recognized by a protective monoclonal antibody. Proc. Natl. Acad. Sci. USA 85, 602.

Bushell, G.R., Ingram, L.T., Fardoulys, C.A., and Cooper, J.A. (1988) An antigenic complex in the rhoptries of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 28, 105.

Camus, D. and Hadley, T.J. (1985) A *PLasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science 230, 553.

Camus, D., J.A. Lyon, T. Reaud-Jareed, J.D. Haynes, and C.L. Diggs. (1987) Characterization of gp195 processed products purified from *Plasmodium falciparum* culture supernates. Mol. Biochem. Paras. 26, 21.

Certa, U., Ghersa, P., Dobeli, H., Matile, H., Kocher, P., Srivastava, J.K., Shaw, A.R., and Perrin, L.H. (1987) Aldolase activity of a *Plasmodium falciparum* protein with protective properties. Science. 230, 1036.

Chang, S.P., Hui, G.S.N., Kato, A., Siddiqui, W.A. (1989) Generalized immunological recognition of the major merozoite surface antigen (gp195) of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA 86, 6343.

Chang, S.P., Kramer, K.J., Yamaga, K.M., Kato, A., Case, S.E., Siddiqui, W.A. (1988) *Plasmodium falciparum*: gene structure and hydropathy profile of the major merozoite surface antigen (gp195) of the Uganda-Palo Alto isolate. Exp. Parasit. 67, 1.

Chang, S.P., H.L. Gibson, C.T. Lee-Ng, P.J. Barr and G.S.N. Hui (submitted) A carboxyl-terminal fragment of *P. falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth.

Charbonneau, H. (1989) Strategies for obtaining partial amino acid sequence data from small quantities (<5nmol) of pure or partially purified protein. In: A practical guide to protein and peptide purification for microsequencing. P. Matsudaira, ed. Academic Press, New York.

Cheung, A., A. Shaw, J. Leban and L. Perrin (1985) Cloning and expression in E. coli of a surface antigen of *Plasmodium falciparum* merozoites. EMBO Journal 4, 1007.

Cheung, A., J. Lean, A.R. Shaw, B. Merkli, J. Stockler, C. Chizzolini, C. Sander, and L.H. Perrin (1986) Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. Proc. Natl. Acad. Sci. USA 83, 8328.

Clark, J.T., Anand, R., Akoglu, T. and McBride, J.S. (1987) Identification and characterisation of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites. Parasitol. Res. 73, 425.

Cohen, S., McGregor, I.A., and Carrington, S. (1961) Gamma-globulin and acquired immunity to human malaria. Nature 192, 733.

Collins, W.E., R.F. Anders, M. Pappaioanou, G.H. Campbell, G.V. Brown, D.J. Kemp, R.L. Coppel, J.C. Skinner, P.M. Andrysiak, F.M. Favaloro, L.M. Corcoran, J.R. Broderson, G.F. Mitchell, and C.C. Campbell. (1986) Nature 323, 259.

Cooper, J.A., Ingram, L.T., Bushell, G.R., Fardoulys, C.A., Stenzel, D., Schofield, L. and Saul, A.J. (1988) The 140/130/105 kilodalton protein complex in the rhoptries of *Plasmodium falciparum* consists of discrete polypeptides. Mol. Biochem. Parasitol. 29, 251.

Emonard, H. and Grimaud J. (1990) Matrix metalloproteinases. A review. Cell. Mol. Biol. 36, 131.

Epstein, N., L.H. Miller, D.C. Kaushel, I.J. Udeinya, J. Rener, R.J. Howard, R. Asofsky, M. Aikawa and R.L. Hess. (1981) Monoclonal antibodies against a specific surface determinant on malarial (*Plasmodium knowlesi*) merozoites block erythrocyte invasion. J. Immunol. 127, 212.

Etlinger, H.M., Caspers, P., Matile, H., Schoenfeld, H.J., Stueber, D., Takacs, B. (1991) Ability of recombinant or native proteins to protect monkeys against heterologous challenge with *Plasmodium falciparum*. Infect. Immun. 59, 3498.

Etzion, Z. Murray, M.E. and Perkins, M. (1991) Isolation and characterization of rhoptries of *Plasmodium falciparum*. Mol. Biocem. Parasit. 47, 51.

Freeman, R.R., Trejdosiewicz, A. and Cross, G.A.M. (1980) Protective monoclonal antibodies recognizing stage-specific merzoite antigens of a rodent malaria parasite. Nature 284, 366.

Freeman, R.R., and A.A. Holder (1983) Characteristics of the protective response of BALB/c mice immunized with a purified *Plasmodium yoelii* schizont antigen. Clin. Exp. Immun. 54, 609.

Freeman, R. and A. Holder (1983) Surface antigens of malaria parasites; A high molecular weight precursor is processed to an 83,000 Mol Wt form expressed on the surface of *Plasmodium falciparum* merozoites. J. Exp. Med. 158, 1647.

Geimann, Q.M., and M. Meagher (1967) Susceptibility of a New World monkey to *Plasmodium falciparum* from man. Nature 215, 437.

Haldar, Kasturi, M.A.J. Ferguson, and G.A.M. Cross (1985) Acylation of a *Plasmodium falciparum* merozoite surface antigen via sn-1,2-diacyl glycerol. J. Biol. Chem. 260, 4969.

Hall, R., J. McBride, G. Morgan, A. Tait, J. Zolg, D. Walliker, and J. Scaife (1983) Antigens of the erythrocytic stages of the human malaria parasite *Plasmodium falciparum* detected by monoclonal antibodies. Mol. and Biochem. Paras. 7, 247.

Hall, R., Hyde, J.E., Goman, M., Simmons, D.L., Hope, I.A., Mackay, M., Scaife, J., Merkli, B., Richle, R., Stocker, J. (1984) Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature 311, 379.

Harte, P.G., N.C. Rogers, and G.A.T. Targett (1985) Role of T cells in preventing transmission of rodent malaria. Immunology 56, 7.

Heidrich, H., W. Strych, and J. Mrema (1983) Identification of surface and internal antigens from spontaneously released *Plasmodium falciparum* merozoites by radioiodination and metabolic labeling. Z.Parasitenkd. 69, 715.

Heidrich, H.G., A. Miettinen-Baumann, C. Eckerskorn and F. Lottspeich (1989) The N-terminal amino acid sequences of the *Plasmodium falciparum* (FCB1) merozoite surface antigens of 42 and 36 kilodalton, both derived from the 185-195-kilodalton precursor. Mol. and Biochem. Paras. 34, 147.

Herrera, S., Herrera, M.A., Perlaza, B.L., Burki, Y., Caspers, P., Doebli, H., Rotmann, D., Certa, U. (1990) Immunziation of *Aotus* monkeys with *Plasmodium falciparum* blood-stage recombinant proteins. Proc. Natl. Acad. Sci. USA 87, 4017.

Herrington, D.A., D.F. Clyde, G. Losonsky, M. Cortesia, J.R. Murphy, J. Davis, S. Baqar, A.M. Felix, E.P. Heimer, D. Gillessen, E. Nardine, R.S. Nussenzweig, V. Nussenzweig, M.R. Hollingdale, and M.M. Levine (1987) Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Nature 328, 259.

Heussen, C. and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. 102, 196.

Hibbs, M.S., Hasty, K.A., Seyer, J.M., Kang, A.H., and Mainardi, C.L. (1985) Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. J. Biol. Chem. 260, 2493.

Hoffman, S.L., D. Isenbarger, G.W. Long, M.Sedegah, A. Szarfman, L. Waters, M.R. Hollingdale, P.H. van der Meide, D.S. Finbloom, and W.R. Ballou (1989) Sporozoite vaccine induces generically restricted T cell elimination of malaria from hepatocytes. Science 244, 1081.

Holder, A.A. and R.R. Freeman (1981) Immunization against blood-stage rodent malaria using purified parasite antigens. Nature 294, 361.

Holder, A. and R. Freeman (1982) Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and monoclonal antibody. J. Exp. Med. 156, 1528.

Holder, A. and R. Freeman (1984) The three major surface antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. J. Exp. Med. 160, 624.

Holder, A.A., Freeman, R.R., Uni, S., and Aikawa, M. (1985) Isolation of a *Plasmodium falciparum* rhoptry protein. Mol. Biochem. Parasitol. 14, 293.

Holder, A., M.J. Lockyer, K.G. Odink, J.S. Sandhu, V. Riveros-Moreno, S.C. Nicholls, Y. Hillman, L.S. Davey, M.L. Tizard, R.T. Schwarz, and R.R. Freeman (1985) Primary structure of the precursor to three major surface antigens of *Plasmodium falciparum* merozoites. Nature 317, 270.

Holder, A.A., R.R. Freeman, S.C. Nicholls (1988) Immunization against Plasmodium falciparum with recombinant polypeptides produced in *Escherichia coli*. Paras. Immunol. 10, 607.

Holder, A.A., J.S. Sandhu, Y. Hillman, L.S. Davey, S.C. Nicholls, H. Cooper and M.J. Lockyer (1987) Processing of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. Parasitology 94, 8.

Holder, A.A. (1988) The precursor to major merozoite surface antigens: structure and role in immunity. Prog. Allergy 41, 72.

Howard, R.F., Stanley, H.A., Campbell, G.H., and Reese, R.T. (1984) Proteins responsible for a punctate fluorescence pattern in *Plasmodium falciparum* merozoites. Am. J. Trop. Med. Hyg. 33, 1055.

Howard, R.J., J.A. Lyon, C.L. Diggs, J.D. Haynes, J.H. Leech, J.W. Barnwell, S.B. Aley, M. Aikawa, and L.H. Miller (1984) Localization of the major *Plasmodium falciparum* glycoprotein on the surface of mature intraerythrocytic trophozoites and schizonts. Mol and Biochem Paras. 11, 349.

Howard, R.J., J.S. McBride, S.B. Aley, and K. Marsh (1986) Antigenic diversity and size diversity of *Plasmodium falciparum* antigens in isolates from Gambian patients. II. The schizont surface glycoprotein of molecular weight _200,000. Paras. Immun. 8, 57.

Hui, G.S.N., and W.A. Siddiqui (1987) Serum from Pf195 protected Aotus monkeys inhibit *Plasmodium falciparum* growth in vitro. Exp. Paras. 64, 519.

Hui, G.S.N., S.P. Chang, L.Q. Tam, A. Kato, S.E. Case, C. Hashiro, S. Kotani, T. Shiba, S. Kusumoto, and W.A. Siddiqui (1989) Characterization of antibody responses induced by different synthetic adjuvants to the *Plasmodium falciparum* major merozoite surface precursor protein gp195. Vaccine 90, 447.

Hui, G.S.N., Tam, L.Q., Chang, S.P., Case, S.E., Hashiro, C., Siddiqui, W.A., Shiba, T., Kusumoto, S., Kotani, S. (1991) Synthetic low-toxicity muramyl dipeptide and monophosphoryl lipid A replace Freund Complete Adjuvant in inducing growth-inhibitory antibodies to the *Plasmodium falciparum* major merozoitte surface protein, gp195. Infect. Immun. 59, 1585.

Kan, S.C., K.M. Yamaga, K.J. Kramer, S.E. Case, and W.A. Siddiqui (1984) *Plasmodium falciparum*: Protein antigens identified by analysis of serum samples from vaccinated Aotus monkeys. Infect. Immun. 43, 276.

Kaslow, D.C, Quakyi, I.A., Syin, C., Raum, M.G., Keister, D.B., Coligan, J.E., McCutchan, T.F. and Miller, L.H. (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature 333, 74-76.

Kemp, D.J., R.L. Coppel and R.F. Anders (1987) Repetitive proteins and genes of malaria. Ann. Rev. Microbiol. 41, 181.

Knapp, B., Hundt, E., and Kupper, H.A. (1990) *Plasmodium falciparum* aldolase:gene structure and localization. Mol. Biochem. Parasitol. 40, 1.

Kohler, G. and C. Milstein (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495.

Kotani, S., H. Takada, I. Takahashi and T. Shimamoto (1986) Immunobiological activities of synthetic lipid A analogues with low endotoxicity. Infect. Immun. 54, 673.

Kramer, K.J., Kan, S.C. and Siddiqui, W.A. (1982) Concentration of *Plasmodium falciparum*-infected erythrocytes by density gradient centrifugation in percoll. J. Parasitol. 68, 336.

Kumar, S.L., H. Miller, I.A. Quakyi, D.B. Keister, R.A. Houghten, W.L. Maloy, B. Moss, J.A. Berzofsky, and M.F. Good (1988) Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium flaciparum*. Nature 334, 260.

La Rosa, G.J., Davide, J.P., Weinhold, K., Watebury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.F., Dreesman, G.R., Boswell, R.N., Sadduck, P. et al. (1990) Conserved sequence and structrual elements in the HIV-1 principal neutralizing determinant. Science 249, 932.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680.

Lyke, E., K. Carlberg and R. Norby. (1975). Interactions between *Toxoplasma gondii* and its hosts cell: function of the penetration-enhancing factor of Toxoplasma. Infect. Immun. 11, 853.

Lyon, J., R. Geller, J.D. Haynes, J.D. Chulay, and J.L. Weber (1986) Epitope map and processing scheme for the 195,000 dalton surface glycoprotein of *Plasmodium falciparum* merozoites deduced from cloned overlapping segments of the gene. Proc. Natl. Acad. Sci. USA 83, 2989.

Lyon, J., J.D. Haynes, C. Diggs, J. Chulay, C. Haidaris, and J. Pratt-Rossiter (1987) Monoclonal antibody characterization of the 195 KD major surface glycoprotein of *Plasmodium falciparum* malaria schizonts and merozoites. Identification of additional processed products and a serotype-restricted repetitive epitope. J. Immun. 138, 895.

Mackay, M., M. Gowan, N. Bone, and J. Hyde (1985) Polymorphism of the precursor for the major surface antigens of *Plasmodium falciparum* merzoites, studies at the gene level. EMBO Journal 133, 3823.

Majarian, W.R., T.M. Daly, W.P. Weindanz, and C.A. Long (1984) Passive immunization against murine malaria with an IgG3 monoclonal antibody. J. Immun. 131, 3131.

McBride, J.S., C.I. Newbold, and R. Anand (1985) Polymorphism of a high molecular weight schizont antigen of the human malaria parasite *Plasmodium falciparum*. J. Exp. Med. 161, 160.

McBride, J.S., and Heidrich, H.G. (1987) Fragments of the polymorphic $M_r$ 185 000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. Mol. Biochem. Parasit. 1987, 23, 71.

McCallum-Deighton, N. and Holder, A.A. (1992) The role of calcium in the invasion of human erythrocytes by *Plasmodium falciparum*. Mol. Biochem. Parasit. 50, 317.

Moscatelli, D. and Rifkin, D.B. (1988) Membrane and matrix localization of proteinases: a common theme in tumaor cell invasion and angiogenesis. Biochim. Biophys. Acta 948, 67.

Murphy, G., Hembry, R.M., McGarrity, A.M., Reynolds, J.J. and Henderson, B. (1989) Gelatinase (type IV collagenase) immunolocalization in cells and tissues: use of an antiserum to rabbit bone gelatinase that identifies high and low $M_r$ forms. J. Cell Sci. 92, 487.

Nichols, B.A., M.L. Chiappino and G.R. O'Connor (1983) Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. J. Ultra. Res. 83, 85.

Oaks, S.C., V.S. Mitchell, G.W. Pearson, and C.C.J. Carpenter, ed. (1991) Malaria: Obstacles and Opportunities. National Academy Press, Washington, D.C.

Ockenhouse, C.F., S. Schulman, and H.L. Shear (1984) Induction of crisis forms in the human malaria parasite *Plasmodium falciparum* by gamma-interferon-activated, monocyte-derived macrophages. J. Immun. 133, 1608.

Patarroyo, M.E., P. Romero, M.L. Torres, P. Clavijo, A. Moreno, A. Martinez, R. Rodriguez, F. Guzman, and E. Cabezas (1987) Induction of protective immunity against experimental infection with malaria using synthetic peptides. Nature 328, 629.

Pattarroyo, M.E., R. Amador, P. Clavijo, A. Moreno, F. Guzman, P. Romero, R. Teascon, A. Franco, L. A. Murillo, G. Ponton, and G. Trujillo (1988) A synthetic vaccine protects humans against challenge with asexual blood stages of Plasmodium falciparum malaria. Nature 332, 158.

Perkins, M.E. (1984) Surface proteins of Plasmodium falciparum merozoites binding to the erythrocyte receptor, glycophorin. J. Exp. Med. 160, 788.

Perkins, M.E. (1989). Erythrocyte invasion by the malaria merozoite: recent advances. Exp. Paras. 69, 94.

Perrin, L.H., Merkli, B., Loche, Chizzolini, C., Smart, J, Richle, R. A. (1984) Antimalarial immunity in Saimiri monkeys, immunization with surface components of asexual blood stages. J. Exp. Med. 160, 441.

Perrin, L.H., B. Merkli, M.S. Gabra, J.W. Stocker, C. Chizzolini, and R. Richie (1985) Immunization with a Plasmodium falciparum merozoite surface antigen induces a partial immunity in monkeys. J. Clin. Invest. 75, 1718.

Peterson, M.G., R.L. Coppel, P. McIntyre, C.J. Langford, G. Woodrow, G.V. Brown, R.F. Anders, and D.J. Kemp (1988) Variation in the precursor to the merozoite surface antigens of Plasmodium falciparum. Mol. and Biochem. Paras. 27, 291.

Pirson, P.J. and M.E. Perkins (1985) Characterization with monoclonal antibody of a surface antigen of Plasmodium falciparum. J. Immun. 134, 1946.

Potocnjak, P., N. Yoshida, R.S. Nussenzweig, and V. Nussenzweig (1980) Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. J. Exp. Med. 151, 1504.

Pouvelle, B., Speigel, R., Hsiao, L., Howard, R.J., Morris, R.L., Thomas, A.P., and Taraschi, T.F. (1991) Direct access to macromolecules by intraerythrocytic malaria parasites. Nature 353, 73.

Raybould, T.J.G. and M. Takahashi (1988) Production of stable rabbit-mouse hybridomas that secrete rabbit MAb of defined specificity. Science 240, 1788.

Redinbaugh, M.G. and R.B. Turley (1986) Adaptation of the Bicinichoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. 153, 267.

Ridley, R.G., Takacs B., Lahm, H-W., Delves C.J., Goman M., Certa, U., Matile, H., Woollett, G.R., and Scaife, J.G. (1990) Characterisation and sequence of a protective rhoptry antigen from *Plasmodium falciparum*. Mol. Biochem. Parasitol. 41, 125.

Ridley, R.G., Lahm, H-W., Takacs,B., and Scaife, J.G. (1991) Genetic and structural relationships between components of a protective rhoptry antigen complex from *Plasmodium falciparum*. Mol. Biochem. Parasitol. 47, 245.

Roger, N., Dubremetz, J.F., Delplace, P., Fortier, B., Tronchin, G., and Vernes, A. (1988) Characterization of a 225 kilodalton rhoptry protein of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 27, 135.

Romero, P., J.L. Maryanski, G. Corradin, R.S. Nussenzweig, V. Nussenzweig, and F. Zavala (1989) Cloned cytotoxic T cells recognize and epitope in the circumsporozoite protein and protect against malaria. Nature 341, 323.

Ruebush, T.K., Campbell, G.H., Moreno, A., Patarroyo, M.E., and Collins, W.E. (1990) Immunization of owl monkeys with a combination of *Plasmodium falciparum* asexual blood-stagesynthetic peptide antigens. Am. J. Trop. Med. Hyg. 43,355.

Saul, A., J. Cooper, D. Hauquitz, D. Irving, Q. Cheng, A. Stowers and T. Limpaiboon (1992) The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. Mol. Biochem. Paras. 50, 139.

Sam-Yellowe, T.Y., Shio, H., and Perkins, M.E. (1988) Secretion of *Plasmodium falciparum* rhoptry protein into the plasma membrane of host erythrocytes. J. Cell Biol. 196, 1507.

Sam-Yellowe, T.Y. and Perkins, M.E. Interaction of the 140/130/110 kDa rhoptry protein complex of *PLasmodium falciparum* with the erythrocyte membrane and liposomes. Exp. Parasit. 73, 161.

Schmidt-Ullrich, R., J. Brown, H. Whittle, and P. Lin (1986) Human-human hybridomas secreting antibody to the Mr 195,000 *Plasmodium falciparum* blood stage antigen. J. Exp. Med. 163, 179.

Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982) A one-step purification of membrane proteins using a high efficiency immunomatrix. J. Biol. Chem. 257, 10766.

Schofield, L., Bushell, G.R., Cooper, J.A., Saul, A.J., Upcroft, J.A., and Kidson, C. (1986) A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. Mol. Biochem. Parasitol. 18, 183.

Schrevel, J., Grellier, P., Mayer, R., and Monsigny, M. (1988) Neutral proteases involved in the reinvasion of erythrocytes by *Plasmodium* merozoites. Biol. Cell. 64, 233.

Seltzer, J.L., Jeffrey, J.J. and Eisen, A.Z. (1977) Evidence for mammalian collagenases as zinc ion metalloenzymes. Biochim. Biophys. Acta 485, 179.

Siddiqui, W.A., D.W. Taylor, S.C. Kan, K. Kramer, and S. Richmond-Crum (1978) Partial protection of *Plasmodium falciparum* vaccinated *Aotus trivirgatus* against a challenge of a heterologous strain. Am. J. Trop. Med. Hyg. 27, 1277.

Siddiqui, W.A. and K.L. Palmer (1981) Propogation of malaria parasites in vitro. In: Advances in Cell Culture. K. Maramorosch (ed.) v.1 New York: Academic Press.

Siddiqui, W.A., L.Q. Tam, S.C. Kan, K.J. Kramer, S.E. Case, K.L. Palmer, K.M. Yamaga, and G.S.N. Hui (1986) Induction of protective immunity to monoclonal antibody defined *Plasmodium falciparum* antigens requires strong adjuvant in Aotus monkeys. Infect. Immun. 52, 314.

Siddiqui, W.A., Tam, L.Q., Kramer, K.J., Hui, G.S.N., Case, S.E., Yamaga, K.M., Chang, S.P., Chan, E.B.T., and Kan, S.C.(1987) Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA 84, 3014.

Towbin, H., T. Staehelin and J. Gordon (1979) Electrophoretic transfer of proteins from polyacyylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76, 4350.

Trager, W. and Jensen, W.(1976) Human malaria parasites in continuous culture. Science 193, 673.

Vartio, T. and Baumann, M. (1990) Human gelatinase/type IV collagenase is a regular plasma component. FEBS Lett 255, 285.

Vartio, T. and Hovi, T. (1987) Polypeptide composition of human macrophage gelatinase. Acta Chem. Scand. Ser. B 41, 754.

Vartio, T., Hovi, T. and Vaheri, A. (1982) Human macrophages synthesize and secrete a major 95,000-dalton gelatin-binding protein distinct from fibronectin. J. Biol. Chem. 257, 8862.

Vermeulen, A.N., T. Ponnudurai, P.J.A. Beckers, J.-P. Verhave, M.A. Smits, and J.H.E.T. Meuwissen (1985) Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. J. Exp. Med. 162, 1460.

Voller, A. and R.S. Bray (1962) Fluorescent antibody staining as a measure of antibody. Proc. Soc. Exp. Biol. Med. 110, 907.

Weber, J.L., W.M. Leninger, and J.A. Lyon (1986) Variation in the gene encoding a major merozoite surface antigen of the human malaria parasite *Plasmodium falciparum*. Nucleic Acids Research 14, 3311.

Woesnner, J.F. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB 5, 2145.

World Health Organization (1969) Re-examination of the global strategy of malaria eradication. A report by the Director General to the 22nd WHO Assembly. May 30, 1969. Annex 13, Part I WHO Official records No. 176. Geneva: World Health Organization

World Health Organization (1991) Tropical Disease Progress in Research 1990-1991. Tenth Program Report of the UNDP/World Bank/WHO Geneva: World Health Organization