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CHARACTERIZATION OF A *SCHISTOSOMA MANSONI* C_3 RECEPTOR AS A POTENTIAL VACCINE CANDIDATE

by

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Department of Zoology

Submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

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ABSTRACT

Schistosomiasis, a chronic and debilitating parasitic disease, infects an estimated 200 million people and causes 750,000 deaths a year. Therefore, the development of an effective vaccine is of high priority for the control of this disease.

The surface of the syncytial epithelium of *Schistosoma mansoni* consists of an apical plasma membrane and an overlying envelope. The rapid turnover of these membranes in response to the host's attack is considered to be one of the mechanisms by which this parasite escapes the effects of the host's immune system. Our approach has been to identify surface components which play a pivotal role in this evasion mechanism and target functional important antigens as possible vaccine candidates against schistosomiasis.

The third component of Complement (C_3) has previously been shown to stimulate the synthesis of the EN via a Ca^{2+}-dependant signal transduction mechanism. The present study was designed to identify and characterize a C_3 receptor on the schistosome surface.

Using rosette assays a C_3 binding site was found present in the older stages of schistosome development, and with immunolabelling microscopy the C_3 binding site was localized on the dorsal surface of male parasites. ELISAs performed on isolated surface membranes shown that the C_3 binding site is restricted to the envelope fraction. Crosslinking experiments demonstrated that

a 130 kD envelope polypeptide is the C_3 receptor, and metabolic labelling studies proved that the receptor is synthesized by the schistosomes and not adsorbed from the host. Antibodies raised against this receptor were able to block envelope synthesis *in vivo*, confirming the function of this receptor in envelope synthesis. Immunological cross-reaction studies have demonstrated the presence of the C_3 receptor on *S. haematobium* and *S. mansoni*.

In conclusion, the C_3 receptor by virtue of its accessibility on the surface, its immunogenicity, and its function in membrane turnover, should be an excellent experimental vaccine candidate against schistosomiasis. Furthermore, it is the first time that an envelope component was identified and can be used as a marker for the envelope.

TO

THE CHILDREN OF KIBWEZI, KENYA

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To Dr. Graham Reid of the Institute of Primate Research (IPR) in Kenya, for introducing me to the human side of schistosomiasis and for transmitting to me his love for Africa. The *Schistosoma haematobium* and the baboon sera were generous gifts from him that helped me complete this project. I am very thankful to Dr. Reid and all the people at IPR for making my stay in Kenya so enjoyable.

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EBV,	Epstein-Barr Virus
ECP,	Eosinophil cationic protein
ELISA,	Enzyme-Linked Immuno-Sorbent Assay
EN,	Envelope
EPO,	Eosinophil peroxidase
Fc,	C terminal portion of an immunoglobulin which may activate complement
$F(ab')_2$,	the two antigen binding portions of an immunoglobulin
FITC,	Fluorescein Isothyocyanate
HSV,	Herpes Simplex Virus
Ig,	Immunoglobulin
IMS,	Infected Mouse Serum
kD,	kiloDalton
KRP,	Kreb's Ringer Phosphate
MαAPM,	Mouse-anti-APM Serum
MαEN,	Mouse-anti-Envelope Serum
Mα130,	Affinity purified anti-130 kD envelope polypeptide
MAC,	Membrane Attack Complex
MCP,	Membrane Cofactor Protein
MEM,	Minimum Essential Medium
MIP,	Membrane attack complex Inhibiting Protein

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LIST OF ABBREVIATIONS

5-HT, 5-Hydroxytryptamine; Serotonin

ABTS, 2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)

ADCC, Antibody-Dependent Cell-mediated Cytotoxicity

AP, Alkaline Phosphatase

APM, Apical Plasma Membrane

BCIP, Bromo-Chloro-Indolyl Phosphate

BSA, Bovine Serum Albumin

C_3, Third Component of the Complement System

DAB, Diaminobenzedine

DAF, Decay-Accelerating Factor

DB, Discoid Bodies

DPBS, Dulbecco's Phosphate Buffered Saline

DSS, Disuccinimidyl suberate

DTT, Dithiothreitol

EA19S, Erythrocytes coupled to anti-erythrocytes IgM antibodies

EAC_1, Erythrocytes coupled to C_1 component of the Complement System

EAC_{14}, Erythrocytes coupled to C_4 component of the Complement System

EAC_{1423}, Erythrocytes coupled to C_3 component of the Complement System

EBV,	Epstein-Barr Virus
ECP,	Eosinophil cationic protein
ELISA,	Enzyme-Linked Immuno-Sorbent Assay
EN,	Envelope
EPO,	Eosinophil peroxidase
Fc,	C terminal portion of an immunoglobulin which may activate complement
$F(ab')_2$,	the two antigen binding portions of an immunoglobulin
FITC,	Fluorescein Isothyocyanate
HSV,	Herpes Simplex Virus
Ig,	Immunoglobulin
IMS,	Infected Mouse Serum
kD,	kiloDalton
KRP,	Kreb's Ringer Phosphate
MαAPM,	Mouse-anti-APM Serum
MαEN,	Mouse-anti-Envelope Serum
Mα130,	Affinity purified anti-130 kD envelope polypeptide
MAC,	Membrane Attack Complex
MCP,	Membrane Cofactor Protein
MEM,	Minimum Essential Medium
MIP,	Membrane attack complex Inhibiting Protein

MLB,	Multilamellar Bodies
Mr,	Relative Molecular Mass
NBT,	Nitroblue Tetrazolium
NBS,	Normal Baboon Serum
NMS,	Normal Mouse Serum
NMSα130,	Normal Mouse Serum anti-130 envelope polypeptide
PAGE,	Polyacrylamide Gel Electrophoresis
pI,	Isoelectric Point
PKC,	Protein Kinase C
PMSF,	Phenyl-Methyl-Sulfonyl-Fluoride
PRV,	Pseudorabies Virus
RBC,	Red Blood Cell
SDS,	Sodium Dodecyl Sulfate
SHBS,	*Schistosoma haematobium* Infected Babbon Sera
SMBS,	*Schistosoma mansoni* Infected Baboon Sera
TBS,	Tris-Buffered Saline
TCA,	Trichloroacetic acid
Tween 20,	Polyoxyethylene sorbitan monolaurate
TTBS,	Tween-Tris Buffered Saline

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CHAPTER 1

INTRODUCTION

1.1 Schistosomiasis- An overview:

Schistosomiasis, because of its prevalence and economic impact, is the second most important tropical disease after malaria. The disease is present in seventy six countries in tropical and subtropical areas of the world (Bergquist,1990). Recent estimates indicate that schistosomes infect 200 million people and claim 500,000 lives a year (Rogers, 1987; Capron, 1992). Six hundred million more people are likely to be exposed to the infection (Bergquist, 1990) in an environment of poverty, lack of potable water and inadequate hygienic practices (Mott, 1987).

Infection with schistosomes results in a debilitating chronic disease known as schistosomiasis or bilhariasis, which is characterized by anaemia, diarrhea, abdominal pain and sometimes death. Between 5 and 10 % of heavily infected people will eventually die as a result of their infection, while others will suffer a chronic disease of varying severity involving enlargement of the liver and spleen with portal hypertension.

Although considerable advances have been made in the control of schistosomiasis over the last two decades, in particular through the development of effective drugs like praziquantel, and by treatment of the snail intermediate host with molluscides, the epidemiology of the disease has remained unchanged (Bergquist, 1990). In addition, drugs which must be

administered continually are prohibitively expensive to the economy of developing countries, and have the threat of producing strains of drug-resistant parasites. Therefore, alternative control measures, especially vaccines, are a high priority with the realistic aim of reducing morbidity and transmission rather than inducing a complete protection.

1.2 The biology of *Schistosoma mansoni*:

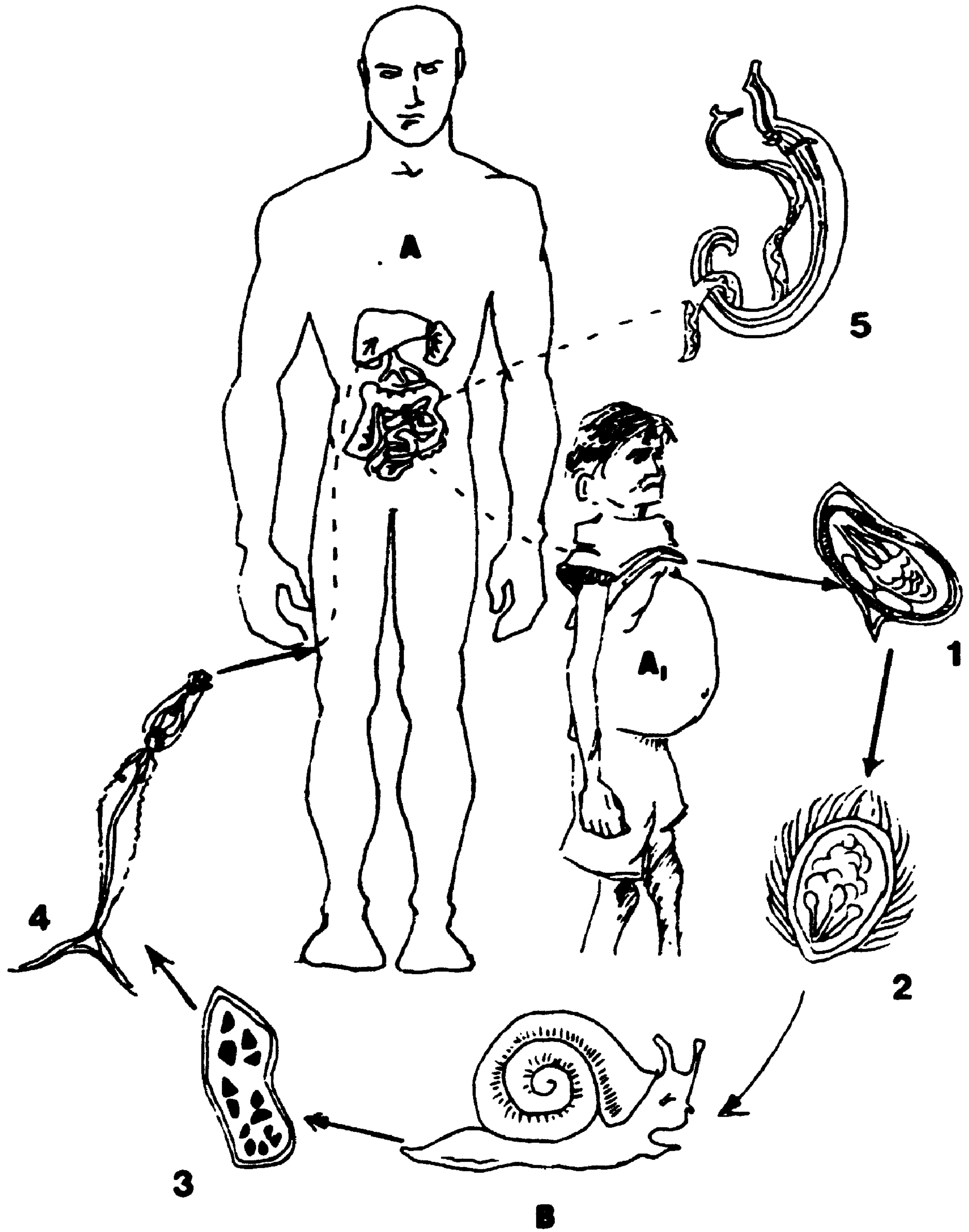
Schistosomes are members of the family Schistosomatidae and of the genus *Schistosoma*. They are dioecious, digenetic trematodes, which live in the blood vessels of vertebrates hosts. Five species are considered important human parasites; *S. mansoni, S. japonicum,* and *S. haematobium*, which are the most significant and widespread, and *S. intercalatum* and *S. mekongi*, which have a more narrow distribution. Some species, like *S. mattheei* are occasionally infectious to man while others like *S. incognitum* are regarded as potential zoonoses (Rollinson and Southgate, 1987). The egg morphology, in conjunction with the genera of the intermediate snail host in which the parasite develops, differentiates the species of schistosomes. For example, *S. haematobium* has terminal-spined eggs and develops in the snail *Bulinus*. *Schistosoma mansoni* has lateral-spined eggs and lives in the snail *Biomphalaria*. *Schistosoma japonicum* has round and small-spined or spineless eggs and develops in *Oncomelania* snails.

Schistosoma mansoni is the most important species in Africa and

America and is the subject of our study. It causes intestinal schistosomiasis in man. People become infected by coming into contact with the infective form of the parasite, the cercariae, by swimming, working, and bathing in contaminated water. Infection is initiated when cercariae penetrate the skin of the host, after which they undergo profound structural, physiological and biochemical adaptations to life inside the host. The schistosomulum undertakes a series of migrations via the lungs (where it develops further and adapts for intravascular migration) and then via the arterial circulation until it reaches the vessels of the hepatic portal system. At this site schistosomula of different sexes are paired, develop into adult worms of 10 mm in length (about 35 days post-infection) and, as pairs, relocate to the mesenteric plexus where the female starts producing eggs. There the worms may live for many years (usually from 3.5 to 12 years), with some worms surviving for 30 years (Vermund *et al.*, 1983)]. Some of the eggs traverse the intestinal epithelium and are expelled to the outside with the faeces. The eggs hatch into miracidia when the appropriate conditions are met, and miracidia start a new cycle by infecting the snail intermediate host in which cercariae develop. After a few weeks (between 4 to 6) cercariae are shed into the aqueous medium ready to infect the next host (See Figure 1.1 for a representation of the schistosome life cycle).

Figure 1.1: Representation of the life cycle of *Schistosoma mansoni*.

A, definitive host; A_1, definitive host showing the effects of schistosomiasis; B, the snail intermediate host; 1, egg (containing a fully formed miracidium) is produced by adult females and is discharged with the host's faeces; 2, miracidium (hatches out when the egg is immersed in water, then penetrates an appropriate snail host); 3, sporocyst (once the miracidium penetrates the snail it develops asexually into two generations of sporocysts from which cercariae develop); 4, cercaria (the cercariae emerge from the snail and infect the definitive host by penetrating the skin); 5, adult worm of both sexes (after penetration cercariae are transformed into schistosomula, migrate to the portal circulation and mature into adults in the mesenteric veins).


A
5
A1
1
2
4
3
B

1.3 The disease:

Schistosomiasis is a chronic disease that persists from childhood (at the time of first water contact) to adult life (von Lichtenberg, 1987). The severity of the disease depends on the number of worms present in the host.

During the period of cercarial penetration and schistosomula migration and development, a number of symptoms are manifested. Dermatitis at the site of contact occurs within 24 to 36 hours after infection, followed by traumatic changes in the lungs, sometimes accompanied by haemorrhages. An acute hepatitis is produced by the arrival and development of the larvae in the hepatic portal blood vessels. At the time of maturation and egg output a few infected persons develop an acute febrile illness which is known as "toxaemic schistosomiasis" in Brazil and as "Katayama fever" in Japan. Most infected individuals have no or very minor early symptoms. Although lesions of internal organs are occurring, there is not much outward manifestation of the disease. Only after 5 or more years of infection, do individuals with heavy parasitic burdens suffer advanced fibrovascular lesions of the target organs such as spleen and liver (pipestem fibrosis) accompanied by portal hypertension. This portal hypertension can result in collateral circulation, or if this extra circulation is absent, liver failure and death can occur. These lesions, which develop over long periods, are caused by schistosome eggs, rather than by the worms themselves. Less than half the eggs laid by the female will reach the lumen of the gut, the rest of the eggs are trapped in host tissues or are swept into the

portal veins or lung arterioles. In addition, worms occasionally leave the portal system into the cava veins and deposit their eggs in the pulmonary arterioles or within the vertebral venous system, with ectopic depositions of eggs in the the spinal cord or kidneys. Eggs that are trapped in host tissues (mainly in the liver) stimulate the host immune response and an intense granulomatous reaction occurs around the eggs. A newly produced granuloma consists of activated macrophages congregated centrally around the egg-shell with some multinucleated Langerhans-type giant cells. This is followed by the arrival of plasma cells along with some mast cells, neutrophils, lymphocytes, fibroblasts, mononuclear phagocytes, and eosinophils. Fibroblasts appear early and gradually replace all other cell types in the granuloma. Mature granulomas range from 350 μm to sizes that are visible by the human eye (von Lichtenberg, 1987). The size of the granulomas peaks by days 6-8 after egg deposition in the tissue.

The production of pseudo-abscesses around the eggs with later transformation into pseudo-granulomas produces fibrosis and obstruction of segments in the digestive tract, liver cirrhosis and portal obstruction leading to ascites, poor digestion, and in some cases esophagic varices. When the granulomas are large or are in groups they can cause obliteration of portal veins. This process continues during the parasite's life and can last for about 25 years if the patient survives. There are some lesions associated with dead worms being swept into the liver or lungs producing focal lesions. But these

focal lesions are minor and of little clinical importance compared to the damage caused by the millions of eggs generated during the course of the infection.

1.4 Epidemiology of schistosomiasis:

Acknowledging the inadequate economic resources of schistosome-endemic countries to achieve complete protection, the current objective of schistosomiasis control is merely to achieve a reduction of the disease (Mott, 1987) in the hope that in the long term schistosomiasis will be eradicated.

In order to increase the efficacy of any control tool against schistosomiasis it is imperative to understand the intricacies of the transmission of this parasitic disease, including the patterns of human contact with infected water.

Although some small animals are considered reservoirs for human schistosomiasis, people are the major source of infection. Consequently, schistosome infections are maintained in areas where human waste is able to directly drain into a water supply where both the snail intermediate host thrives and where there is human contact with the infested water. However, not all of the infected population is responsible for transmitting the disease. Previous studies have found that schistosome eggs are concentrated in small areas throughout the endemic areas, suggesting that eggs are passed by only a small number of infected people (Wilkins, 1987). In Nigeria, males under 21 years of age were found to have a relative transmission potential of 77 %

(Pugh, 1979) while in St. Lucia, children between the ages of 5 to 14 were responsible for 58 % of potential transmission (Upatham *et al.*, 1976). In addition, in an schistosome-endemic area, the intensity and prevalence of infection also varies among members of the community. Children are the most affected (Pesignan *et al.*, 1958) and males 15 to 19 years of age have the highest prevalence (Domingo *et al.*, 1980). The intensity of infection declines with age, which suggests that older subjects have acquired a degree of immunity. However, some adult populations subjected to very high exposures, (e.g. canal workers in Gambia and fishermen in Kenya) were shown to have high infection rates (Wilkins, 1987), indicating that the intensity of exposure is also important. Again, intense exposure was suggested to be the major factor in studies where there had been rapid reinfection after chemotherapy (Wilkins, 1987). In view of these factors, control programmes should be directed to selected groups (children) or occupational groups (canal workers).

People not only are the source of infection but are also the vector in the spread of the disease, since refugees from famine, human conflict and natural disasters are moving to and from endemic areas (Weniger and Schantz, 1984). Human intervention has also introduced the snail intermediate host into new areas by altering the water flow for irrigation and personal use and by developing more agricultural areas with the consequent increase of sites of transmission (Hunter *et al.*, 1982).

To summarize, transmission of human schistosomiasis is influenced by the distribution of the snail intermediate host, by contamination of the water supply with human faeces or urine (for *S. haematobium*), by the patterns of water contact by humans and by the role of protective immunity.

1.5 The immune response to Schistosomes in experimental hosts:

The fact that common laboratory animals are susceptible to schistosome infection has facilitated the study of the immunology of schistosomiasis. Mice, hamsters, rats, birds, guinea pigs, and primates have been used as experimental hosts (McLaren and Smithers, 1987). Mice and primates allow *S. mansoni, S. haematobium, and S. japonicum* to develop into adults that produce eggs. Rats on the other hand, allow *S. mansoni* to develop for only 4 weeks, after which most of the worms are expelled (McLaren and Smithers, 1987). The few remaining worms are stunted and do not produce viable eggs (McLaren and Smithers, 1987). Guinea pigs permit the development of adult *S. mansoni*, however, the eggs that these parasite produce are non-viable and are not excreted in the faeces (McLaren and Smithers, 1987).

Each animal model has advantages and disadvantages. The rat model will not be appropriate to study egg-induced pathology, but because resistance to infection does develop in the rat, it can therefore be used to examine some immune mechanisms. The mouse model, although producing egg-induced pathology and showing partial resistance to reinfection, suffers from the

disadvantage that the small level of infection leading to pathology (1 worm pair) is equivalent to 2000-3000 worm pairs in a 60 kg man. Since it is difficult to encounter such a high level of infection in man, murine schistosomiasis represents at best only the most extreme end of the spectrum in the human-schistosome relationship. Although a light infection in guinea pigs is more comparable to that commonly seen in humans, its use as an experimental host has only recently been exploited.

Immunity to schistosomes in an experimental host can be induced after exposure to a natural primary infection, or after immunizations with irradiated cercariae or with antigenic preparations (Capron *et al.*, 1987a). Protective immunity following a natural cercarial infection is induced by the presence of adult worms, but it is directed against the larval stages of secondary infections. The adult worms remain unharmed by the immune processes they have elicited. This phenomenon has been called "concomitant immunity" (Smithers and Terry, 1967). Resistance develops at the time of egg-laying and peaks 4-6 weeks later. In contrast, immunity that develops after infection with irradiated cercariae (vaccine immunity), develops rapidly after exposure to the parasites, peaks at week 5 and remains high. Since irradiated parasites cannot mature into adults, the immunity that the irradiated cercarial infection elicits is not directed to adult worms or eggs, but is induced by damaged larvae. The target of this vaccine immunity is also the migrating larvae of a challenge infection. Another difference between these two types of immunity is that concomitant

immunity is not species specific (Smithers and Doenhoff, 1982), whereas vaccine immunity is specific to the schistosome species which was used in the vaccine (Moloney and Webbe, 1987).

Immunity in animals has also been induced by vaccination with at least ten different partially protective antigens such as paramyosin (Lanar *et al.*, 1986), enzymes such as cercarial proteinase (Newport *et al.*, 1988), glutathione S-transferase (Smith *et al.*, 1986), and acetylcholinesterase (Goldlust *et al.*, 1986). Antigens reported to have homology with the snail intermediate host or molluscan antigens such as keyhole limpet haemocyanin (Grzych *et al.*, 1987) are reported to confer partial protection. Homogenates of schistosomes of various stages with varying adjuvants have been tested as vaccines. Homogenates mixed with alum were shown to induce an IgE response (Horowitz *et al.*, 1982). Intradermal injections of homogenates with BCG have resulted in antibody independent T-cell responses (James *et al.*, 1985). Immunizations with subcutaneous injections of homogenates with saponin resulted in both antibody and delayed-type hypersensitivity responses (Simpson and Cioli, 1987). These approaches have given levels of protection comparable to immunization with irradiated cercariae. Good immunity has also been achieved with repeated immunizations of mice with frozen and thawed schistosomula in the absence of an adjuvant (Simpson and Cioli, 1987).

1.6 The human immune response to Schistosome infection:

Schistosomiasis is characterized by the long term survival of adult worms in the portal and mesenteric veins. It is this continued presence of adult worms from a primary infection that makes it difficult to determine the degree to which an individual is becoming reinfected, and consequently, it is difficult to evaluate levels of immunity to new infections. It has been reported that the severity of infection (heavy egg output) declines with age, but it was also difficult to distinguish between immunity and lack of exposure as reasons for lack of reinfection after treatment or for the absence of superinfections in older people (Capron *et al.*, 1987a). Both explanations have had their supporters. The decline in prevalence and intensity of infection was attributed to the slow development of immunity by Bradley and McCullough (1973), while other investigators have suggested that as people increase in age, the pattern of their contact with cercariae in water changes (Warren, 1973).

Different studies have demonstrated that immunity plays an important role in determining the levels of infection in humans. A report by Walker (1970) showed that children who had played in a schistosome-infected pool during their very early years showed an absence of susceptibility to infection compared to other school children who became infected upon exposure. The interpretation given for this finding was that these children had become infected and reinfected until the age of ten years, by which time they had acquired a degree of immunity that prevented further infection. However, they were unable

to abort the existing infection, similar to the concomitant immunity model proposed by Smithers and Terry in 1967.

The most convincing evidence for existence of acquired immunity in humans has come from studies of reinfection by Butterworth *et al.* (1984, 1985). After twelve months of chemotherapy, some children that subsequently had high exposure to schistosome-infected water were heavily reinfected, consequently they were classified as susceptible. Another group of children with the same amount of exposure were not reinfected or had mild reinfections. This group was classified as resistant. Because it was found that the resistant children were older than the susceptible children, it was suggested that the acquisition of immunity is gradual and increases with age. Subsequent studies have demonstrated immunological differences between the two groups. In a related study, groups of children of 5-9 and 10-14 years showed a correlation between exposure and reinfection, however adults resisted reinfection in spite of high levels of exposure. The level of infection acquired by subjects aged 25 years or more are several hundred times less than in children aged 5-8 years. Although it was observed that water contact declines in older children and adults, this decline was not responsible for the marked reduction with age in intensities of reinfection after treatment (Butterworth *et al.*, 1984; Butterworth *et al.*, 1985). In addition, a study by Wilkins *et al.* (1987) demonstrated that adult women, after chemotherapy, have lower levels of reinfection as compared with children with the same level of exposure. These studies present convincing

evidence of acquired immunity in humans which is manifested as a resistance to reinfection with schistosomes that cannot b . explained solely by the degree of exposure to contaminated water. An age dependent-factor is important with the assumption that there is a development of a protective immunity with age. Immunity develops at the age of ten, after which established worm burdens are killed at a constant rate until the last worms are lost in the fourth decade of life. At this point susceptibility to reinfection is regained (Bradley and McCullough, 1973). However, the level of exposure to infected water also determines the degree of infection. since children with high levels of exposure were more heavily infected than children with less water contact (Butterworth *et al.*, 1984; Butterworth *et al.*, 1985).

Other factors besides age and exposure might also affect the infective rate of schistosomes. Recently, in a study performed in Brazil, Abel *et al.* (1991) reported that variations in intensities of schistosome infections that could not be accounted for by differences of sex, age, or exposure to infection are the result of a genetic component that predisposes the individual to high infection.

1.7 Effector mechanisms and immunity in human infections:

The immune response against schistosomiasis can be divided in two parts. The first is a resistance to reinfection which is directed against the original infecting worms but prevents or attenuates subsequent infections. The second response is directed against the parasite eggs and results in the

hepato-intestinal granuloma formation characteristic of this disease. The granuloma inflammation results in T Helper-cell-mediated delayed type hypersensitivity to egg antigens (Stadecker, 1992).

Since research in our laboratory is directed towards developing a vaccine to induce immunity against schistosome infections I will concentrate on the first response.

Although the cellular and humoral components of immunity to schistosomiasis are not totally understood, the role of some effector mechanisms have been reported with a dynamic balance between protective and regulatory or blocking mechanisms (Capron and Dessaint, 1985; Capron *et al.*, 1982; 1987a).

All stages in the life-cycle of the schistosomes are recognized by antibodies in human serum (Butterworth *et al.*, 1985; Hagan *et al.*, 1987). The effect of these antibodies on the different stages of the parasite are not well determined. It appears that not all the antibodies are protective, in fact some appear to be irrelevant and others obstruct binding of specific antibodies to the surface of the parasites (blocking antibodies). In the absence of complement or cells, human antibodies appear to have no direct effect on schistosome parasites *in vitro* (Hagan *et al.*, 1987). Cytotoxic antibodies lethal for schistosomula have been found *in vitro* assays. These antibodies, mainly of the IgG class, bind to the surface of schistosomula and activate complement leading to extensive damage to the surface and ultimately to parasite death

(Torpier *et al.*, 1979; Hagan *et al.*, 1987). Freshly prepared human serum has been shown to kill schistosomula by complement activation via the alternative pathway. The susceptibility of this parasite to killing by this mechanism decreases as the parasite matures.

It is generally agreed that the major role of antibodies is inducing cytotoxic destruction of schistosomulum via antibody-dependent cell-mediated cytotoxicity (ADCC), in the presence or absence of complement (Torpier *et al.*, 1979). This involves monocytes, macrophages, eosinophils and platelets as the cellular components and IgE or anaphylactic subclasses of IgG as the humoral components (Ellner and Mahmoud, 1979; Capron, 1992; Capron *et al* 1982; Capron and Dessaint, 1985). Young schistosomula are fully susceptible to ADCC, however, lung and post-lung developmental stages of schistosomes appear to be completely resistant to ADCC (Tavares *et al.*, 1978; 1980). The nature of the ADCC is still under investigation. However, each cell type was reported to release different mediators that contribute to the cytotoxicity. Monocytes and macrophages released interleukin-1 and lysosomal enzymes (Mazingue *et al.*, 1987). Eosinophils are reported to release purified major basic protein (MBP) and eosinophil cationic protein (ECP) as well as specific eosinophil peroxidase (EPO) (McLaren *et al.*, 1981; Capron *et al.*, 1987b). These factors might affect the parasite directly or through enzymatic activities (Capron *et al.*, 1987b). Gamma-interferon, and tumour necrosis factors from $CD4^+$ T lymphocytes were identified as increasing the cytotoxic activity. In

contrast, $CD8^+$ cells release a suppressive lymphokine against platelet activation (Pancre *et al.*, 1986). Therefore, the cytotoxic activity can be modulated by antagonistic activities between $CD4^+$ and $CD8^+$ cells.

Killing of schistosomula by neutrophils and eosinophils can also occur without the participation of antibody, provided that a source of serum is present, since neutrophils have C_3 receptors, and can thus bind to schistosomula coated with C_3 once activation has taken place. The effect of complement appeared to be mediated through the production of complement-peptide fractions which activate the neutrophils. Neutrophils activated in this way were shown to adhere more non-specifically and to increase the release of effector molecules.

The ADCC observed *in vitro* is correlated with studies of human infections. Human infections are associated with a strong IgE antibody response (Dessaint *et al.*, 1975). In the presence of IgE-rich serum from infected individuals, human monocytes, platelets, and eosinophils can damage schistosomula *in vitro* (Capron *et al.*, 1987a; Hagan *et al.*, 1985a; Joseph *et al.*, 1983). In reinfection studies it was determined that high eosinophil counts were important in preventing reinfection (Hagan *et al.*, 1985b; Wilkins *et al.*, 1987). Studies performed in Gambia by Hagan *et al.* (1991) demonstrated that after treatment for schistosomiasis there was a correlation between the production of anti-schistosome IgE and the acquisition of immunity to reinfection. In contrast, IgG_4 antibodies were correlated with susceptibility to reinfection, possibly by

blocking IgE effector pathways and delaying the development of protective immunity.

Iskander *et al.* (1981) in a study of Egyptian patients infected with *S. mansoni* and *S. haematobium*, found that a great proportion of the antibodies produced were of the IgG_4 type and these antibodies were inefficient in activating complement and binding to monocytes and macrophages. These antibodies might interfere with complement activation by IgG_1. They also suggested that by competing with IgE antibouies for allergenic worm antigens, IgG_4 could also block mast cell degranulation. Similarly, Rihet *et al.* (1991) reported, in a study of Brazilian adolescents, that IgE levels were six-to eight-fold higher in the sera of resistant adolescents. Further studies reported that low levels of IgE antibodies in combination with high IgG_4 levels resulted in over a 100-fold increase in susceptibility to reinfection (Capron, 1992; Dunne, *et al.*, 1992).

A correlation was also reported between reinfection rates and levels of both IgM anti-schistosomulum antibodies and IgM and IgG anti-egg antibodies. When serum was depleted of IgM there was an increase in IgG-dependant eosinophil killing of schistosomula. IgM antibodies isolated from the sera of various individuals blocked the eosinophil-dependent killing of schistosomula mediated by IgG antibodies of the same sera. In addition, IgM antibodies are present in higher levels in younger susceptible children and lower levels in the older resistant children (Khalife, *et al.* 1986).

IgG_2 antibodies against egg antigens have been also reported as mediating blocking activities. Thus, resistance to *S. mansoni* reinfection may be, in part, due to specific and protective anti-parasite antibodies, and also due to the absence of blocking antibodies. Butterworth *et al.* (1987) suggested that early in the infection egg antigens promote IgM antibodies and maybe some IgG antibodies that are not protective. These antibodies cross-react with glycoproteins on the surface of the schistosomulum. Potentially protective IgG and IgE antibodies may be also mounted against the same antigens, but as blocking antibodies are more abundant early in the infection children remain susceptible. Later on the antibody production switches to more protective antibodies and children become immune to reinfection. An alternative hypothesis was suggested by Hagan *et al.* (1991). They suggested that high levels of anti-parasite IgG protective antibodies develop only after many years of infection, and this factor alone may explain the slow development of immunity and it may not be necessary to speculate on the role of blocking antibodies.

In summary, immunity to reinfection involves the interaction of several antibody isotypes, complement components, T cells, and various cells components.

1.8 Schistosome evasion of the immune system:

Adult schistosomes are able to survive for years within the mesenteric

veins of the definitive host, while new invading juvenile parasites (schistosomula) are unable to become established (Smithers and Terry, 1969). This phenomenon by which the host immune system is ineffective in dealing with resident parasites while rejecting subsequent infections is called "concomitant immunity". To explain the concomitant immunity phenomenon, different mechanisms of immune evasion have been attributed to schistosomes which may operate simultaneously or in succession. The nature of these mechanisms is under current investigation by different laboratories including our own.

Since most of the host-parasite interaction occurs at the surface of the schistosome it is important to understand the nature of the schistosome tegument.

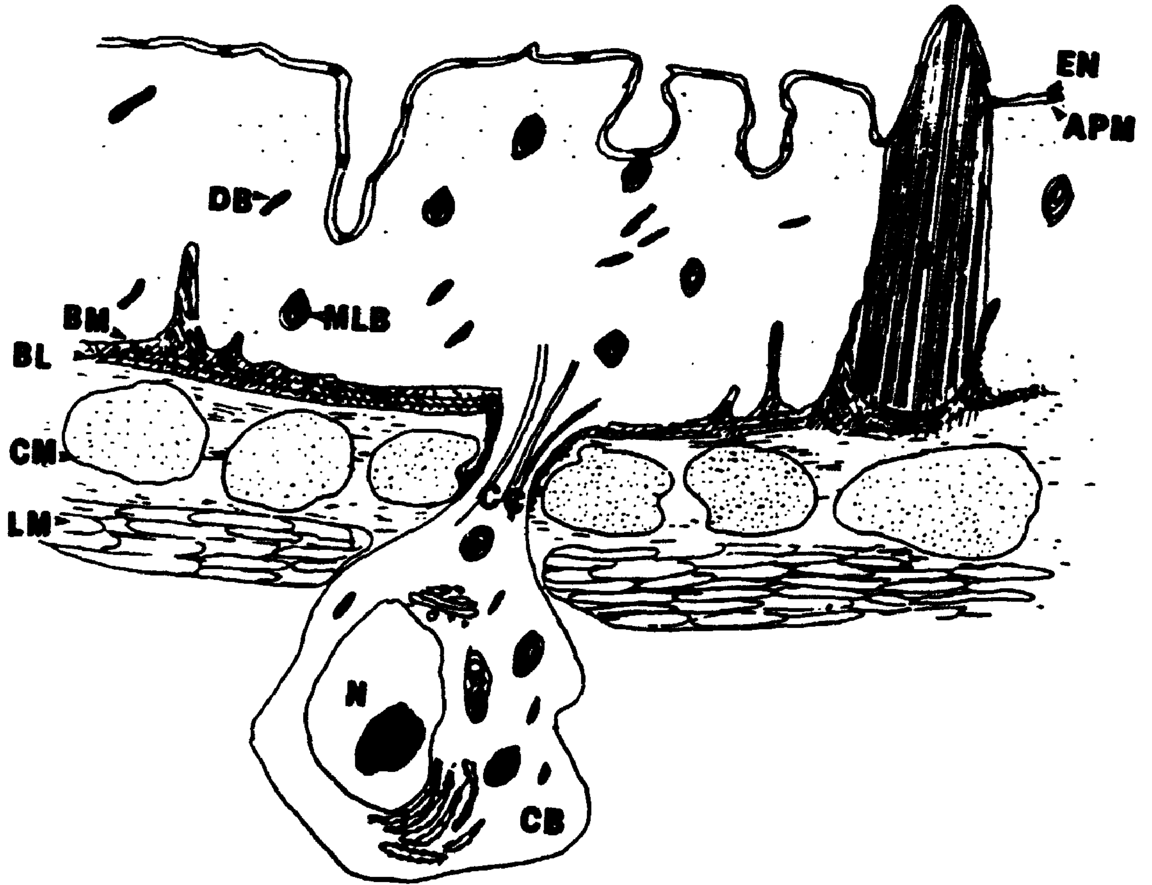
1.8.1 The schistosome tegument:

The tegument of cercariae, schistosomula, and adult schistosomes (represented in Figure 1.2) is a syncytial epithelium with a cytoplasmic layer containing mitochondria, discoid and multilamellar bodies, spines and sensory cells. This layer is defined by an interior fibrous basal lamina and on the exterior by an outer surface plasma membrane. They are connected by microtubule-lined processes to the cell bodies which lie below a muscle layer underneath the basal lamina. These cell bodies contain the nucleus, endoplasmic reticulum, Golgi complex and mitochondria. The tegument of the adult worm is derived directly from the tegument of the penetrating cercariae,

which has a single trilaminate outer surface membrane covered by a glycocalyx. After cercarial penetration into the host, the parasite undergoes physiological and morphological changes and becomes a schistosomulum. Within 48 hours following transformation the schistosomulum has gained a double outer surface membrane (Hockley and McLaren, 1982) with an heptalaminar appearance (Hockley, 1973). This double membrane is composed of an envelope (EN) and an apical plasma membrane (APM). The envelope is unique to blood-dwelling flukes (McLaren and Hockley, 1977), and is correlated with parasite resistance to immune attack. The discoid bodies and multilamellar bodies, which are thought to be precursors of the apical plasma membrane and envelope, respectively, are synthesized in the cell bodies and may be transported to the surface by moving along the cytoskeleton (Zhou and Podesta 1989).

Figure 1.2: Representation of the surface epithelial layer of *Schistosoma mansoni*.

EN, envelope; APM, apical plasma membrane; S, spine; DB, discoid bodies; MLB, multilamellar bodies, BM, basal membrane; BL, basal lamina; CM, circularly arranged muscle fibers; LM, longitudinal muscle fibers; N, nucleous; CB, cell bodies; CC, cytoplasmic connections.


EN
APM
DB
BM
MLB
BL
CM
LM
N
CB

1.8.2 Proposed mechanisms of Schistosome immune evasion:

The accelerated membrane turnover hypothesis states that the ability of the worms to survive in an immunologically hostile environment is related to their capacity to repair damaged membranes. Thus protection from host immune attack may be accomplished by the rapid synthesis, modulation and turnover of the outer membrane complex (EN and APM) of the epithelial syncytium, allowing damaged membrane to be shed and replaced before the integrity of the cytoplasm becomes affected (Podesta, 1982). The unique structure of the schistosome tegument can be explained in terms of this hypothesis, since the lack of cellular compartments of the tegument may be an adaptation which allows synchronous and metabolic cooperation in the syncytium, allowing rapid and efficient repair in the surface (Podesta, 1982). Thus, when antibody binds to the outer membrane, only the bound membrane portion is shed and newly synthesized membrane will fill the gap. Podesta, (1982) has postulated that it is energetically more feasible to turn over the surface membrane of a syncytium rapidly than it is to replace entire cells of a cellular epithelial layer.

The accelerated turnover hypothesis is supported first, by the findings that adult worms incubated *in vitro* renew their APM and EN with half lives of 6 and 3 hours, respectively (Dean and Podesta, 1984). Secondly, schistosomula progressively develop resistance to the host immune system over a period of several days (Dessein, *et al.*, 1981). Schistosomula are susceptible, *in vitro*, to

humoral as well as to complement-mediated cellular cytotoxicity very early after infection (Butterworth, 1977; Butterworth *et al.*, 1982; Santoro, 1982). In contrast, parasites recovered from the host after 4 to 5 days of infection are resistant, *in vitro*, to the same immune mechanisms (Butterworth *et al.*, 1982; Smithers *et al.*, 1977; Santoro *et al.*, 1979). This resistance is correlated with the synthesis on the schistosomula surface, three days after penetrating the host's skin, of a new membrane (Smithers *et al.*, 1977; McLaren and Hockley, 1977; McLaren, 1980; Hockley and McLaren, 1973) more properly called an envelope, EN (Locke, 1982), giving the surface an heptalaminar appearance (Hockley, 1973; Smithers *et al.*, 1977; McLaren and Hockley, 1977; McLaren, 1980; Hockley and McLaren, 1973). Susceptible schistosomula possess only the apical plasma membrane (APM) whereas resistant adult worms have the EN overlying the APM. Supportive evidence for the role of the envelope in immune evasion is found in studies performed in rats. In this non-permissive host, schistosomes fail to synthesize the EN completely and the infection is aborted after 4 weeks (Senft *et al.*, 1978).

This evasion mechanism necessarily involves responses by the parasite to signals originating within the host, particularly signals of an immune nature (Podesta *et al.*, 1987). Tavares *et al.* (1978) suggested that serum increases the turnover rate of most of the proteins or glycoproteins of the schistosome surface and McLaren and Nino-Incani (1982) reported that schistosomula resistance is connected with or prompted by exposure to host molecules.

Young and Podesta (1984) investigated possible modulators of the APM and EN formation and found that the addition of 5-hydroxytryptamine to the incubation medium increased the synthesis of the APM. In contrast, the complement factor, C_3, increased incorporation of choline into phosphatidylcholine in the envelope of *S. mansoni*, and increased the synthesis and transport to the parasite surface of multilamellar bodies which are the precursor organelles for the envelope (Podesta *et al.*, 1987; Young and Podesta, 1986; Zhou and Podesta, 1989; McDiarmid *et al.*, 1982). In this case the binding of complement may provide the stimulus for surface renewal (Podesta, 1982; Young and Podesta 1986).

Another mechanism used to explain immune evasion ignores the presence of a syncytial epithelium and suggests that parasite target epitopes are masked by molecules of host origin. During the 1960s a prevalent view was that host and parasites share antigens and that the cross-reactivity of these antigens was in part responsible for the survival of the parasites in immune-competent hosts (Sprent, 1962; Damian, 1979). This phenomenon was called " molecular mimicry" (Damian, 1979). This hypothesis has been further modified by taking into account the origin of the antigens. According to the original theory of Damian (1964) the parasite possesses in its genome, genes that express host-like antigens that have arisen by chance with natural selection acting as a force to stimulate the production and maintenance of successful mutations. Thus the sharing of antigens was a preadaptation to parasitism.

Capron *et al.* (1968) presented the "antigen induction" hypothesis. This hypothesis also required the presence in the parasite genome of genes controlling the synthesis of host-like antigens. However, in this case, the phenotypic expression of the host-like antigen genes is determined by the particular host in which the parasite finds itself. Finally the "masking hypothesis" of Smithers, *et al.* (1968) postulated that the "shared" antigens are actually host antigens acquired by direct contact between the host and the parasite, and these antigens serve to mask potentially vulnerable parasite antigens.

Evidence in support of "antigen mimicry" has not been overwhelming. To qualify as an evasive mechanism used by the schistosome, the shared antigens must be synthesized by the parasite and must be important to the parasite's survival. Most of the reported antigens have failed at the first task. The induced synthesis hypothesis has been criticized on the grounds that the parasite would need to possess an extremely large number of antigenic genes to code for cross-reacting antigens for all the different species that the parasite might infect. The most favoured hypothesis has been the "masking" hypothesis and is now more accepted as "disguise" (McLaren, 1985). This hypothesis was supported by Smithers and Terry (1967). These investigators reported that adult schistosomes, which were grown in one host species and then were subsequently surgically introduced into different host species, were killed when the second host had been previously immunized against the first host's erythrocytes or other tissues. Thus, monkeys immunized against mouse

antigens rejected worms of mouse origin but not worms derived from monkeys. Electron microscopic studies confirmed that mouse antigens were present on the surface of mouse-derived schistosomes prior to transfer, and also revealed that the immune attack mounted against the parasite by the pre-immunized monkeys was directed against the surface (McLaren, 1985). Further research has confirmed the presence of host antigens on the parasite surface. Among the host-like antigens that have been detected on the schistosome surface are: heterologous antibodies bound to the surface by the Fc portions (Kemp *et al.*, 1977), the Forssman antigen (Dean and Sell, 1972), α_2-macroglobulin (Damian *et al.*, 1973), blood group determinants such as the ABH and Lewis blood group antigens (Clegg *et al.*, 1971; Dean, 1974; Goldring *et al.*, 1976), major histocompatibility antigens (Gitter *et al.*, 1982; Simpson *et al.*, 1983), non-MAC host alloantigens (Sher *et al.*, 1978) as well as Decay-Accelerating Factor (DAF; Fatima *et al.*, 1991).

Although several host-like antigens have been identified on the parasite surface, proof of the functional significance of these antigens remains undetermined. Evidence for a protective function of acquired host antigens is circumstantial (Smithers and Terry, 1967). Many types of cells in culture pick up host antigens and little significance is attributed to this. All these antigens are thought to be acquired via hydrophobic interactions of their ceramide groups with parasite lipid-rich structures such as cell membranes (Clegg *et al*, 1971). McDiarmid and Podesta (1983) suggested that the association of host

antigens with the parasite surface is non-specific and is due to the "stickiness" of the parasite surface. In addition, resistant schistosomes that have been shown to bear host antigens also bind anti-schistosome antibodies (MacLaren, 1985). Thus, if protection is indeed rendered by disguising host antigens, it may be less than complete and hence ineffective or only partially effective. Moreover, resistance to immune attack in juvenile worms has been reported to develop in the absence of host molecules (Dessein *et al.*, 1981). Moser *et al.* (1980) have reported that the loss of susceptibility by parasites cannot be attributed solely to antigen disguise. These investigators have shown that worms that were surface-labelled with trinitrophenol (TNP) groups, to bypass the host antigen disguise and render the parasite foreign, survived the anti-TNP antibody attack.

Clearly then, the disguise hypothesis does not provide all the answers to the mechanisms of immune evasion by blood flukes.

An alternative hypothesis to explain the role of host antigens in the parasite surface was postulated by Fatima *et al.* (1991). Instead of providing a non-specific disguise from the host immune system, the molecules adsorbed onto the schistosome surface could provide a specific functional protection against immune attack. In fact, it was recently reported that schistosomes, *in vitro,* acquire a 70 kD Decay-Accelerating Factor (DAF) from human erythrocytes. DAF inhibits complement activation by destabilizing the C_3 convertases (Walter *et al.*, 1992). DAF, *in vitro*, renders schistosomes resistant

to complement damage (Fatima *et al.*, 1991).

After different subclasses of immunoglobulins were detected on adult schistosomes (Kemp *et al.*, 1976; 1977; Sogandares-Bernal, 1976), immunological blockade was also postulated as an immune escape mechanism, where masking of surface antigens by antibodies directed against the target antigens renders them inaccessible to aggressor lymphocytes (Sogandares-Bernal, 1976; McLaren, 1980). Khalife (1986) and Butterworth (1987) and their associates postulated that at the beginning of a schistosome infection, egg antigens elicit production of IgM antibodies or in some cases a subtype of IgG antibody that is not protective. Instead, they cross-react with glycoproteins on the schistosome surface. Because these antibodies are predominant early in the infection, children became more susceptible to schistosome reinfections. But as the antibody production switches to a predominantly protective response older children become immune to infection.

Although less accepted, alternative mechanisms of schistosome resistance have been postulated. Among them are the notion that schistosomes failed to be killed because worm antigens suppress the host's immune system (Kayes *et al.*, 1979). Non-specific immune suppression was also observed (Kelley *et al.*, 1976). It was reported that suppressor cells, thought to be Ia and T cells, are regular components of the peripheral blood of patients suffering from chronic schistosomiasis (Ottensen 1979, Chensue and Boros, 1979) and that immune complexes contribute to immune suppression

during schistosomiasis (Attallah *et al.*, 1979). Gazzinelli *et al.*, (1985) have described suppressive factors in the serum of humans infected with *S. mansoni*. Also, the decrease in the ratio between helper and suppressor T lymphocytes ($T4^+$:$T8^+$) was suggested to be at least partly responsible for the immune suppression observed in schistosomiasis (Colley *et al.*, 1983; Gastl *et al.*, 1984).

Reduction of surface antigen expression (Butterworth *et al.*, 1982) and the non-antigenicity of the outer membrane of the surface of *Schistosoma mansoni* (McLaren, 1985) have also been suggested as alternative mechanisms for parasite survival and the phenomenon of concomitant immunity.

One of the non-specific mechanisms to explain concomitant immunity was postulated by McHugh *et al.*, (1987). These investigators suggest that resistance to reinfection in infected mice was due to damage to the integrity of the hepatic portal vascular system as a result of schistosomiasis. Thus, new infecting schistosomula would be prevented from settling in the liver and instead, migrate to less favourable sites for their development.

Undoubtedly then, it is possible that these different mechanisms may act simultaneously in schistosome-host interactions to enhance the parasite's successful immune resistance.

1.9 Rationale and Thesis Objectives:

Since *Schistosoma mansoni* has developed immune evasion

mechanisms based on surface membrane turnover, the study of surface components of *Schistosoma mansoni* will provide useful information about components that might be critical in protecting the parasite against the host immune mechanisms and promote parasite survival. Membrane components are important not only because of their antigenic characteristics, but also because of their location of intimate contact between the host and the parasite, and hence they are the sites where the equilibrium between host and parasite is maintained. Our approach to identifying and characterizing functional surface proteins implicated in immune evasion is an important step for the development of an anti-schistosome vaccine and, or for the design of targets for chemotherapy.

The rapid membrane turnover of schistosomes as an evasion mechanism necessarily involves responses by the parasite to signals originating within the host, particularly of an immune nature (Podesta *et al.*, 1987). Previous studies have suggested that the sloughing of surface membrane components is stimulated by serum factors (Kemp *et al.*, 1977; Samuelson *et al.*, 1980; Tarleton and Kemp; 1981). For the envelope, in particular, the third component of the complement system (C_3) has been shown to stimulate EN synthesis (Young and Podesta, 1986) and the synthesis of the multilamellar bodies which are the precursor organelles for the envelope (Zhou and Podesta, 1989). Envelope biogenesis in response to C_3 would necessarily require surface receptors. The presence of C_3 receptors on the surface of

schistosomes has been suggested previously (McGuinness and Kemp, 1981).

The present study was designed to carry out the following objectives:

1) To investigate the existence of a C_3 receptor on the schistosome surface and, if present, determine whether it is localized in the envelope or apical plasma membrane.

2) To characterize biochemically and immunochemically the C_3 receptor.

3) To produce antibodies against the C_3 receptor and use them to elucidate the function of the C_3 receptor

4) To use the antibodies against the C_3 receptor to screen a cDNA *Schistosoma mansoni* library.

5) To determine the primary sequence of the C_3 receptor, provided that positive clones are obtained in objective 4.

CHAPTER 2

BINDING OF C_3 TO THE SURFACE OF ADULT SCHISTOSOMES

2.1 Introduction

The disease produced by the parasite *Schistosoma mansoni* is characterized by the long term survival of the adult schistosomes originating from a primary infection, concomitant with the immune rejection of juvenile schistosomes of secondary and subsequent infections (Smithers and Terry, 1969). The ability of adult schistosomes to survive in an otherwise immuno-competent host has led investigators to suggest that schistosomes have evolved defense mechanisms that allow them to evade the effects of the host's immune system (discussed in Chapter 1). In our laboratory, we believe that this mechanism involves a signal transduction process leading to rapid turnover of the surface membranes in response to immune attack. Previous studies involving adult *S. mansoni* have shown that this parasite recognizes various host components as signals for membrane renewal (Young and Podesta, 1986). For the envelope (EN) in particular, the third component of the complement system (C_3) has been shown to stimulate envelope synthesis (Young an Podesta, 1986), as well as the synthesis of multilamellar bodies (MLB) which are the precursor organelles of the envelope (Zhou and Podesta, 1989). Envelope synthesis is mediated by a Ca^{2+} dependent signal

transduction mechanism (Young and Podesta, 1986; Podesta *et al.*, 1987). If the synthesis of the EN is a physiological response that involves C_3 as a signal, then the schistosome must possess receptors for C_3 on its surface. The existence of C_3 receptors on the schistosome surface has been previously suggested (McGuinness and Kemp, 1981). However, studies that attempted to demonstrate the presence of these receptors have produced conflicting results (Kabil, 1976; Ouassi *et al.*, 1980; Rasmussen and Kemp, 1987).

The experiments presented in this chapter were designed to confirm the probable existence of C_3 receptors on the parasite surface.

2.2. Materials and Methods

2.2.1. Maintenance of *Schistosoma mansoni* life cycle

The life cycle of a Puerto Rican strain of *S. mansoni* was routinely maintained in male Syrian hamsters (Charles River, Que) and in *Biomphalaria glabrata* snails (University of Lowell, Lowell, MA). Infected snails were shipped monthly from Lowell University and were maintained in the dark in shallow horticultural bedding trays containing dechlorinated tap water. The room temperature was approximately 25°C. Snails were fed Romaine lettuce *ad libitum*. Twice a week, shedding of cercariae from the snails was induced by placing the snails in a beaker containing a small volume of dechlorinated tap water. The beaker was left under fluorescent light for two hours, after which the snails were returned to the bedding trays. Cercariae were concentrated by centrifugation at 1000 xg for 30 seconds in 15 ml conical centrifuge tubes using an IEC centrifuge. The cercariae in the pellet were removed with a pasteur pipette and placed in a 15 ml beaker. Aliquots of 10 μl were stained with iodine on a microscope slide and cercariae were counted using a dissecting microscope (E. Leitz Wetzlar, Germany).

2.2.2. Preparation of mechanically transformed schistosomula

Freshly harvested cercariae were transferred into conical centrifuge tubes containing Minimum Essential Medium with Earle's salts and glutamine (MEM, Flow Laboratories Inc, McLean, VA). To separate the tails from the cercariae bodies, the tubes were centrifuged five times, for 30 seconds each at 1000 xg,

and the pellet resuspended in MEM. The last pellet resulting from the fifth centrifugation step was repeatedly passed through a syringe with a 23 G needle until the majority of cercariae had lost their tails as determined by light microscopy.

2.2.3. Recovery of lung-stage schistosomula

Male Syrian hamsters were kept under a 12 hour light-dark cycle and fed Purina rat chow and water *ad libitum*. To obtain lung stage schistosomula hamsters were injected subcutaneously in the abdominal region with 5000 - 6000 cercariae in less than 1 ml of dechlorinated water. Five days post-infection, the hamsters were killed by cervical dislocation. Their lungs were removed, cut into 2 mm^3 pieces, and cultured in MEM medium at 37°C for three hours. The lung pieces were then removed from the culture medium using a fine mesh. The medium which contained schistosomula was then transferred into 15 ml conical centrifuge tubes and centrifuged at 1000 xg for 30 seconds. The pellet was then resuspended in 1 ml of MEM and lung schistosomula were detected using an inverted microscope.

2.2.4. Recovery of liver stage schistosomula

Male Syrian hamsters were injected subcutaneously with 5000 - 6000 cercariae in dechlorinated water, and killed 10 days post-infection by cervical dislocation. A ventral incision was made extending from the abdominal region towards the thoracic region to expose the hepatic portal vein and the heart. Liver stage schistosomula were recovered into a fine mesh by cutting the

hepatic portal vein and perfusing the left ventricle of the heart with Krebs's Ringer Phosphate (KRP, pH 7.4; 0.12 M NaCl, 0.016 M Na_2HPO_4, 1 mM KH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, 1mM $CaCl_2$). The liver was then removed, cut into 2 mm^2 pieces and incubated at 37°C for three hours. Liver schistosomula were processed as the lung schistosomula, described in section 2.2.3.

2.2.5. Recovery of adult worms

Male Syrian hamsters were injected subcutaneously with 1500 - 1600 cercariae in dechlorinated water. Forty days post-infection the hamsters were killed by cervical dislocation, after which, the abdominal and part of the thoracic cavities were exposed by making a ventral incision. After cutting the hepatic portal vein, perfusion was carried out by applying KRP to the left ventricle. Worms were flushed and collected onto a fine mesh and kept on cold KRP at 4°C. Worms trapped in the mesenteric veins were removed manually with the use of a spatula.

2.2.6. Isolation of Envelope (EN) and Apical plasma membrane (APM) fractions

Freshly collected adult worms were washed three times in KRP and incubated in Dulbecco's Phosphate Buffered Saline (DPBS pH 7.4; 0.137 M NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 1.47 mM KH_2PO_4, 2.68 mM KCl) for 30 min at room temperature to facilitate the release of adsorbed host proteins from the parasite surface. EN and APM fractions were obtained

following the method of McDiarmid *et al.* (1982). Briefly, the EN was obtained by incubating worms in Tris-digitonin solution (0.1% digitonin and 0.01 M sucrose in 0.1 M Tris buffer, pH 7.4) for 5 min at 4°C with shaking, followed by a rinse with an equal volume of Tris-sucrose (0.1 M Tris, pH 7.4 and 0.2 M sucrose). These two fluids were pooled. To subsequently remove the APM fraction the EN stripped worms were then incubated for 20 minutes in Tris-digitonin at 4°C with shaking, followed by a rinse in Tris-sucrose. The EN and APM fractions were centrifuged (Sorvall RC-5B) at 35,000 xg for 1 hour. The resulting pellets were suspended in DPBS and washed by centrifugation at 15,000 xg for 20 min at 4°C in an Eppendorf microcentrifuge. The protein content of the fractions was estimated by the method of Bradford (1976) using bovine serum albumin (BSA, Fraction V, Boehringer Mannheim, Laval, Que) as a standard.

2.2.7. Alkaline phosphatase assay

Each time that the membrane fractions were removed from the surface of adult schistosomes, 5 and 10 μl aliquots of APM and EN fractions were used to assay for alkaline phosphatase (AP) activity. Since AP is a marker for the APM, this assay was used to assess the level of cross contamination of the membrane fractions. AP activity was determined colorimetrically using a kit supplied by Sigma (Chemical Company, St. Louis, MO) utilizing p-nitrophenyl phosphate as substrate according to the manufacturer's instructions. Briefly, aliquots were brought up to 0.1 ml in Tris- sucrose and mixed with 0.5 ml of

glycine buffer [0.1 M glycine (pH 10.5), and 1 mM $MgCl_2$] and 0.5 ml of p-nitrophenyl phosphate (4 mg/ml). The mixture was incubated for 30 min at 37°C. The reaction was then stopped with 5 ml of 0.04 N NaOH. The liberated p-nitrophenol was read at an absorbance of 405 nm using an ELISA plate reader. To estimate the amount of alkaline phosphatase in the samples, the absorbance readings were plotted on a graph with a standard curve prepared using known amounts of p-nitrophenol.

2.2.8. Rosette assay

Approximately 50 parasites of different stages of schistosome development were incubated for either 30 min at 37°C or 1 hr at 4°C in 1 ml DPBS or Veronal Buffer (pH 7.4), with coupled erythrocytes (Dimension Laboratories, Mississauga, Ont). Erythrocytes were coupled to IgM antibodies against erythrocytes (EA19S), to the C_1 component of the complement system bound to IgM antibodies (EAC_1), to the C_4 component of the complement system attached to IgM antibodies (EAC_{14}), and to the C_3 component of the complement system (EAC_{1423}) attached to IgM antibodies. Positive control experiments contained peritoneal cells instead of parasites. Peritoneal cells were obtained from C57 Black Bl/6 mice (Charles River, Que) 4 days after they were stimulated with thioglycollate by following the method previously described (Gallily and Feldman, 1967). All incubations were done in sixteen well plates (Nunc, Gibco Laboratories, Burlington, Ont). Rosette formation was detected by light microscopy with an inverted microscope.

2.2.9. Indirect Immunofluorescence of live adult worms

Adult worms were incubated with 140 μg/ml of human C_3 in DPBS for 60 min at 4°C. After three washes in DPBS, they were incubated for 30 min in a 1% (w/v) solution of BSA/DPBS, washed once in BSA/DPBS, and incubated further in a 1:10 dilution of $F(ab')_2$ goat anti-human C_3 (Cappel Laboratories, West Chester, PA) for 45 min at 4°C. Parasites were then washed three times in BSA/DPBS and incubated for 45 min at 4°C in a 1:25 dilution of fluorescein isothyocyanate (FITC)-$F(ab')_2$ rabbit anti-goat IgG (Cappel). This was followed by three washes in DPBS. Parasites used in the negative control groups were incubated in DPBS containing 5% (v/v) normal sheep serum instead of the primary antibodies. Other negative controls included incubations in the absence of C_3 or $F(ab')_2$ goat anti-human C_3. The parasites were then examined for fluorescence via light microscopy.

2.2.10. Indirect Immunofluorescence in fixed adult worms

Adult worms were incubated in a 1:10 dilution of heat-inactivated normal rabbit serum (56°C for 30 min) for 50 min at room temperature in an end over end shaker. After 3 washes in DBPS, the worms were incubated with human C_3 (140 μg/ml) in DPBS for 45 min at room temperature. Control groups were incubated in DPBS alone. The worms were then washed once in DPBS and fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature. The worms were washed three times in DPBS for 30 min total, and then incubated with $F(ab')_2$ goat anti-human C_3 at a 1:100 dilution in DPBS containing 1% (v/v)

rabbit serum for 1 hour at room temperature. After three washes in DPBS, parasites were incubated with a 1:25 dilution of FITC-conjugated $F(ab')_2$ rabbit anti-goat IgG for 1 hour at room temperature. Worms were then washed in DPBS, mounted on slides and examined using an MRC-600 laser scanning confocal microscope (Bio-Rad, Toronto, Ont).

2.2.11. Determination of C_3 binding to fixed adult worms by peroxidase immunocytochemistry

Freshly collected and washed adult worms were rinsed in 0.1 M phosphate buffer (PBS, pH 7.4) and incubated in 1 ml of 10 % (v/v) heat-inactivated rabbit serum in PBS for 50 min at room temperature in an end over end shaker. The worms were washed in PBS and separated into different groups which were then incubated with human C_3 at a concentration of 140 μg/ml in PBS for 5, 15, 30, and 45 minutes at room temperature. In later experiments the optimum incubation time of 30 min was used. Negative control groups were incubated in PBS alone for 30 min. All groups were then incubated in 4% (w/v) paraformaldehyde for 30 min at room temperature, and subsequently washed in several changes of PBS for a total of 30 min. Parasites were then incubated in 1 ml of a 1:100 dilution of $F(ab')_2$ goat anti-human C_3 containing 10 μl of rabbit serum (to block non-specific binding sites), for 1 hour at room temperature. After three, 10 min washes in PBS, the worms were incubated in 1 ml of rabbit anti-sheep antibodies (1:100 dilution in PBS) containing 10 μl of rabbit serum. After three washes in PBS, worms were

incubated in 1 ml of a 1:100 dilution of goat peroxidase anti-peroxidase (PAP) complex in PBS containing 10 μl of rabbit serum for 1 hour at room temperature. After three washes in PBS, the worms were mounted on microscope slides and observed with a light microscope.

2.2.12. Enzyme-linked immunoabsorbent assay (ELISA) to detect C_3 binding to the EN of adult worms

Ninety-six microwell plates (Nunc, Gibco Laboratories, Burlington, Ont.) were coated overnight at 4°C with EN or APM fractions (0.1 ml/well) at a concentration of 10 μg protein per ml of DPBS. Any remaining binding sites were blocked with 200 μl BSA/DPBS for one hour at room temperature. Plates were then washed with 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20, Sigma) in DPBS (DPBS/Tween), followed by a one hour incubation with human C_3 (10 μg/ml-100 μl/well) at room temperature. The plates were washed three times with DPBS/Tween. Goat $F(ab')_2$ anti-human C_3 was then added at a 1:200 dilution (100 μl/well) and further incubated for 1 hour at room temperature. The plates were washed three times with DPBS/Tween and subsequently incubated with peroxidase-labelled rabbit $F(ab')_2$ anti-goat IgG (Cappel) at a 1:1000 dilution (100 μl/well) for 1 hour at room temperature. This incubation was followed by five washes in DPBS/Tween. The enzyme substrate 2,2'-azino-di-3-ethyl-benzthiazoline sulphonate (ABTS, Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added (100 μl/well) and incubated in the dark for 45 min. Negative controls included incubations in the absence of

membrane proteins, C_3, or first antibody. Readings were performed at an absorbance of 405 nm using an ELISA plate reader (Bio-Teck Instruments, Inc.).

2.2.13. Effect of neuraminidase on C_3 binding

EN and APM fractions were treated with 3.3 U of neuraminidase/ml (*Clostridium perfringes* type V, Sigma) as described by McDiarmid and Podesta (1984). The treated EN and APM fractions were used to coat 96-well plates and ELISA assays were carried out as described in section 2.2.12.

2.3. Results

Since preliminary immunofluorescence and ELISA assays gave positive results even in the control groups, and because Kemp *et al.* (1977) reported that schistosomes bind to the Fc portion of immunoglobulins, it was necessary to use only $F(ab')_2$ antibodies for the subsequent C_3 binding experiments. This modification proved to be effective in preventing non-specific binding.

Indirect immunofluorescence was used to determine whether C_3 binds to live adult schistosomes. Results from these experiments (Table 2.1) demonstrated that fluorescence could be seen on the surface of the adult worms. However, the pattern of fluorescence was not distributed evenly on the surface of the worm, instead it appeared to be localized in small patches. In addition, these areas of fluorescence disappeared from the surface of live parasites within 30 min, apparently through shedding into the incubation medium. Patchy fluorescence was observed only on the surface of male worms. Female worms showed no surface fluorescence. Nonspecific fluorescence was observed around the region of the mouth, gastric cavity, and eggs of the female worms in both the experimental and control groups. Since the level of fluorescence disappeared rapidly from the surface of worms, the parasites were fixed in subsequent experiments. However, previous work (McGuinness and Kemp, 1981; Tarleton and Kemp, 1981) had shown that binding of complement to the parasite surface can be abolished when the worms are fixed in formalin, which suggested that the schistosome C_3 receptor

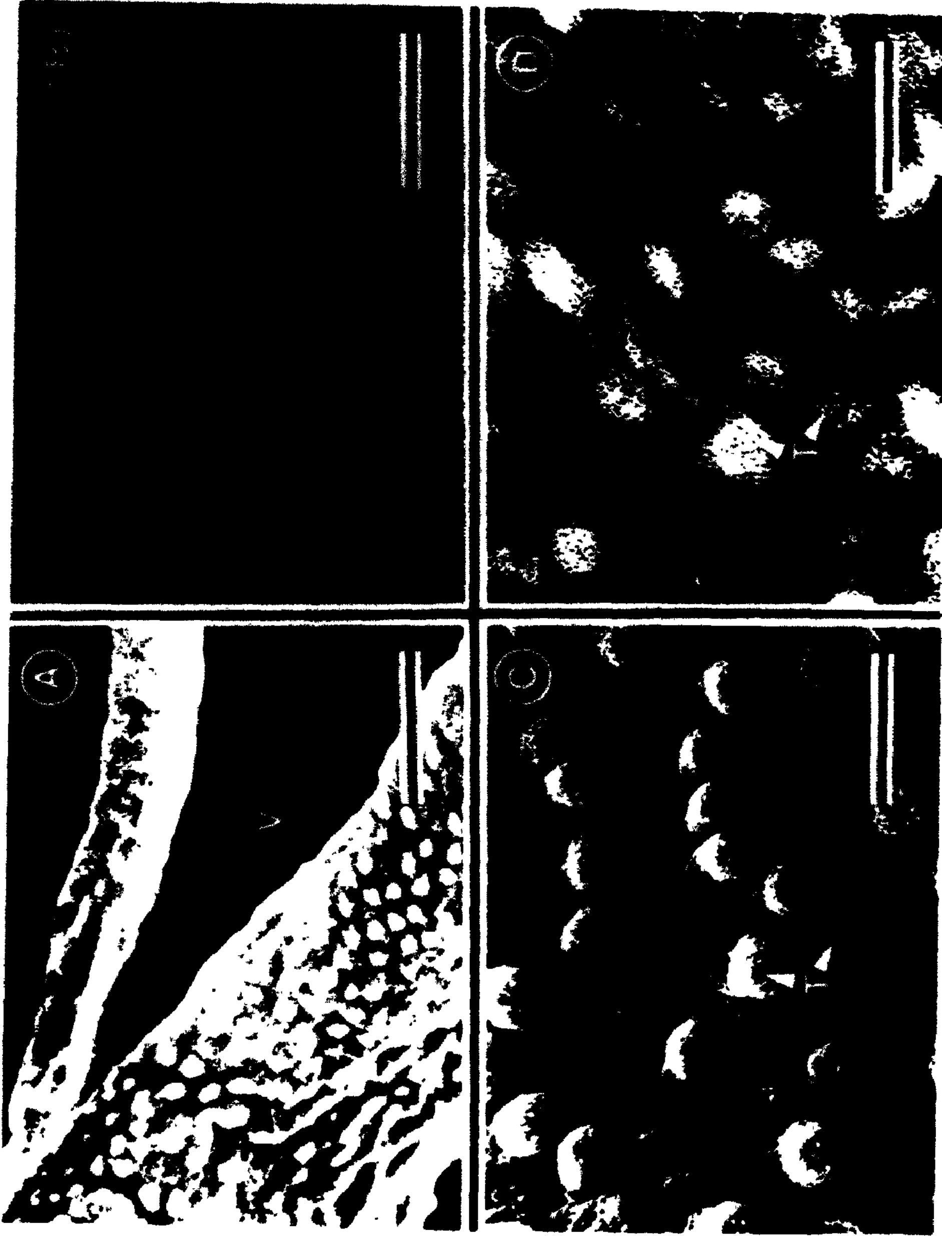
according to the wet transfer method of Towbin *et al.* (1979). The gel, the membrane, the filter paper and the support pads were soaked in transfer buffer [25 mM Tris (pH 8.5), 192 mM glycine, 20 % (v/v) methanol] and then assembled into a transfer sandwich. Transfer proceeded for 1 hour at 100 mV using a cooling pack in a Bio-Rad apparatus with platinum wire electrodes.

3.2.3. Cross-linking of C_3 to the EN with disuccinimidyl suberate (DSS)

Human C_3 was radiolabelled with ^{125}I using Iodobeads (immobilized chloramine-T molecules on polystyrene beads, Pierce, Rockford, Il) according to the manufacturer's instructions. Unreacted ^{125}I was separated from radiolabelled C_3 by gel filtration on a G-25 Sephadex Column (Pharmacia, Uppsala, Sweden). Eluted material was collected in ten-drop fractions. An aliquot of each fraction was read in a gamma counter, and the fractions corresponding to the eluted radiolabelled C_3 were pooled. Envelope proteins (185 μg) were reacted with 3 nM radiolabelled C_3. Another sample containing a 100-fold excess of unlabelled C_3 in addition to labelled C_3 was prepared in parallel. Each sample was incubated for 1 hr at room temperature with gentle shaking. Unbound C_3 was removed by centrifugation at 15000 x g for 15 min. The samples were washed twice in DPBS (pH 7.4), resuspended in 500 μl of DPBS, and each sample was then aliquoted in 5 tubes. DSS (Pierce) was dissolved in dimethyl sulfoxide and was added to each tube at 2 % of the membrane suspension volume and at a final concentration of 0 mM, 0.1 mM,

Figure 2.1 C_3 binding to the surface of male *Schistosoma mansoni* adult worms detected by immunofluorescence using a confocal microscope. Live adult worms were incubated with C_3 prior to fixing in formalin. Bound C_3 was detected with FITC-labelled goat anti-human C_3 antibodies. A. Scanning electron micrograph of adult untreated male *Schistosoma mansoni* showing the ventral (V) and dorsal surface (D). Numerous tubercles (T) are present on the male dorsal surface. B. Confocal images of the ventral surface syncytium of male adult *Schistosoma mansoni* that was treated with C_3. No fluorescence was observed on the male ventral surface. C. Scanning electron micrograph of the dorsal surface of untreated male adult *Schistosoma mansoni* showing the tubercles (T). D. Confocal images of the dorsal surface syncytium of male adult *Schistosoma mansoni* that was treated with C_3. Fluorescence is shown in the tubercles (T) of the surface syncytium.

Bars A = 75 μm; B, C, D = 25 μm



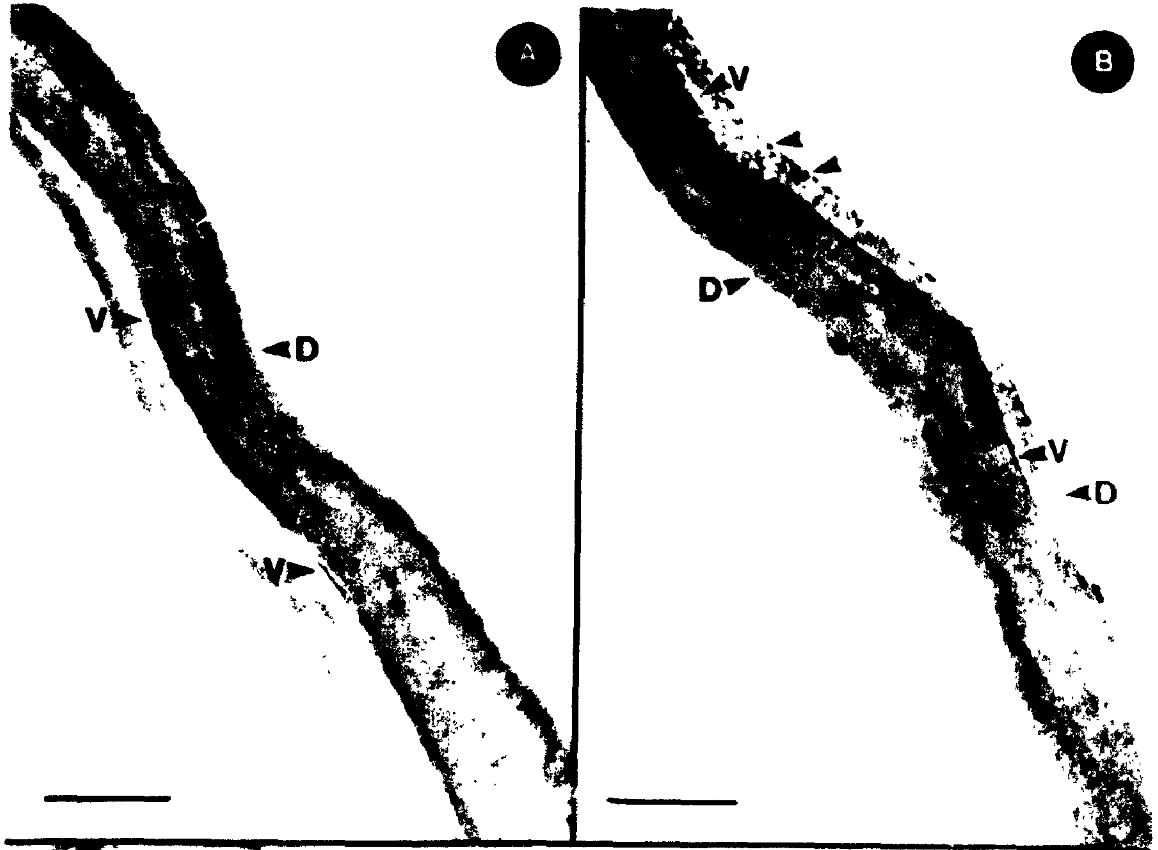
is formalin-sensitive. To overcome this problem, the worms were fixed after C_3 was bound to the parasite surface. Under these conditions, fluorescence was observed in the tuberculated dorsal surface but not on the ventral surface of adult male worms (Figure 2.1. C and D). In addition, shedding of fluorescence into the medium was also prevented by fixation. No fluorescence was noted on the surface of the female worms but non-specific fluorescence was evident on the region of the oral suckers (Results not shown). Parallel experiments using immunocytochemistry confirmed the results obtained by immunofluorescence (Figure 2.2). In these experiments, the peroxidase label was associated with the surface of the male worm, and was restricted to the tuberculated dorsal surface. The ventral surface of the male schistosome as well as the entire surface of the female schistosome (not shown) were negative for peroxidase staining.

The results of the rosette assay are presented in Table 2.2. Similar results were observed when experiments were performed either at 4° or 37°C. This assay showed that only erythrocytes that have been coupled to C_3 bound to adult male schistosomes. Erythrocytes coupled to C_1 or C_4 did not bind to the surface of the adult worms. In this assay only mature parasites were able to bind C_3. Cercariae and mechanically transformed schistosomula did not bind any component of the complement system. These results correlate with morphological differences in the surfaces of adult and juvenile parasites. Cercariae and young schistosomula, which lack an envelope overlying the

apical plasma membrane, did not bind C_3, whereas, lung, liver and adult schistosomes which posses an envelope, bound C_3. Since C_3 binding to the surface of the schistosomes occurred only in those stages of the parasite that possess an envelope as part of their surface epithelium, the possible role of the envelope on C_3 binding was investigated further. With this objective in mind, a series of enzyme-linked immunoabsorbent assays using the envelope and apical plasma membrane fractions of the adult parasites were developed. The results of these studies are shown in Figure 2.3. The results on C_3 binding indicated that the EN is the major fraction with C_3 binding activity.
Hence, binding of C_3 can be used as a marker for the EN fraction. In contrast to the C_3 binding activity, and consistent with previous studies (Dean and Podesta, 1984), we have confirmed that alkaline phosphatase is a suitable marker for the APM. We further studied the C_3 binding activity of the EN using a series of immunoassays and the results are shown in Table 2.3. To eliminate the possibility that C_3 was binding non-specifically to sialic acid residues on the parasite surface (McDiarmid and Podesta, 1984) we treated the membranes with neuraminidase. This treatment did not affect binding of C_3. In addition, binding of C_3 to the EN fraction was saturable and specific (Figure 2.4), suggesting the presence of a receptor for C_3 in the EN of adult worms.

Figure 2.2. C_3 binding to the surface of adult *Schistosoma mansoni* detected by immunoperoxidase. Worms were incubated with human C_3 prior to formalin fixation. Bound C_3 was detected with peroxidase-labelled goat anti-human C_3. (A) Male *Schistosoma mansoni* of the control group. No peroxidase deposition was observed on the schistosome surface. B. Male *Schistosoma mansoni* of the experimental group. The dorsal surface (D) demonstrated specific deposition of the peroxidase reaction as a granular precipitate (see arrows). No peroxidase label was observed on the male ventral (V). surface. C. Higher magnification micrograph of the tuberculated surface in B showing the peroxidase deposition.

Bars: A, B = 1 mm; C = 250 μm.


A
B
V
D
V
V
D
D
V
D

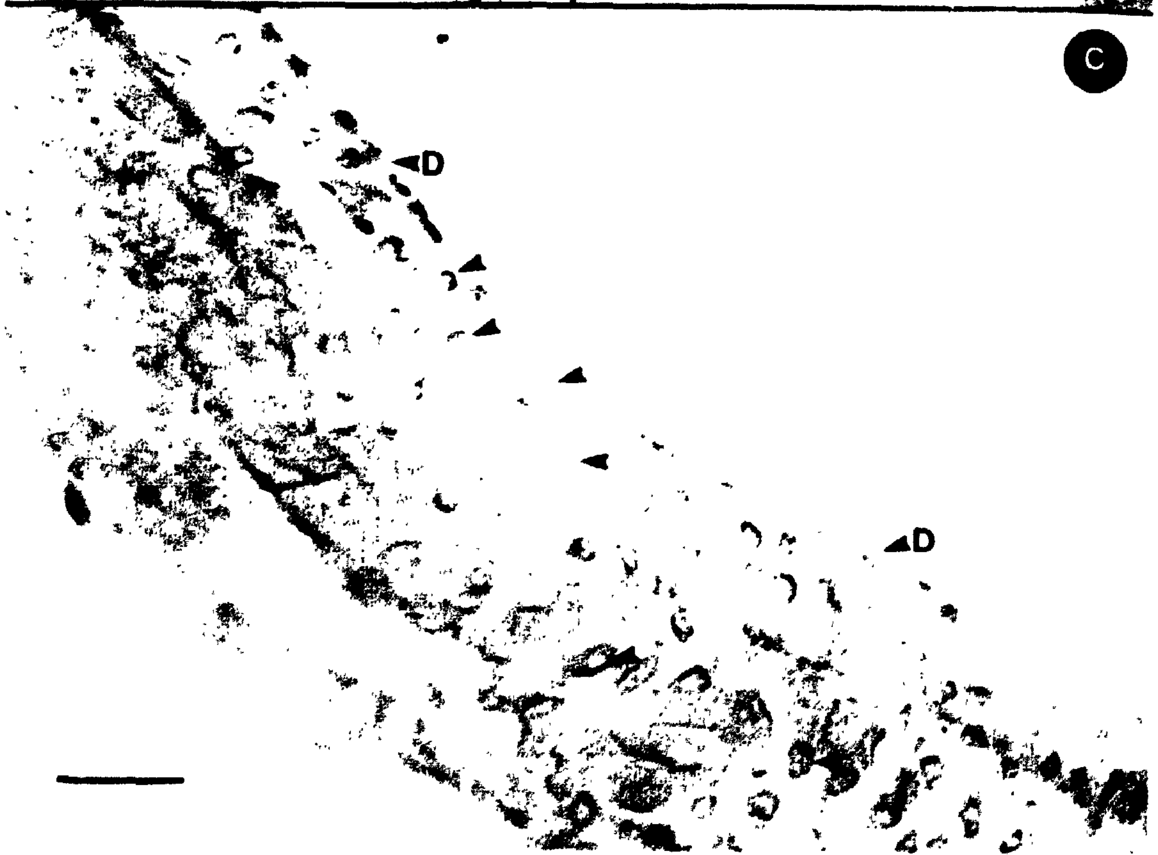

C
D
D

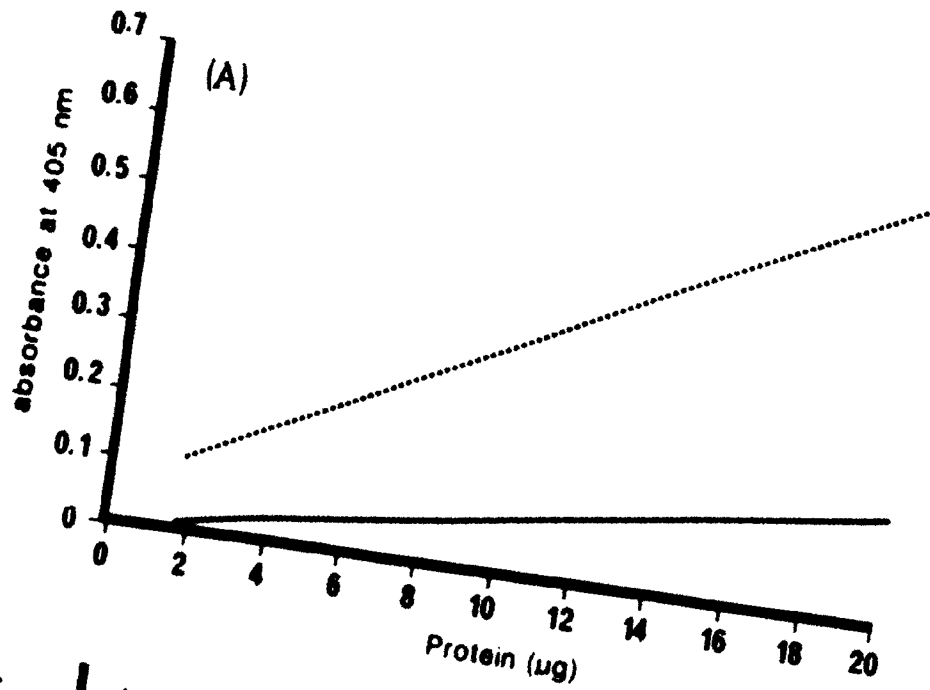
Table 2.2. Rosette assay of C_3 binding to different developmental stages of *Schistosoma mansoni*

	Rosette Formation			
	E19	EC_1	EC_{14}	EC_{1423}
Cercariae	-	-	-	-
Schistosomula	-	-	-	-
Lung stage	-	-	-	+
Liver stage	-	-	-	+
Adults	-	-	-	+
Peritoneal Cells	ND	ND	ND	+

\+ Rosette formation
\- Absence of rosette
ND Not done
E19 Erythrocytes coupled to IgM,
EC_1 Erythrocytes coupled to Complement 1
EC_{14} Erythrocytes coupled to Complement 4
EC_{1423} Erythrocytes coupled to Complement 3

Figure 2.3. (A) C_3 binding activity in the envelope (EN) and apical plasma membrane (APM) of *Schistosoma mansoni*. The results represented in this graph were obtained by ELISA using different concentrations of EN proteins and reacted with C_3 as described in Materials and Methods. The slopes represent C_3 binding to the EN (broken line) and APM (solid line). The lines indicated are defined by the following equations: EN, $Y = 0.060\ (\pm\ 0.017) + 0.028\ (\pm\ 0.002)\ X$; APM $Y = 0.004\ (\pm\ 0.006) + 0.008\ (\pm\ 0.001)$, $n = 82$. The two slopes are significantly different at $p < 0.001$.

(B) Specific activity of alkaline phosphatase in the APM and EN. Alkaline phosphatase activity is expressed as Sigma units (σ) per 100 ml. The slopes of the regression lines are equivalent to the specific activity of alkaline phosphatase. For the EN (broken line), $Y = 0.37\ (\pm\ 0.20) + 0.23\ (\pm\ 0.12)\ X$, $n=33$. For the APM (solid line), $Y = 4.16\ (\pm\ 1.39) + 2.59\ (\pm\ 0.32)\ X$, $n = 33$.


(A)
absorbance at 405 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
20
Protein (µg)

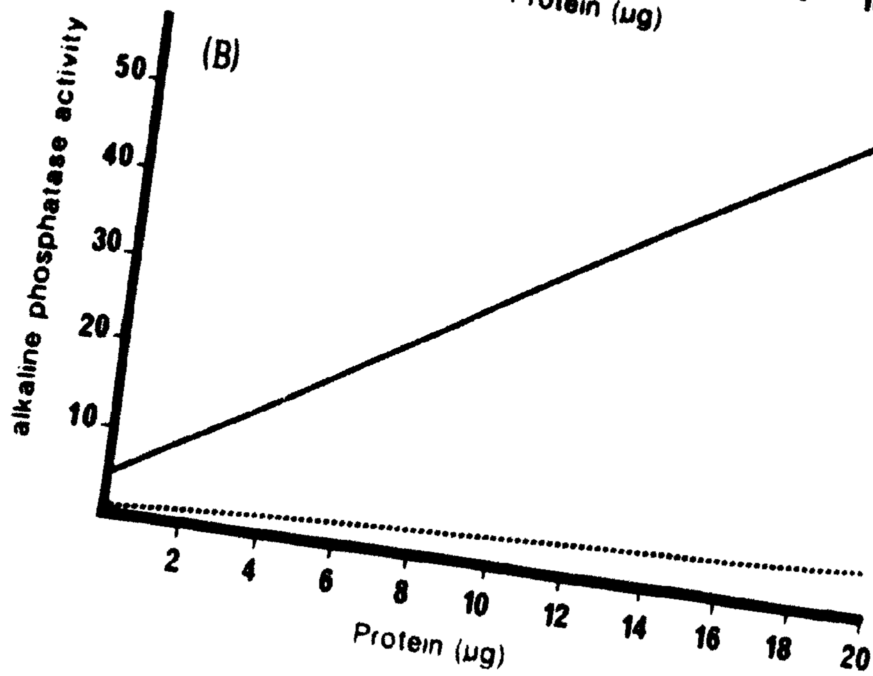
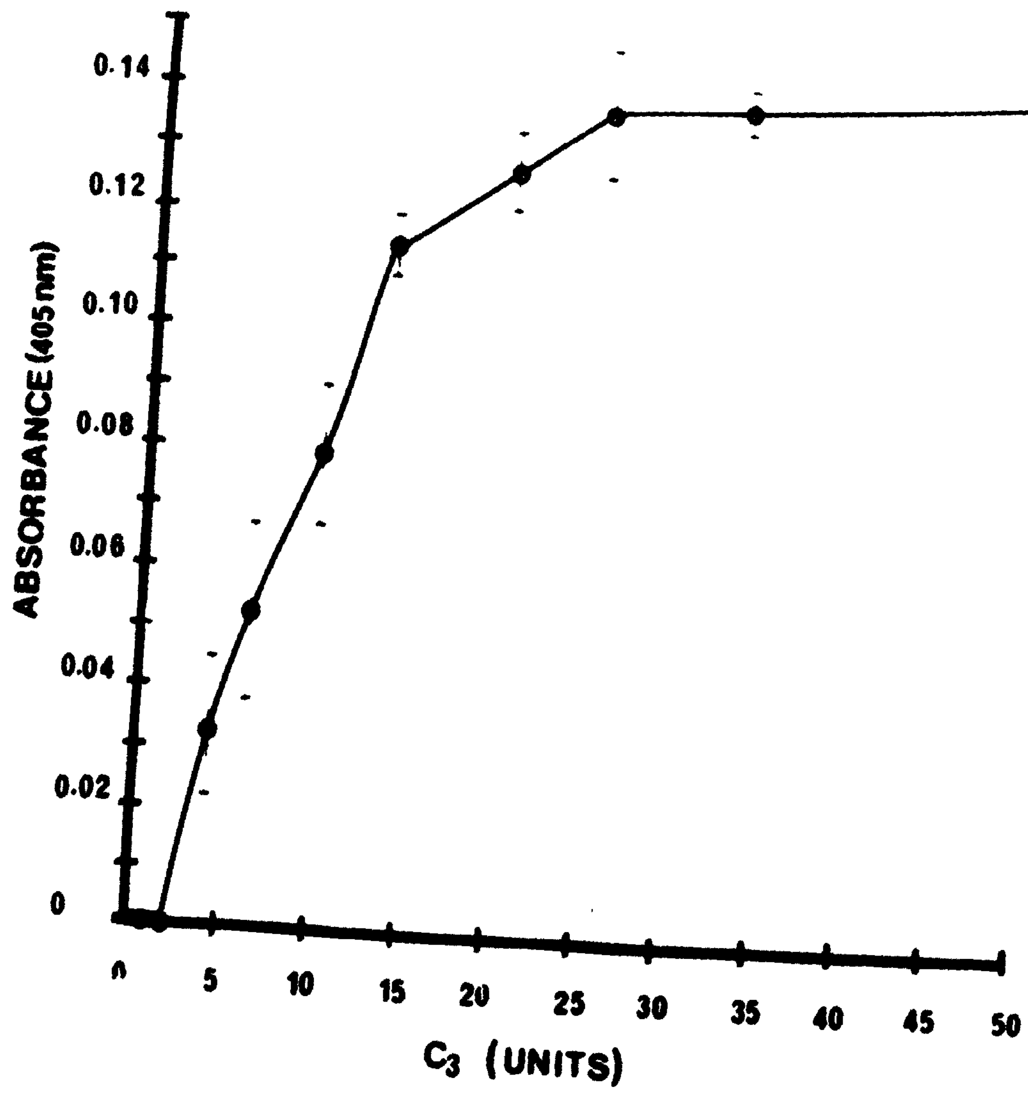

(B)
alkaline phosphatase activity
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
Protein (µg)

Table 2.3. C_3 binding to the envelope and apical plasma membrane fractions of the surface of adult *Schistosoma mansoni*.

ELISA Reactants	O.D (405 nm)[a]	
	EN	APM
Control[b]	0	0
Experimental[c]	0.423 ± 0.097[#*]	0.087 ± 0.041[#]
Experimental + Neuraminidase	0.480 ± 0.025[*]	0.081 ± 0.031

[a] n= 15 for each group
[b] Negative control, absence of membranes, or C_3 or goat anti-C_3 $f(ab)^2$
[c] C_3, goat anti-C_3 $f(ab)_2$, peroxidase rabbit anti-goat $f(ab)_2$
[*] Significance between these two values is $p < 0.01$
[#] No significant difference.

Figure 2.4. Saturability of C_3 binding sites on *Schistosoma mansoni* (EN) analyzed by ELISA. EN samples (10 μg of protein per ml; 1 μg/well) were incubated in DPBS and reacted with different concentrations of C_3. Bound C_3 was detected with $F(ab)_2$ goat anti-human C_3, and peroxidase-labelled $F(ab')_2$ rabbit anti-goat IgG. Negative controls lacked the EN proteins or the primary antibody. Absorbance readings were taken at 405 nm. The results are expressed as a mean of five different experiments. Vertical bars represent standard errors. Saturation of C_3 binding sites for 1 μg of envelope proteins is reached with 20 units of C_3.



ABSORBANCE (405 nm)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
30
35
40
45
50
C_3 (UNITS)

2.4 Discussion

The experiments described in this chapter were designed to determine the possible existence of a C_3 receptor on the surface of adult *Schistosoma mansoni*. This investigation was prompted by the following observations. First, cercariae and newly transformed schistosomula activate the alternative pathway of the complement system and are susceptible to immune killing by this mechanism. In contrast, several day-old schistosomula, lung stage, and adult schistosomes are resistant to complement attack while still retaining both their ability to bind C_3 (Ruppel 1986; Ruppel *et al.*, 1983; 1984) and a morphologically intact surface (Ruppel, 1986). Second, bound C_3 has been shown to be shed from the parasite surface (Samuelson *et al.*, 1980; Kemp *et al.*, 1980; Tarleton and Kemp, 1981; McGuinness and Kemp, 1981) which suggests that C_3 binding to the surface of schistosomes has an effect on membrane turnover. This is consistent with the research performed by Podesta *et al.* (1987), Young and Podesta (1986), and Zhou and Podesta (1989). These investigators reported that C_3 accelerates the synthesis of the EN on adult worms and increases the synthesis of multilamellar bodies, the precursor organelles of the EN. Previously, membrane turnover has been suggested as a mechanism by which schistosomes contend with the host immune system (Podesta, 1982). Since a C_3-dependent physiological response has been correlated to the survival of adult schistosomes, it is likely that these worms possess a signal transduction mechanism which is responsible for EN

renewal. Previously, the presence of C_3 receptors (Tarleton and Kemp, 1981; McGuinness and Kemp, 1981) or C_3 acceptor sites (Marikosvky *et al.*, 1990) have been suggested on the surface of adult schistosomes. However, C_3 binding to the parasite surface using immunofluorescence microscopy has proved difficult to demonstrate since such studies have generated conflicting data. For example, Kabil (1976) reported the presence of C_3 on the surface of female but not male schistosomes. McGuinness and Kemp (1981) detected binding of C_3 on the surface of adult male schistosomes. Sogandares-Bernal (1976) could not detect C_3 associated with adult worms at all. In contrast, Ruppel *et al.* (1983) found that several-day-old or adult schistosomes bound C_3 to their surfaces when incubated *in vitro* with fresh normal human serum, but did not bind C_3 from other animal sera. In the latter study it was reported that adult schistosomes retained a morphologically intact surface. Finally, several investigators have reported that adult worms possess complement-binding antigens that are shed into the incubation medium (Van Egmond *et al.*, 1981; Rasmussen and Kemp, 1987; McCormick and Damian, 1987).

In the present study, different experiments utilizing various techniques were designed to determine whether the third complement component, C_3, binds to the surface of any of the developmental stages of *S. mansoni*. Results from the rosette assay indicate that C_3 can bind to the surface of lung and liver stage schistosomula as well as adult worms. However, no rosettes were formed when C_3-coupled erythrocytes were incubated with cercariae or

mechanically transformed schistosomula. In addition, binding of C_3 appeared to be specific since erythrocytes coupled to the complement components C_1 or C_4 failed to form rosettes with any of the developmental stages of the parasite that are present in the vertebrate host. These results suggest that older schistosomes possess C_3 binding sites, while earlier developmental stages do not (Table 2). Previous studies have demonstrated that C_3 exhibits different effects on juveniles and adult schistosomes. Incubation of juvenile schistosomes with C_3 results in lysis (Butterworth *et al.*, 1982; Butterworth, 1977; Santoro, 1982), but does not appear to have an effect on membrane synthesis (Podesta *et al.*, 1987; Young and Podesta, 1986). In contrast, in the complement-resistant adult worms (Butterworth *et al.*, 1982; Smithers *et al.*, 1977; Santoro *et al.*, 1979) C_3 stimulates the synthesis of the envelope and its precursor organelles (Podesta *et al.*, 1987; Young and Podesta, 1986; Zhou and Podesta, 1989). This envelope synthesis is thought to lead to an enhanced shedding and replacement of membranes which had bound serum components before damage to the integrity of the schistosome surface could occur, and hence allowing adult schistosomes to evade the host immune attack. If C_3 receptors are part of a mechanism evolved to allow the parasites to evade the effects of immune attack, their presence in a schistosome developmental stage should be correlated with immune resistance. The experiments performed using rosette assays appear to substantiate this hypothesis. Furthermore, since the surface epithelium of older parasites differs

from that of juveniles by the presence of an envelope (Hockley and McLaren, 1973; Senft *et al.*, 1978), we have suggested that these C_3 receptors, if present, will be located in the EN.

The results of the immunofluorescence experiments which were designed to detect C_3 binding by live parasites, showed that fluorescence was associated with the surface of male parasites (Table 1). However, the fluorescence was not distributed evenly over the entire surface of the males, instead it appeared to be present in patches. In addition, the fluorescence was lost from the parasite surface very rapidly (within 30 minutes of incubation, results not shown). Similar results were obtained by McGuinness and Kemp (1981), using C_3 bound to *Salmonella typhi*. These investigators observed that bacteria can associate with the dorsal surface of live male adult schistosomes, but this association was transient, since the bacteria were shed from the parasite surface after 20 minutes of incubation at room temperature. In addition, Samuelson *et al.* (1980) found that C_3 bound to the surface of lung stage schistosomula and adult worms, was shed into the incubation medium with a half-life of 5 hours.

Since C_3 binding could only be detected on the surface of live parasites for a short period of time due to the shedding of membrane-bound C_3 into the incubation medium, it was necessary to prevent this by fixation of the worms. Furthermore, since the schistosome C_3 receptor has been shown to be sensitive to formalin (McGuinness and Kemp, 1981), the worms were fixed after

C_3 was bound to the surface of the parasite. This step did not prevent binding of C_3. Binding of C_3 was detected with fluorescein-labelled antibodies using a confocal microscope or with peroxidase-labelled antibodies using a convensional light microscope. Consistent with the results presented by McGuinness and Kemp (1981), we found in both fluorescein- and peroxidase-labelling studies that C_3, binds only to the dorsal surface of adult male worms. The ventral surface of male worms and the surface of female worms were negative for C_3 binding. Previous studies by Podesta *et al.* (1987) have demonstrated differences in the properties of the dorsal and ventral surface of the male schistosomes. The dorsal surface is relatively hydrophilic, non-adhesive, and contains a thick, continuous glycocalyx, whereas the ventral surface of the male as well as the entire surface of the female are relatively more hydrophobic, adhesive and the glycocalyx is thinner and aggregated. These investigators suggested that these differences in surface properties aid in the sexual pairing process by allowing close contact between the female and the male ventral surface. In contrast, the non-adhesive male dorsal surface, which is exposed to the host immune system, will prevent immunocompetent cells from adhering. In addition to this basic protective mechanism, it is reasonable to expect that any immune evasive mechanism that the schistosome has evolved will be more concentrated in or possibly restricted to the surface of the parasite that is in contact with the host immune system. This could explain the absence of C_3 receptors on the female surface and male ventral surface.

Having detected binding of C_3 to the surface of adult schistosomes ELISA assays were carried out to determine which fraction of the schistosome surface is able to bind C_3. The results obtained revealed that C_3 binding sites are present in the EN and not in the APM fraction. Furthermore, binding of C_3 to the EN was saturable and specific, (Figure 2.4) suggesting that specific receptors in the EN are involved. However, fractionation of these two membranes often results in some contamination of EN proteins in the APM fraction and vice versa (McDiarmid *et al.*, 1983). This contamination could explain the low levels of binding observed in the APM fraction.

In addition, previous studies involving bacterial surfaces have demonstrated that C_3 binds non-specifically to surface sialic acid (Joiner, 1986). and since McDiarmid and Podesta (1984) reported that the adult schistosomes have a surface glycocalix that contains sialic acid, the possibility that C_3 was binding non-specifically to sialic acid residues needed to be investigated. This was tested by treating the EN fractions with neuraminidase prior to their use in an ELISA assay. Neuraminidase was previously shown to be effective in removing sialic acid residues from the surface of *S. mansoni* (McDiarmid and Podesta, 1984). Treating membrane fractions with neuraminidase did not affect C_3 binding (Table 3), suggesting further that the binding of C_3 to the schistosome surface is specific.

In conclusion, the results presented in this chapter provide strong evidence to suggest the presence of C_3 receptors on lung and liver

schistosomules and adult worms of *Schistosoma mansoni*. These receptors appear to be restricted to the dorsal surface of male schistosomes and located in the envelope. In addition, shedding of C_3 from the surface of live parasites is consistent with previous studies that reported an increase of envelope turnover in response to C_3, and hence supporting the hypothesis that rapid membrane turnover may be a mechanism by which schistosomes escape attack by the host immune components.

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF A C_3 RECEPTOR ON THE ENVELOPE OF *SCHISTOSOMA MANSONI*

3.1. Introduction

Unlike prokaryotic and protozoan parasites residing inside host cells, schistosomes are constantly exposed to the components of the immune system, and yet are able to survive in such an inhospitable environment. This observation has led investigators to believe that schistosomes possess mechanisms connected with their surface, to either evade or combat the host immune system. Since schistosomes have a syncytial epithelium, a feature that is common in organisms that must deal directly with immune attack, it has been suggested that the syncytial epithelium is an adaptation that allows the schistosomes to evade the host immune system (Podesta, 1982). The syncytium may facilitate signal transduction leading to rapid and efficient turnover of the schistosome surface membranes when the integrity of the membranes are compromised. For example, it has been shown that the third component of the complement system increases the synthesis of the schistosome envelope (Young and Podesta, 1984) and of the envelope precursor organelles, the multilamellar bodies (Zhou and Podesta, 1986). Therefore C_3 may be the signal ligand component of a signal transduction mechanism affecting envelope turnover. A response by the parasite that uses

C_3 as a signal will necessarily involve receptors for C_3 on the surface of the schistosome.

The work presented in the previous chapter demonstrated that C_3 binds to the surface of adult schistosomes, more precisely, to the envelope fraction. The binding was shown to be saturable and specific, suggesting the presence of C_3 receptors. The experiments outlined in this chapter were designed to identify which protein in the schistosome envelope is responsible for C_3 binding.

3.2. Materials and Methods

3.2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed according to Laemmli (1971) using a mini-gel system (Bio-Rad). Samples of known amounts of protein in solution were solubilized in 2 x SDS sample buffer [2 % (w/v) SDS, 100 mM Tris (pH 6.8), 20 % (v/v) glycerol, 0.05 % bromophenol blue, and 5 % (v/v) 2-mercaptoethanol]. The samples were boiled for 5 min, centrifuged briefly to precipitate insoluble material, and the supernatant loaded into gels and electrophoresed. Polypeptides were resolved using polyacrylamide separating gels that varied from 5-12 % according to the needs of the experiment. Stacking gels ranged from 3-5 % polyacrylamide. Electrophoresis was carried out at 200 mV (constant voltage) for 45 minutes. Some gels were stained directly with Coomassie Brilliant R Blue or double stained with Coomassie blue and silver (Merrit *et al.*, 1981). For autoradiography, gels were dried for 2 hours onto Whatman No 1 filter paper and exposed to Kodak XAR-5 film at -70°C using a Dupont Cronex Lighting Plus intensifying screen. For immunoblot analysis, gels were immediately blotted onto nitrocellulose (0.2 μm pore size) after electrophoresis. Molecular weight standards in the range of 18 to 200 kD were purchased from Bethesda Research Laboratories, Gaithersburg, MD.

3.2.2. Electroblotting of proteins resolved by gel electrophoresis

Transfer of proteins onto nitrocellulose membranes was carried out

according to the wet transfer method of Towbin *et al.* (1979). The gel, the membrane, the filter paper and the support pads were soaked in transfer buffer [25 mM Tris (pH 8.5), 192 mM glycine, 20 % (v/v) methanol] and then assembled into a transfer sandwich. Transfer proceeded for 1 hour at 100 mV using a cooling pack in a Bio-Rad apparatus with platinum wire electrodes.

3.2.3. Cross-linking of C_3 to the EN with disuccinimidyl suberate (DSS)

Human C_3 was radiolabelled with ^{125}I using Iodobeads (immobilized chloramine-T molecules on polystyrene beads, Pierce, Rockford, Il) according to the manufacturer's instructions. Unreacted ^{125}I was separated from radiolabelled C_3 by gel filtration on a G-25 Sephadex Column (Pharmacia, Uppsala, Sweden). Eluted material was collected in ten-drop fractions. An aliquot of each fraction was read in a gamma counter, and the fractions corresponding to the eluted radiolabelled C_3 were pooled. Envelope proteins (185 μg) were reacted with 3 nM radiolabelled C_3. Another sample containing a 100-fold excess of unlabelled C_3 in addition to labelled C_3 was prepared in parallel. Each sample was incubated for 1 hr at room temperature with gentle shaking. Unbound C_3 was removed by centrifugation at 15000 x g for 15 min. The samples were washed twice in DPBS (pH 7.4), resuspended in 500 μl of DPBS, and each sample was then aliquoted in 5 tubes. DSS (Pierce) was dissolved in dimethyl sulfoxide and was added to each tube at 2 % of the membrane suspension volume and at a final concentration of 0 mM, 0.1 mM,

0.5 mM, 1 mM or 5 mM. The reaction was allowed to proceed for 20 minutes at room temperature, then stopped with 40 mM glycine (final concentration). The tubes were then spun at 15,000 x g for 15 min and the pellets resuspended in 40 μl of 2 x SDS sample buffer. The samples were analyzed by SDS-PAGE using 7.5 % separating and 5 % stacking polyacrylamide gels. When this experiment was repeated, 5 % separating and 3 % stacking polyacrylamide gels were used. The gels were fixed, dried onto filter paper and exposed to Kodak X-ray film (XAR-5) with intensifying screens (Du Pont Canada Inc. Mississauga, On) for 4 to 30 days at -70°C.

3.2.4. Photoaffinity Labelling of a C_3 binding polypeptide

A photoactivatable ^{125}I-labelled cross-linking reagent N-[4-(p-azido-m-{^{125}I}iodophenylazo) benzoyl]-3-aminopropyl-N'-oxysuccinimide ester (Denny-Jaffe Reagent, NEX-227, Du Pont) was used to derivatize C_3 according to the manufacturer's instructions. All steps up to the point of a photolytic reaction were carried out in the dark in a ventilated hood. The cross-linker was mixed with a 5000 molar excess of C_3 and incubated for 1 hour at room temperature with occasional agitation. The reaction was then stopped with 0.2 M glycine (pH 8.5). The unreacted cross-linker was removed by gel filtration on a Bio Gel PD6 column (10 cm high). Fraction tubes of 12 drops were collected and a 5 μl aliquot of each tube was read in a gamma counter. Fraction tubes containing the derivatized C_3 were pooled. The derivatized C_3 (6 nM) was

reacted with 200 μg of EN proteins for 1 hour at room temperature with occasional agitation. Unbound C_3 was removed by centrifugation for 15 min at 15000 x g. The preparation was washed three times in DPBS (pH 8.0), resuspended in 400 μl of DPBS, and aliquoted into 100 μl samples (two experimental and two controls). In preparation for photolysis, nitrogen was bubbled into the reaction for 5 minutes, followed by 4 or 8 minutes of ultraviolet irradiation with a long wave UV lamp (UVL.56, Ultraviolet Products, San Gabriel, CA) with brief mixing every two minutes. Sodium dithionite (pH 8.0) was then added every 15 minutes (45 minutes total) to a final concentration of 0.2 M. Excess sodium dithionite was removed by centrifugation, followed by 3 washes in DPBS. Control samples were treated in the same manner but were not subjected to UV light and/or sodium dithionite cleavage. Samples were then resuspended in 50 μl of 2 x Laemmli sample buffer and resolved by SDS-PAGE. Autoradiographs were exposed from 4 to 30 days at -70°C.

3.2.5. C_3-sepharose affinity chromatography

3.2.5.1 Coupling C_3 to activated CNBr beads:

Human C_3 was coupled to CNBr-activated Sepharose 4B (Sigma Chemical Company, Mississauga, On) according to the manufacturer's instructions. Five hundred milligrams of beads were swollen in 10 ml of HCl (1 mM) for 15 min, washed and then reswollen on a glass filter with a total of 100 ml of HCl (1 mM) in several aliquots. The supernatant was removed between additions. One

milligram of C_3 was dissolved in 600 μl of coupling buffer [0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.3)]. The gel suspension was washed with coupling buffer and immediately mixed with the C_3 solution and incubated overnight at 4°C in an end-over-end mixer (Labquake Shaker, Labindustries, Inc, CA). The remaining active groups were blocked with 0.2 M glycine (pH 8) for 2 hours at room temperature. Excess adsorbed proteins were washed away by alternating washes of high and low pH buffer solutions [coupling buffer, (pH 8.3) followed by 0.1 M acetate buffer, 0.5 M NaCl (pH 4)]. Beads were stored at 4°C in DPBS until used. For the control samples, BSA was coupled to CnBr-activated beads in the same manner.

3.2.5.2. Radiolabelling of envelope proteins:

Envelope proteins were labelled with ^{125}I by following the method described by Markwell and Fox (1978). Glass test tubes (12x75 mm) were coated with 30 μg of iodo-gen [1,3,4,6-Tetrachloro-3α-6α-diphenylglycoluril (Pierce)] and stored in a desiccator over Drierite until used. An iodo-gen coated tube was rinsed twice with DPBS to remove any loose microscopic flakes. Four hundred μg of EN proteins in 400 μl of DPBS were added to the tube along with 1.5 mCi of $Na^{125}I$. The reaction was allowed to proceed for 15 min with gentle agitation. The sample was removed from the reaction vessel to terminate the iodination reaction and NaI (0.25 M) was added to the sample. Unreacted iodine was removed by centrifugation at 35,000 x g for 15 min followed by three washes in DPBS.

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