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SCHISTOSOMA MANSONI : CHARACTERIZATION AND COMPARISON OF

THE SURFACES OF DEVELOPING SCHISTOSOMULA AND ADULT WORMS

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ABSTRACT

An animal infected with schistosome worms is stimulated by antigen associated with the living, adult parasite to mount an immunological reaction against the surface of invading schistosomula. The adult worms, however, remain unaffected by the immune response of the host and the young schistosomula develop resistance to the host response during the 5 days in which they migrate from the skin to the lungs. The aim of the experiments described in this thesis was to compare the biochemical composition and antigenicity of the surface of the parasite at different stages of development from the cercaria to the adult, in order to understand more about how resistance to the host is acquired and maintained.

The initial step was to establish a method for producing sufficient quantities of schistosomula which were uniformly developed and uncontaminated by host material. Comparative studies were made of several types of artificially transformed schistosomula and the prototype organisms, namely schistosomula resulting from cercarial penetration of isolated skin (SS). An organism (MS) produced by mechanically separating cercarial tails from bodies with subsequent incubation of the bodies in a defined medium was shown to undergo surface changes similar to those observed during cercariae-SS transformation. Surface microvilli were produced in the 1st hour, the glycocalyx was lost within 2 hours, there was a change from a trilaminate to a heptalaminate surface membrane by 2-3 hours and the inclusions seen in the tegument were similar to those previously described in the SS tegument.

Only one morphological difference was noted between MS and SS: the pre-acetabular glands of MS retain their contents for up to 48-72 hours while SS secrete the granular contents within 3 hours of skin penetration.

MS were therefore used as the reference organisms in all further studies. Comparisons were made with cercariae, the _in vivo_ stages of schistosomula collected from the skin (SS) and from the lungs (LS), the _in vitro_ schistosomula (CS) and adult worms.

Immunological techniques and reagents, i.e. immunofluorescence, ^{125}I-Protein A binding, and radio-labelled myeloma proteins and anti-globulin were adapted to the study of living schistosomes. The use of triple-layer antibody techniques and the use of radioimmunoassays are considered to be significant improvements over previous studies.

A combined study of antibody class and subclass development and the binding capacity of the surfaces of the various organisms revealed differences both in the host response of different mice strains and in the quantity and distribution of antibody bound by the various schistosome stages. With identical cercarial infections, Parkes mice produced detectable levels of schistosome surface specific antibody by 10 days while antibody was not detected until 2-3 weeks after infection in CBA mice. IgM levels were shown to appear late and remained high throughout the course of the infection. IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgD levels were only slightly increased while IgA levels were moderately increased over that observed with normal mouse sera. Furthermore, the various schistosome stages differed in the binding of antibody:cercariae, MS and SS bound significantly more antibody than did the later stages, namely, CS, LS and the adult schistosomes.

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A phenomena observed during the immunofluorescence studies was the sloughing of the fluorescent surface coat of MS as the organism moved across the microscopic field. Sloughing could be prevented at $4^{o}C$; electron microscopy revealed no damage to the tegument, but microvilli were observed in the sloughing surface coat. Attempts to restain "sloughed organisms" with immune serum were unsuccessful. Sloughing may represent a mechanism for eliminating host antibodies but further studies are necessary to prove this.

The use of radioimmunoassays revealed the adsorption of host immunoglobulins onto the surface of all the schistosomula stages. The use of iodinated myeloma proteins and $F(ab)_2$ fragments indicated that this adsorption was non-specific and did not require the presence of an Fc receptor.

Schistosome synthesis of a mouse α_2- macroglobulin-like determinant was confirmed using the artificially prepared MS. However adsorption was also observed in studies using ^{125}I-Mo α_2-macroglobulin.

Correlations between parasite antigens and the presence of host antigens on the schistosome surfaces have been observed. The expression of human blood group-like antigens and mouse erythrocytes antigens was studied by mixed agglutination, immunofluorescence and immunoradioassay. With all schistosome stages the binding of specific antibody is relative to the amount of host material detected on the surface.

A variety of biochemical techniques were used to compare the tegumental surfaces of cercariae, schistosomula and adults. These techniques included lactoperoxidase iodination and galactose oxidase-tritiated borohydride labelling of surface proteins and glycoproteins,

followed by detergent solubilization and separation of components by polyacrylamide gel electrophoresis.

Similarities and differences in the total proteins and surface components of the various schistosome stages were noted. The number of proteins obtained by 1° SDS-PAGE was between 45-55, which was considered minimal. The majority of these proteins were common to all stages, two of these proteins migrated at rates similar to actin and myosin. The actin-like component was labelled by the lactoperoxidase technique, indicating that it is exposed to the external surface.

The proteins unique to a particular developmental stage included a 160,000-180,000 molecular weight component of cercariae, three components (58,000-60,000 daltons, 23,000 daltons and 11,000 daltons in adult schistosomes and a protein of $\simeq$ 29,000 daltons in adult females.

Cercariae had 8 surface components, which were iodinated by the lactoperoxidase technique, 3 of these components were also labelled by the galactose oxidase- ^{3}H borohydride procedure.

MS and SS shared 8 lactoperoxidase labelled surface components, 3 of these were similar to cercarial surface components. Two surface components were also labelled by the galactose oxidase- ^{3}H borohydride procedure.

LS had 7 components which were iodinated by the lactoperoxidase procedure; 3 components are also labelled by tritium.

CS possess 9 lactoperoxidase labelled components, three of which were tritiated.

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Adult schistosomes had 8 iodinated surface components,
3 of which were labelled by tritium.

It is concluded that the schistosomes maintain a complex
interaction of acquisition, elimination and mimicry of host antigenic
material and thus evade the action of the host immune response. It
is suggested that the development of these evasive mechanisms occurs
during the period between skin penetration and migration through the
lungs.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CHAPTER I: INTRODUCTION

A.   The Human Disease: Schistosomiasis

Schistosomiasis is a trematode infection affecting more than
200 million people in tropical and subtropical areas of the world.
The major schistosome species which infect man have different geo-
graphic distributions: Schistosoma mansoni (Sambon, 1907) occurs in
Africa, Arabia, South America and the Caribbean, Schistosoma japonicum
(Katsurada, 1904) is found in Japan, China, Thailand and the Philippines,
and Schistosoma haematobium (Bilharz, 1852; Weinland, 1858) in North
Africa, parts of West Africa, the Middle East, Mauritius and Madagascar.
Figure 1 shows the world distribution of human schistosomiasis.

Schistosomiasis has been designated the world's second most
important tropical disease by the 1976 Special Program for Research
and Training in Tropical Diseases, a joint project of the World Health
Organization and the United Nations Development Programme. Several
major factors have contributed to this classification and other
recent interests in the disease. Firstly schistosomiasis occurs
mainly in developing countries, where the loss of human labour due
to the chronic debilitating nature of the disease can be of great
economic importance. Secondly, the development and extension of
irrigation projects have led to increased prevalence and distribution
of the disease and this trend is likely to continue. Finally, the
molluscidal and chemotherapeutic measures at present available have
not proved satisfactory or promising in terms of eradication of
schistosomiasis.

The fact that schistosomes, in contrast to many infectious agents,

1

FIGURE 1.

A. World distribution of Schistosomiasis due to S.mansoni.

B. World distribution of schistosomiasis due to S.japonicum
and S.haematobium

Taken from: Epidemiology and Control of Schistosomiasis (Bilharziasis)
pp 34-35, ed. by N. Ansari (Geneva), 1973.

.mansoni.

.japonicum

miasis (Bilharziasis)

1973.

A

S. mansoni
INCIDENCE
HIGH   LOW

B

S. haematobium
INCIDENCE
HIGH   LOW

S. japonicum
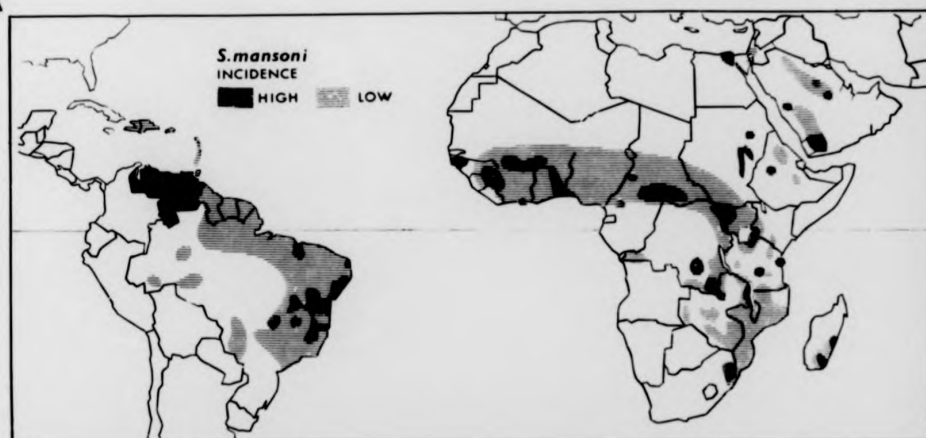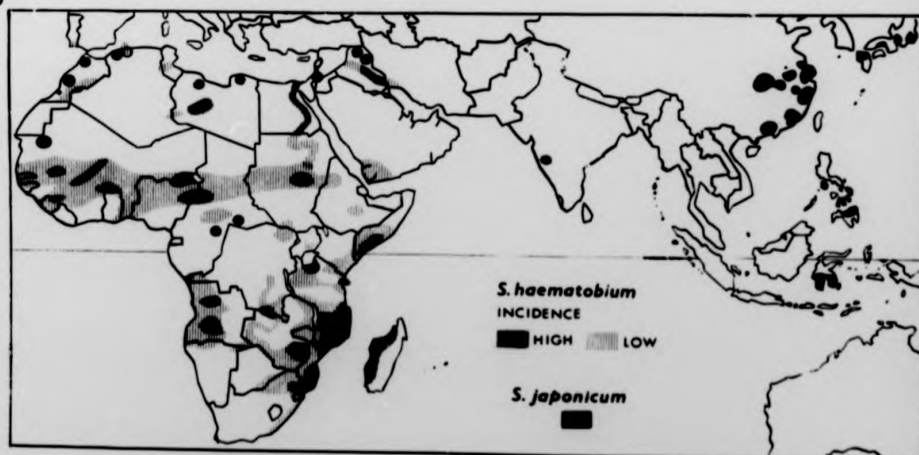
do not multiply in the human host has profound implications with
respect to epidemiology, control and possible eradication of the
disease. The major factors governing the worm burdens of individuals
are the rate of acquisition of new worms, schistosome longevity, which
is probably in the range of 5-10 years, and the effects of acquired host
resistance (Warren et al., 1974). Recent studies suggest that even
in endemic areas human exposure to cercariae is usually very sporadic
and seasonal; furthermore, the level of infectivity of most bodies
of water is relatively low, because few snails are infected and
cercariae are dispersed in large volumes of water. Thus frequent
and/or prolonged contact with contaminated water appears necessary
for persons to acquire worm burdens which produce significant
disease (Warren, 1973).

Epidemiological studies in man have shown that in endemic areas
schistosomiasis is mainly a disease of the young, and that with
advancing age there is a decreased passage of eggs and a lessening
of the associated symptoms. The fall in prevalence and intensity of
infection in adults is generally believed to result from the gradual
development of resistance (for reviews see Smithers & Terry, 1969;
Smithers, 1976). However, in many cases this fall could be explained
equally well by the less frequent contact with water by the older
members of the population. McCullough and Bradley (1973) have
offered evidence for the existence of concomitant immunity to
S.haematobium in man. Their studies of egg output in children over
a period suggest that schistosomes acquired during the first decade

3

of life are tolerated and that a substantial resistance to further infection develops. They (Bradley and McCullough, 1973) suggest that in later decades some people lose their immune status, probably due to natural death of worms and subsequent loss of antigenic stimulus, and can then be reinfected.

The severity of schistosomiasis and its clinical symptoms depend on the Schistosoma species involved and on the intensity of the parasite infection. The major pathological manifestations are due to the host inflammatory response to the schistosome eggs which become entrapped within tissues and organs. Based on observations of periportal round cell infiltration in the liver, Andrade (1965) has suggested that cell-mediated mechanisms are involved in the host response. With S.mansoni infections, the granulomatous and fibrotic response of the host creates an intrahepatic presinusoidal block, leading to portal hypertension and eosophageal varices (Ramos et al., 1964). The liver parenchymal cells are not directly damaged and hepatic blood flow is maintained within normal limits by arterial neovascular formation, thus providing adequate hepatocyte perfusion (Andrade and Cheever, 1971). The major complication of hepato-splenic schistosomiasis is the occurrence of bleeding eosophageal varices. However, in some cases pulmonary hypertension with cor pulmonale may develop, following massive embolization of eggs and worms to the pulmonary vasculature. The most severe forms of intestinal schistosomiasis occur in Egypt, where inflammatory polyps are a major complication (for review see: Marcial-Rojas, 1971).

4

In S.japonicum infections the same organ systems are involved
but the resulting pathology is quite different and more pronounced
due to certain biological aspects of the parasite. The females
produce 10 times as many eggs as S.mansoni females and, furthermore,
the eggs are produced in large aggregates and have a tendency to
calcify. The pathology surrounding the eggs suggests a greater degree
of exudative granulomatous lesions with more infiltration of neutro-
phils than is seen with S.mansoni. Another unusual aspect of
S.japonicum is that a worm pair tends to deposit the large masses
of eggs in a relatively confined area, which can result in intestinal
obstruction, or, if ectopic organisms reach the brain, diffuse cerebral
lesions.

With S.haematobium infections, the urinary tract is involved and
granulomas which surround the masses of calcified eggs can physically
diminish the working volume and efficiency of the bladder. This
condition may lead to hydronephrosis, impaired renal function and
eventually uremia.   Furthermore, an increased incidence of bladder
cancer in schistosomiasis haematobium has been reported from Egypt
and Iraq.

B. The Parasite

The three schistosome species which infect humans have similar
life cycles involving a succession of stages: egg, miracidium, first-
stage sporocyst, second-stage sporocyst, cercaria, schistosomulum
and adult (S.mansoni adults are shown on Plate 1). As shown in Figure 2 the
basic life cycle has an alternation of reproductive modes; the sexual genera-

5

PLATE 1.

S.mansoni adults in copula, female lying in gynecophoral canal of male.

FIGURE 2.

Diagrammatic representation of the life cycles of the <u>Schistosoma</u>

species which infect man.

Paired mature Worms

Veins of bladder - S. haematobium

Mesenteric vessels - S. mansoni
of bowel - S. japonicum

**DEFINITIVE HOST
MAN**

Hepatic portal vessels

Arteries

Heart

RETAINED IN TISSUES

Lungs

6—12 weeks

Heart

Veins

Schistosomula

Eggs excreted with urine or faeces

**COMMON EXTERNAL ENVIRONMENT
IN WATER**

Cercariae
penetrate skin

Free swimming
Cercariae

EGGS

S. mansoni

S. haematobium   **S. japonicum**

48 hours

INTERMEDIATE HOSTS

Bulinus sp.

in fresh water

Cercariae
2nd-stage sporocysts
1st-stage sporocyst

within snail

Free swimming
Miracidium

24 hours

4—7 weeks

Biomphalaria sp

if appropriate snail species is penetrated

Oncomelania sp.

tion of adult schistosomes is in the definitive mammal host and the asexual multiplicative stage is in the intermediate molluscan host. In the human host, mature schistosomes mate and then migrate from the liver to the mesenteric or vesical venules, where each worm pair produces 300-3000 eggs per day, depending on the species. The eggs penetrate the vessel walls by the combined action of enzymatic secretions and peristalsis and pass through the tissues into the lumen of the intestine or the bladder. Many of the eggs fail to escape and are entrapped in various tissues, especially in the bowel and liver in the case of S.mansoni and S.japonicum, and in the bladder and urogenital tract in the case of S.haematobium. The eggs that do escape contain a fully developed miracidium and are voided in the excreta (faeces or urine).

Once in fresh water, the miracidium becomes active and emerges through a break in the egg shell caused by its own activity and by osmotic effects. The ciliated miracidium swims actively until it makes contact with and penetrates the appropriate snail species. Lytic enzymes, as well as muscular effort, may be involved in penetration.

After penetration of the snail, the miracidium looses its cilia and develops into a first stage sporocyst near the entry site. This development occurs only within the appropriate snail species; in other species the miracidium is destroyed by phagocytosis. Second-stage sporocysts develop from the germinal cells within the first-stage sporocyst and upon maturation, migrate to the digestive glands

and the ovotestis of the snail, where further asexual multiplica-
tion occurs. The resulting cercariae break out of the second-stage
sporocysts and migrate through the tissues and blood sinuses to
the edge of the mantle. Regeneration occurs within the second-
stage sporocysts and cercariae may be produced on a daily basis
for 1-2 months (McClelland, 1965). The developmental cycle from
miracidium to cercariae requires 4-5 weeks for S.mansoni, 5-6 weeks
for S.haematobium and 7 or more weeks for S.japonicum.

Cercariae escape from the snail mantle by an active enzymatic
process and become free-swimming organisms. The cercaria has a dis-
crete head (the future schistosomulum), to which an elongated bi-
furcated tail is attached as a locomotory organ. Upon contact with
the skin or mucus membranes of a definitive host, the head section
penetrates rapidly (within 3-10 mins for S.mansoni and as quickly as
10 sec for S.japonicum) while the tail section is shed at the skin
surface. Attachment,and penetration of the host epidermis are facilitated
by mucilagous substances from the postacetabular glands and by lytic
enzymes secreted from the preacetabular glands (Clegg and Smithers,
1968; Ghandour and Webbe, 1973).

The transition from cercaria to schistosomulum occurs in 1-2 hours
within the dermal layer of the skin. The physical process includes
loss of the cercarial glycocalyx and replacement of the trilaminate
tegumental outer membrane with a multilaminate (5-7 layers) membrane,
similar or identical to that of the adult worm (Hockley and McLaren,
1973). The organism also becomes adapted to serum and unable to

9

survive in water (Clegg and Smithers, 1968; for review see Stirewalt, 1976).

The schistosomulum remains in the dermis for 48 to 72 hours, enters the venous circulation and is passively transported through the right heart to the lungs via the pulmonary artery. Craig and Faust (1970) report that S.japonicum and S.haematobium organisms enter the venous circulation by penetrating the cutaneous capillaries. Entry via the lymphatics was reported by Standen (1953) for S.mansoni schistosomula. However, recent studies suggest the more direct route through the capillaries (Miller and Wilson, 1976). The time required to reach the lungs varies among the host species; the highest concentration of schistosomula in the lungs occurs in mice between days 4-7, depending on the strain, and on day 5 for most rat strains. No detectable growth takes place in the lungs and only a small proportion of the lung schistosomula feed on red blood cells (Clegg, 1972). However, the shape of the schistosomulum changes and the organism becomes elongated and very thin prior to penetration of the pulmonary capillaries. Between days 8-26, the schistosomulum migrates via the pulmonary vein to the left heart, the aorta, the capillaries of the mesenteric artery and finally enters the liver via the hepatic portal vein (Sadun et al., 1958; Wilks, 1967). Maturation and pairing occur in the liver. There is evidence to suggest that the presence of males is essential for the full development of the female (for review see Armstrong, 1965; Michaels, 1969). The minimum

period between successful cercarial penetration and the appearance of eggs in the faeces and urine of the definitive hosts is between 30-40 days, but can be much longer.

C. Immunology of schistosomiasis

Experimental hosts

A variety of mammals have been studied for their appropriateness as experimental hosts for the three species of schistosomes affecting man. With S.mansoni which is the species most easily maintained in the laboratory, the rhesus monkey, mouse and rat have been used extensively to study acquired host resistance. Each of these animals presents unique host responses, some of which parallel the human responses.

Much of the original research was done using rhesus monkeys, because S.mansoni infections in these animals can be manipulated to demonstrate the extremes of host response. Monkeys are readily infected with a low dose (100) of cercariae, and tolerate the maturation of the worms and subsequent egg production for many months. However, by 16 weeks the same animals develop a solid resistance to a challenge infection (Smithers and Terry, 1965). In contrast, if a rhesus monkey is heavily infected (>500 cerc.) a large proportion of the worm burden is eliminated soon after the 13th week of infection. Monkeys are being used less frequently due to expense and inconvenience in handling.

The use of mice and rats as experimental hosts has obvious advantages in that these rodents are relatively inexpensive, a convenient size, inbred strains are available and many of the immunological parameters have been determined for these animals. In mice as in

11

lightly infected monkeys, S.mansoni worms reach full maturity and produce chronic infections. Mice acquire detectable resistance to percutaneous cercarial challenge 6 weeks after exposure to small numbers (10-30) of cercariae, with maximum resistance at 12-16 weeks (Sher, Mackenzie and Smithers, 1974). Unisexual cercarial infections induce a similar level of resisistance (Sher, Mackenzie and Smithers, 1974) and immunity is not species specific (Amin and Nelson, 1969). The adult worms of the primary infection are not affected by the development of resistance to reinfection, a situation which is called concomitant immunity (Smithers and Terry, 1969). Concomitant immunity has also been demonstrated in the rhesus monkey (Smithers and Terry, 1965) and in baboons infected with S.mansoni (Taylor et al., 1973) and with S.haematobium (Webbe and James, 1973).

The response of the rat to S.mansoni is similar to that of the heavily infected rhesus monkey but in this case the worms fail to reach maturity and the majority are eliminated between 4 and 6 weeks after exposure to cercariae (Perez, Clegg and Smithers, 1974). The mechanisms of 'spontaneous cure' are not known; Cioli and Dennert (1976) have offered evidence that both immunological and non-immunological factors are involved. Perez and colleagues studying the development of resistance to challenge infections in the rat, observed that resistance peaks at 6-7 weeks after infection and then declines gradually to undetectable levels. Evidence of an anamestic protective response in animals re-exposed to low doses of cercaria suggests that resistance in rats requires constant antigenic stimulation in the form of the living adult worm. The level and time of spontaneous cure and

12

resistance to reinfection may vary among different strains of
rat (Perez, Clegg and Smithers, 1974).

Mice and rats have proved to be poor hosts for S.haematobium
infections. The hamster has been used for both S.mansoni and
S.haematobium with some success (Smithers et al., 1975) although
there are indications that the ability to develop resistance varies
with the strain of hamster (Smith and Clegg, 1976; Brink, personal
observations). The hamster remains the animal of choice for transfer
of schistosomes directly into the hepatic portal system (Brink,
personal observations; Cioli, 1976), although mice have been used
for this procedure (Blum, 1976; Boyer and Ketchum, 1976).

Mechanisms of immunopathology and modulation

The chronically infected mouse (35 cercariae yielding a mean of 3
adult worm pairs) has been shown to be an excellent experimental model
of hepatosplenic schistosomiasis mansoni (Warren, 1966). Many of the
pathophysiologic changes occuring during acute and chronic human
schistosomiasis are also encountered in murine infections (Warren
and DeWitt, 1957); therefore this model has been used to study the
etiology and histopathology of the granuloma and the resultant heptato-
splenic schistosomiasis. The egg was incriminated as the parasite
factor responsible for the pathology by studies involving unisexual,
untreated bisexual, or bisexual infections successfully treated prior
to egg layings. Warren (1961) demonstrated that a significant degree
of heptosplenomegaly, portal hypertension and oesophageal varices
were observed only in mice with bisexual infections which produced
mature viable eggs.

The granuloma surrounding an individual egg trapped in host tissue is a circumscribed avascular lesion, consisting of eosinophils, macrophages, lymphocytes, epithelioid cells and giant cells. The granulomatous lesion may be more than 100 times the volume of the egg and its subsequent fibrosis can markedly block portal blood flow.

A model for studying the aetiology of the egg granuloma has been developed. In this system, known as the 'lung granuloma model', viable eggs isolated from the livers of mice infected with S.mansoni for 8 weeks are injected into the tail veins and become entrapped in the capillaries of the lungs of the recipient mice and elicit granulomatous lesion formation (von Lichtenberg, 1962). Infected mice or those sensitized with whole eggs or antigenic fractions of eggs produce more pronounced and accelerated granuloma formation (Warren, Domingo and Cowan, 1967). Sensitization for granuloma formation by intraperitoneal injection of eggs has been correlated with in vivo and in vitro parameters of delayed hypersensitivity (Boros and Warren, 1970; Colley, 1971; Colley, 1972). Sensitization can be transferred with lymphoid cells but not with serum (Warren et al., 1967) and is inhibited by immunosuppressive drugs (Warren, 1973) neonatal thymectomy (Domingo and Warren, 1967) and antilymphocytic serum (Domingo and Warren, 1968). These characteristics suggest that this type of granuloma formation is a result of cell-mediated, delayed type hypersensitivity response to eggs.

Additional evidence suggests that although antigen and antibody can be demonstrated in the granuloma lesions (Andrade et al., 1961;

von Lichtenberg et al., 1966) antibody mediated inflammation plays
little or no role in the development of the S.mansoni granuloma (see
Warren, 1976). However, the role of antigen antibody complexes
in the pathogenesis of granuloma formation may differ in S.mansoni
and S.japonicum infections. The Hoeppli phenomenon, an antigen-
antibody reaction surrounding the egg, is observed more frequently,
and polymorphonuclear leucocytes are often present in larger numbers
in the S.japonicum granuloma (von Lichtenberg et al., 1966; von
Lichtenberg et al., 1971). Warren et al., (1975) suggest that the
highly localized concentration of antigen which results from the
large aggregates of eggs in S.japonicum predisposes the host reac-
tion toward that of an Arthus-like phenomenon. Many of the questions
concerning the S.japonicum granuloma should be more easily studied
with the recently developed S.japonicum infected rabbit model. This
model appears to parallel the human disease very closely: the in-
fected rabbit develops large granulomas with the Hoeppli phenomenon,
diffuse portal fibrosis simulating pipe stem lesions, as well as
nephropathy between 2½ and 4 months after infection (von Lichtenberg,
1972). Furthermore, the infection is now being standardized in a
mini-rabbit, which will make the use of this model more realistic
for extensive studies (von Lichtenberg, personal communication).

Although antibody does not appear to be necessary for the forma-
tion of the S.mansoni egg granuloma, the occurrence of spontaneous
diminution in the size of the granulomas in chronic infections suggests
that humoral mechanisms may be involved in the suppression of the

cell-mediated response (Domingo and Warren, 1967). Boros, Pelley and Warren (1975) have used the lung granuloma model to confirm the modulation of granulomatous hypersensitivity and have demonstrated increased circulating antibody coincident with modulation and decreased spleen cell responsiveness to soluble egg antigen (SEA). However, Colley (1975) has shown that adoptive transfer of lymphoid cells (either lymph node or spleen cells) from chronically infected mice to syngeneic mice in the early stages of infection suppresses granuloma formation, actively and effectively. Passive transfer of serum under the same conditions has no effect upon on-going granuloma formation.

Further physical and chemical characteristics of SEA have been demonstrated by Boros and Warren (1970) and Pelley et al., (1977). The antigenicity of SEA can be destroyed by trypsin and RNAase, but DNAase has no effect. SEA is stable for 2 hours at 23° and 37°C, but activity is destroyed at 56°C. Disc electrophoresis of SEA reveals 8 to 9 protein bands and preparative zone electrophoresis on Pevikon demonstrates that the activity is contained in two fractions eluted from the anodic area. Molecular sieving on Sephadex columns suggests that the molecular weights of the antigenic moieties are in the range of approximately 10,000 daltons. It has also been shown that the activities responsible for the induction of delayed hypersensitivity bind to a Sepharose-ConA affinity column and can be eluted with alpha-methylmannoside, suggesting that the active moieties contain carbohydrate.

SEA has also recently been extracted from S.haematobium and S.japonicum eggs by homogenization and ultracentrifugation. S.haematobium SEA elicits moderate delayed footpad swelling in mice sensitized by intraperitoneal injection of S.h. eggs. In contrast, S.japonicum SEA elicits massive immediate footpad swelling but no delayed reactions in mice infected for 5-10 weeks.

Innate resistance

Innate resistance to schistosomiasis has been related mainly to the host skin barrier. There appears to be no selectivity in the penetration of the epidermis of mammals by the cercariae; the numbers of cercariae penetrating the skin of hamsters, mice, guinea pigs, rabbits and rats are almost identical (Warren and Peters, 1967). However, Clegg and Smithers (1968) showed that up to 50% of the cercariae which enter the skin of rats die shortly afterwards, whereas about 30% die in mouse skin and only 10% in hamster skin. The deaths occur within 10 minutes of penetration, when the cercariae are still within the epidermis. After 15 minutes many of the cercariae have penetrated the Malpighian layer and entered the dermis where few deaths occur.

Increased host resistance with age has been correlated with death of cercariae in the skin by Ghandour and Webbe (1973). They showed that the level of cercarial mortality in the skin of 2 day old mice is less than one third the mortality in adult skin. During the first month of life, the number of cercariae dying in the skin rises steadily and reaches the adult level when the mouse is 28-35

days old.

Skin penetration is largely dependent upon cercarial enzymes
(Lewert, 1958) acting on the acellular, non-fibrillar ground sub-
stance of the dermis and the subepithelial basement membrane.
Lewert and Mandlowitz (1963) suggested that age resistance in mice
is due to older skin being more resistant to enzyme attack than younger
skin. This explanation was strengthened by the demonstration that
old mice of the LAF strain, which age slowly and have connective
tissue characteristic of much younger mice, are as susceptible as
CF strain mice less than 1 month old. Smithers (1976) therefore
proposed that cercarial death in the epidermis is most likely related
to exhaustion of energy reserved during the period of intense activity
in the early phase of penetration. He suggested that more energy
was required to penetrate the skin of older mice and consequently
fewer cercariae survived compared to cercarial recoveries from skin
of very young mice.

Lower energy reserves may also account for Oliver's (1966)
observation of decreased infectivity with increased ageing of the
cercariae. Glycogen is reduced to about a quarter of the initial
value in S.mansoni cercariae which have been swimming in water for
18 hours (Bruce et al., 1969). Ghandour and Webbe (1973) demonstrated
that the age of cercariae after emergence from the snail is directly
related to the number which die in the skin. In mouse skin,
approximately 30% of 2 hour-old cercariae die, but as the cercariae
age the percentage dying rises steadily and after 8 and 18 hours

18

mortality is almost doubled and trebled respectively. Further-
more, Ghandour has shown that cercarial mortality within mouse
skin is approximately halved if the snail hosts are maintained
in a 2% glucose solution for 48 hours prior to shedding.

The skin barrier is the most obvious and best understood
example of innate resistance. However, other examples exist.
Purnell (1966) found that male hamsters and mice were more suscep-
tible to S.mansoni than female animals; castration of mice or injec-
tion of testosterone lowers the survival rate of female worms (Berg,
1957); and Robinson (1960) showed that massive doses of stilbesterol
in mice cause a delay in maturation of S.mansoni and a reduction
in the length of male worms.

Recent studies have demonstrated a possible correlation be-
tween natural resistance and the inflammatory response of various
experimental hosts. von Lichtenberg (personal communication)
has used "the schistosomula lung model" in which schistosomula are
injected in the tail vein, collect in the lungs and evoke a granulo-
cytic reaction (as described below), to show that primary lung
responses parallel host species susceptibility: hamsters show the
least cell reaction in the lung and are the most susceptible to
infection; mice are intermediate in both parameters, and rats have
the most cell reaction and the lowest susceptibility of the three
animals studied.

Mechanisms of Acquired Resistance

Stimulation of host response. The 1974 report of the World Health
Organization stated that the general failure to provoke immunity by

injecting non-living parasite homogenates has inhibited progress towards identifying the antigenic stimulus to immunity, and our understanding of the stimulation of immunity has necessarily been confined to the living parasite (WHO, 1974). Today the situation remains unchanged.

During the course of infection, the host is exposed to various schistosome stages, each of which could stimulate the immune system by their intrinsic antigens. Possible antigens include: secretions, especially the histolytic secretions of the penetrating cercariae and the escaping egg; digestive enzymes and excretory products of the adult worm; proteins released during turnover of the tegument; and the breakdown products of any stages that, for various reasons, die within the host.

The antigens of the egg elicit both humoral and cellular host responses and, in fact, the immunopathology of schistosomiasis is due to these reactions. However, there is convincing evidence that the egg plays no role in stimulating a protective response. Unisexual infections do not lead to egg production, but do induce resistance in rhesus monkeys (Smithers, 1962). Similar findings in mice following unisexual infections have been reported by Olivier and Schneiderman (1953) and Lin, Ritchie and Hunter (1954). Furthermore, heterologous immunity can be induced by unisexual infections; Amin and Nelson (1969) found that an all male infection with S.mattheei protected mice against S.mansoni challenge. The injection of viable eggs into the circulation also fails to stimulate resistance to S.mansoni in mice (von Lichtenberg et al., 1963; Moore et al.,

1963) and in monkeys (Smithers, 1962). This is true even when as many as half a million eggs are injected directly into the mesenteric veins of rhesus monkeys (Smithers and Terry, 1967). However, Kagan (1952) has presented evidence that eggs may stimulate immunity in mice infected with S.douthitti.

The schistosomula can elicit an immune response; evidence for this is based on infectionswith irradiated (2000 R) cercariae which do not mature past the schistosomula stage. Several exposures to large numbers of irradiated cercariae are necessary and usually only a partial immunity can be demonstrated in rhesus monkeys and mice (WHO, 1974). However, Smithers and Terry (1965) suggest that irradiated cercariae are as immunogenic as normal cercariae in the rat.

The adult worm is considered to be the most important source of immunogenic stimulation, although the specific antigens which elicit the immune response have not been isolated. Smithers and Terry (1967) surgically transferred adult worms into the hepatic portal systems of normal monkeys, thereby establishing adult infections without exposing the monkeys to the earlier schistosome stages. Monkeys receiving as few as 80 pairs of worms were almost completely resistant to a challenge infection 8-14 weeks later. Immunity could not be induced if the worms were killed by snap-freezing immediately before transfer.

In vivo studies.   It is generally accepted that in rodents
the effector mechanisms of acquired resistance are directed against
the young schistosomula. This view is supported by observations
of decreased schistosomula recoveries from the lungs of immune mice
(Sher, Mackenzie and Smithers, 1974), rats (Perez, Clegg and
Smithers, 1974) and hamsters (Smith et al., 1975) following cercarial
challenge. Several investigations have demonstrated that a humoral
component is involved in acquired resistance in rodent models.
Approximately 50% of the capacity to resist infection after cercarial
challenge can be passively transferred with serum from immune mice
(Sher, Smithers and Mackenzie, 1975) and slightly less with serum
from immune rats (Perez, 1974; Phillips et al., 1975). The serum
factor involved in passive transfer is an antibody of the IgG class
(Sher et al., 1977; Perez, 1974; Phillips, Reid and Sadun, 1977).
However, resistance cannot be transferred if the schistosomula have
been established in the recipients for 3-4 days;   this is further
evidence that resistance is directed at the early schistosomula
stage (Sher, Smithers and Mackenzie, 1975; Phillips, Reid and Sadun,
1977).

Phillips and colleagues (1975) studied the adoptive transfer
of protective resistance with thymus derived lymphocytes in rats.
They observed that the initial protective response of the lympho-
cytes could be blocked by the simultaneous administration of serum
from rats infected for four weeks. Perez (1974) suggested that
intravenous injection of normal bone marrow cells would reconstitute
immunity in irradiated rats;   she was not able to transfer pro-

tection to irradiated recipients with immune serum.

Recently, von Lichtenberg, Sher and McIntyre (1976) suggested
a new experimental model for characterizing the kinetics of acquired
resistance, and the specific schistosome stage affected by the host's
immune response. This technique, known as "the schistosomula lung
model", was first used by Blum and Cioli (1975) for studying varia-
tions in schistosomula migration patterns. It involves the injection
of _in vitro_ prepared schistosomula into the tail veins of mice and
their subsequent entrappment in the lungs. Injected schistosomula
evoke granulocytic reactions in much greater number and at a faster
rate in lung tissue of immune rather than normal mice. Maximal
parasite destruction is evident at 24 hrs in immune animals, versus
6 days in the unsensitized host. Furthermore, the cellular composi-
tion of the granulocytic reaction is different in the two types of
animals; it is primarily neutrophilic in normal mice but is heavily
enriched with eosinophils in immune animals. A point of interest
in the schistosomula lung model is that when schistosomula are pre-
cultured _in vitro_ for 24-44 hrs prior to injection, both the immune
cellular reaction and immunity as measured by adult worm perfusion
are reduced. Similarly, schistosomula injected approximately 30 hrs
prior to passive transfer of serum are refractory to immune attack.
This evidence again suggests that schistosomula become progressively
less susceptible to the host's immune response, regardless of
whether their maturation is accomplished _in vivo_ or _in vitro_.

The schistosomula lung model has been used to confirm the involve-

23

ment of both humoral and cellular components in acquired resistance in mice. Significant immunity to injected schistosomula can be passively transferred with immune serum or restored to irradiated recipients of immune serum by reconstitution with normal bone marrow cells. Furthermore, studies using nude mice have indicated that immunity to injected schistosomula is thymus dependent and that it is the humoral component rather than the cellular element which is thymus regulated (Sher, McIntyre and von Lichtenberg, 1977).

In addition to the humoral and cellular components necessary for destruction of schistosomula in the immune mouse lung model, it has been suggested that 5-hydroxytryptamine (serotonin) may be required. Sher. et al., (1977) have shown that immune mice pretreated with the monoamine-depleting drug reserpine do not respond to injected schistosomula. The effect of reserpine can be reversed if the animals are simultaneously treated with a monoamine oxidase inhibitor, pargyline.

Histological evidence suggests that the eosinophil is the most likely effector cell of the immune response (von Lichtenberg et al., 1976; Hsu et al., 1975). Eosinophilic inflammatory responses have been observed both in the skin (Hsu et al., 1975; von Lichtenberg et al., 1976) in response to cercarial challenge, and in the lungs in response to intravenous schistosomula challenge (von Lichtenberg, Sher and McIntyre, 1977). In the immune mouse schistosomula lung model, eosinophil degranulation is frequently observed at the surface of parasites, in addition to eosinophil invasion of damaged organisms.

Further evidence for the role of the eosinophil as the effector cell was offered by Mahmoud et al.,(1975) who treated immune mice with a variety of antisera raised in rabbits against different mouse leukocytes. Only the anti-eosinophil antiserum was effective in suppressing resistance to reinfection.

To summarize, the evidence from the passive and adoptive transfers and the irradiation and reconstitution experiments described above strongly suggests that the effector mechanisms of acquired resistance in rodents are thymus regulated, antibody dependent and cell mediated. The antibody appears to be of the IgG_1 subclass and the cell most likely to be involved is the eosinophil.

In vitro studies. As described above, in vitro experimentation has supported the hypothesis that the host immune response is directed at the young schistosomula of a challenge infection. Most of these studies have used in vitro prepared schistosomula as the test organism and damage has been assessed by microscopical examination or by ^{51}Chromium (^{51}Cr) release. Microscopically, death of the schistosomula is associated with loss of motility and a granular, opaque appearance. Dead organisms fail to exclude eosin, Evans blue or fluorescein conjugated antibody. Living schistosomula labelled with ^{51}Cr retain most of their label during culture; damaged organisms lose up to 60% of the isotope (Butterworth et al., 1974).

A number of in vitro systems have been used to study antibody mediated killing or damage of schistosomula. However, the presence

25

of these antibodies does not correlate with the development of protective immunity, suggesting that other factors, such as cells and complement must be required in vivo.

Clegg and Smithers (1972) cultured young schistosomula in immune monkey serum and observed that the organisms were killed within 4 days. The lethal activity of the serum was present in the IgG fraction and was dependent on labile factors in normal serum, probably components of complement. Lethal antibody has also been demonstrated in the sera of infected rats (Perez et al., 1974), and guinea pigs (Dean and Wistar, 1973) and in sera from human schistosomiasis cases (Smith and Webbe, 1974). The surface of the schistosomula is damaged in the presence of lethal antibody and complement (McLaren et al, 1975). Lethal antibody can be absorbed from immune sera with adult schistosome surface membranes, and these membrane preparations induce high levels of lethal antibody but do not confer immunity when injected into rats (Sher et al., 1974). Perez et al., (1974) have also observed that the presence of lethal antibody alone does not correlate with immunity in rats; high levels of lethal antibody are present in rats when immunity has diminished.

A combination of IgG antibody, complement and rat neutrophils rapidly kills schistosomula, probably by the release of cellular lysosomal enzymes onto the schistosomular surface. In vitro killing in this system does not appear to correlate with immunity, and the antibody titre remains high while resistance in the rats

declines (Dean, Wistar and Murrell, 1974; Dean, Wistar, and
Chen, 1975).

Capron et al., (1975) have observed that in the presence of
fresh immune serum, macrophages will adhere to and damage young
schistosomula. The serum activity can be abolished by heating
at $56^{\circ}C$ and is not restored by complement. The activity can be
removed with an anti-IgE immuno-absorbant column but not with anti-
IgG_1, IgG_{2a}, IgG_{2b}, IgM or IgA. This is the first demonstration
of macrophages being sensitized by IgE. An IgE enriched fraction
of immune rat serum, however, failed to transfer immunity when ad-
ministered to normal rats (Smithers and Perez, unpublished results).

Recently several in vitro systems have been reported in which
the development of the antibody can be correlated with protection.
Perez (1974) sensitized normal rat peritoneal exudate cells with heat-
inactivated immune rat serum and observed that the washed cells
caused damage to the surface of schistosomula. In this system the
effector cell is a macrophage and presence of the IgG antibody parallels
the evolution and decline of immunity in the rat.

In an. in vitro killing assay employing human cells and anti-
bodies, normal human leukocytes cooperate with IgG antibodies from
schistosomiasis patients in mediating damage to schistosomula as
measured by ^{51}Cr release (Butterworth et al., 1974; 1975; 1977).
The effector cells of this reaction have been purified and are
eosinophils (Butterworth, 1977). Ultrastructural studies have
demonstrated that eosinophils adhering to the target schistosomula
discharge their granule contents directly onto the worm surface

(Butterworth, 1972). The reaction of human cells with schisto-
somula can be inhibited with drugs which block microtubule function
(cytochalasin A and B) or glycolysis (sodium fluoride or 2-deoxy-
glucose), raise cyclic AMP levels (aminophilline) or inhibit esterase
activity (tosyl-lysl-chloromethyl ketone) (David et al., 1977).

An IgG fraction in serum of infected rats and mice is responsible
for eosinophil adherence to schistosomula and stimulates these cells
to secrete peroxidase onto the parasite surface (Mackenzie et al.,
1977; McLaren, Mackenzie and Ramalho-Pinto, 1977). The develop-
ment of this antibody correlates closely with the development of
protective immunity; and when protection in the rat declines, the
titre of antibody also declines.

D. Parasite Evasion of Host Resistance

As described above, an important feature of acquired immunity
in experimental hosts is that established adult worms from the initial
infection persist long after resistance has developed against a
challenge infection (Smithers and Terry, 1972). Furthermore, the
major stimulus for the development of resistance is provided by the
adult worms, although they remain unaffected by the immune mechanism.

Evidence has been presented which suggests that the surface of
the young schistosomulum is the target of immune attack. Therefore
evasion of the immune response must involve changes in the surface.
Recent studies indicate that these changes occur rapidly (within
30 hours after penetration), and certainly by the time the schisto-
somula reach the lungs (Clegg and Smithers, 1972; Goldring et al.,

1977; von Lichtenberg, Sher and McIntyre, 1977).

Initially the schistosomulum is immunogenic, it induces high levels of helper T cell activity (Ramalho-Pinto, De Souza and Playfair, 1976) and promotes a variety of antibody responses (Clegg and Smithers, 1972; Capron et al., 1975; Mackenzie et al., 1977). As the schistosomulum develops, it is no longer recognized as foreign (Clegg and Smithers, 1972; Smithers, McLaren and Ramalho-Pinto, 1977), nor is it susceptible to immune attack (Clegg and Smithers, 1972; Sher, 1977). These adaptations occur concurrently with 1) loss of capacity to activate complement (Ramalho-Pinto, pers comm.) 2) the acquisition of host antigens (Goldring et al., 1977), 3) a redistribution of intramembraneous particles in the surface membrane (McLaren et al., 1977), 4) an apparent loss of inclusion bodies associated with membrane turnover (McLaren et al., 1977) and 5) migration from the skin to the lungs (Smithers, 1977). Thus it would appear that some mechanism for masking surface antigens has occurred.

Several mechanisms have been suggested which could enable the later schistosomula stages and adult worms to circumvent the effects of host resistance.

The existence of shared antigens between schistosomes and their hosts has been demonstrated by immuno-electrophoretic techniques. Capron et al., (1965) demonstrated the existence of four common antigens between S.mansoni and their hamster hosts. They also isolated five cercarial antigens which were immuno-precipitated by

a rabbit antiserum produced against Biomphalaria glabrata, the
intermediate snail host. Damian (1967) raised an antiserum against
adult S.mansoni worms and established by immuno-diffusion the existence
of at least four common antigens between S.mansoni and uninfected
mouse serum. All workers agreed with Sprent's original hypothesis
(Sprent, 1959) that the host-like antigens have evolved during the
process of adaptation to the host. This theory, however, does not
allow for the fact that schistosomes are equally well adapted to a
variety of host species.

Further evidence that shared antigens are of parasite origin
has been provided by Damian and his colleagues. Kemp and Damian
(1974) describe an antigen which cross reacts with mouse alpha 2
macroglobulin (α2M) and is present on the surface of adult worms
grown in rhesus monkeys as well as on those grown in mice. Since
primate α2M does not cross react with mouse α2M, the authors suggest
that this antigen is of parasite origin and has evolved as a result of
long contact between host and parasite (Damian, Green and Hubbard,
1973). However, Clegg (1974) states that since S.mansoni is mainly a
parasite of man the evolution of a primate α2M would appear more
likely.

Smithers, Terry and Hockley (1969) have suggested that antigens
synthesized by the host are incorporated into the surface membrane
of the schistosome. They transferred adult worms from mice into the
hepatic portal systems of monkeys immunized against mouse erythrocytes
and observed that most of the worms die within 24 hours and all were

30

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synthesized by the host are incorporated into the surface membrane
of the schistosome. They transferred adult worms from mice into the
hepatic portal systems of monkeys immunized against mouse erythrocytes
and observed that most of the worms die within 24 hours and all were

dead by 48 hours although "mouse" worms survive well in normal monkeys.
Further studies have revealed that the lethal effect is species specific
and can be passively transferred with serum. Electron microscopy
has shown that worm death is caused by rapid destruction of the sur-
face membrane while other tissues are unaffected.

The acquisition of host antigens appears to be a gradual process
both in vivo and in vitro. Worm transfers of schistosomula grown
for 15 days in mice showed that these schistosomula possess host
antigens while only a proportion of 7 day worms and no cercariae
possess them. The antigens could not be removed by extensive
washing nor did they represent an IgG contaminant obtained from the
mouse serum.   Similarly, schistosomula cultured for 15 days in a medium
containing human serum and erythrocytes were destroyed when trans-
ferred into monkeys immunized against human erythrocytes.   Appropriate
control experiments using blood of A Rh-positive and B Rh-negative
types demonstrated that the schistosomula acquired antigens which
were common to the two blood types and possibly acquired, to a
lesser degree, specific blood group antigens (Clegg, Smithers and
Terry, 1970: Clegg, Smithers and Terry, 1971).

The host antigen phenomenon has also been demonstrated by use
of other animal systems. In order to compare the acquisition of
host antigens, Cioli and Neis (1972) transferred worms grown in mice
and rats (Cioli, 1976) into hamsters immunized against mouse or rat
erythrocytes. The results of the mouse to hamster transfers were
similar to those observed by Clegg et al., (1971); destruction

31

was specific and could be transferred with serum. In contrast, although small amounts of rat antigen could be detected, rat worms were not rejected by anti-rat-hamsters. The possession of rat erythrocyte antigens by worms grown in rats has also been demonstrated by Perez (1974).

Recent in vitro work on the host-antigen phenomenon has indicated that glycolipids or megalolipids are acquired from the serum of the host and inserted into the schistosomula surface. The acquisition of A and B antigenic specificities were first demonstrated indirectly by Clegg, Smithers and Terry (1971) when they cultured schistosomula in medium containing blood group antigens. Subsequently, Dean (1974) has shown that schistosomula cultured in the presence of human erythrocytes express both the A and B antigens but not the H antigen. He also showed that the schistosomula did not express the rhesus antigens, the M, N, Duffy[a] or P antigen when grown in the presence of erythrocytes positive for these antigens. In addition when Dean cultured schistosomula in medium containing an alcoholic extract of erythrocyte membranes he could show that the worms became A blood group antigen positive. He was not able to show this with a glycoprotein of A specificity.

Dean's results provide the first documented evidence that schistosomes can acquire blood group antigens which are likely to be glycolipid in nature. The original suggestion, however, that host antigens were likely to be glycolipids was made by Clegg (1972),

who envisaged a situation where the hydrophobic ceramide portion of a glycolipid would insert between other lipid molecules on the schistosome surface. The antigenicity of the glycolipid would be due to the carbohydrate moiety, which would differ in sugar chain lengths, and combinations of sugars (glucose, galactose, fucose, n-acetyl glucosamine, n-acetyl-galactosamine and sialic acid).

Recent studies by Goldring (1976) have confirmed the results of Dean (1974) and have offered further evidence that only specific blood group glycolipids are acquired by schistosomula during in vitro cultivation. His studies indicated that schistosomula did not acquire erythrocyte glycoproteins; nor did they acquire or synthesize intermediate compounds associated with blood group glycolipids. He therefore supports the acquisitive blood group theory.

INTRODUCTION

An important feature of immunity to S.mansoni infections in experimental hosts, and probably in humans, is that established adult worms from the initial infection persist long after resistance has developed against a challenge infection. This form of acquired resistance induced by an infection which survives the host response has been termed'concomitant immunity' by analogy with a similar situation occuring in tumour bearing animals (Smithers and Terry, 1969). Evidence has been presented that the adult worms survive the immunity they induce by 'disguising' themselves in a coat of host molecules (Smithers, Terry and Hockley, 1969; Clegg, Smithers and Terry, 1971; McLaren, Clegg and Smithers, 1975; Goldring, Clegg and Smithers, 1976). It is assumed then that the targets of concomitant immunity are the early schistosomula before they have acquired their host-like disguise (Smithers and Terry, 1976). This hypothesis has been supported by experiments demonstrating a reduction in the recovery of young schistosomula of challenge infections from the lungs of immune animals (Perez, Clegg and Smithers, 1974; Sher, Mackenzie, Smithers, 1974; Smith and Clegg, 1976). Furthermore, the host reponse appears, in most instances, to be directed at the surface of young schistosomula.

Ultrastructural studies have shown that the surface of the schistosomula is damaged when the organisms are cultured in vitro either with immune serum and complement (McLaren, Clegg and Smithers, 1975) or with immune serum and eosinophils (Mackenzie

et al., 1977). Von Lichtenberg and colleagues have offered histological
evidence for in vivo surface damage to the schistosomula at the
skin stage (von Lichtenberg et al., 1976) and in the lungs (von
Lichtenberg, Sher and McIntyre, 1977).

Evidence suggests that the target antigens of the immune
responses are associated with the tegument of young schistosomula,
and that by some mechanism these antigens are disguised as the
organism develops. Therefore a comparative biochemical and
immunological study of the schistosomes at different stages of
development seemed timely.

Previous studies had used schistosomula produced by allowing
cercariae to penetrate isolated rodent skin in vitro (Clegg and
Smithers, 1972). Hockley and McLaren (1973) found that identical
structural and physiological changes took place on the same time
scale with both in vivo and in vitro skin penetrated schistosomula.
Furthermore, other parameters, including water and serum sen-
sitivities, evacuation of penetration glands and loss of
cercarial tail have been reviewed by Stirewalt (1976) and found
to be very similar. However, the skin penetration technique
is a time consuming procedure giving low yields of organisms
possibly contaminated by host material from the skin used in
the technique.

In order to obtain the quantities and type of schistosomula
needed for these immunological and biochemical studies, it
was necessary to use a more efficient technique. I therefore
decided to examine two recently reported procedures for

artificially transforming cercariae to schistosomula. In
this chapter, the tegumental ultrastructure, the development
and the antigenicity of these artificially prepared schistosomula
are compared with those of schistosomula which have transformed
after penetrating isolated mouse skin.   Their viability and
development in vivo and in vitro are also compared and their
use as reference organisms is justified.

MATERIALS AND METHODS

1. Maintenance of the Experimental Model

The Parasite. A Puerto Rican (NIH) strain of S.mansoni
maintained in Biomphalaria glabrata snails and Parkes or CBA
mice was used throughout this study. The parasite, snail and
rodents have been described in detail by Smithers and Terry (1965).

Cercariae. Snails were infected by individual exposure to
10 miracidia overnight at 27°C. 5-10 weeks later cercariae
were stimulated to escape from the infected snails by placing the
snails in 25 ml dechlorinated water in bright light. The free-
swimming cercariae were always used within 2-4 hours.

The Hosts.

Outbred male Parkes mice - 20 g

Inbred female CBA mice - 18-20 g

The mice were anaesthetized by an intraperitoneal injection
of Nembutal (Abbot Laboratories, 60 mg/ml). The Nembutol was diluted
1:10 vol/vol with 10% ethanol and injected to a level of 0.1 ml/10g
body weight. Mice remained anaesthetized for approximately 1 hour.
The fur was clipped from the lower abdomens and each animal was

placed on its back between wooden strips set $\simeq$ 3 cm apart.
Each bare abdomen was moistened with wet cotton wool and a
heavy nickel-plated brass ring was placed in position. The
rings had the following dimensions: I.D. 1.3 cm., depth, 2.0 cm
weight 12 g and volume held 1.2 ml.

The number of cercariae was estimated by diluting an aliquot
of the cercarial suspension with Lugol's iodine and counting the
stained cercariae under a dissecting microscope. The suspension
was carefully mixed and a volume containing the required number
of cercariae was placed in the ring. Animals were exposed to the
infecting dose of cercariae for 10 minutes, after which the
suspension and ring were removed and the animal was allowed to
recover from the anaesthetic.

Preparation of miracidia. Intestines (segment between
stomach and caecum) were removed from 10 Parkes mice which had
been infected 7 weeks previously with 150 cercariae. The intestines
were rinsed with Sorensons buffer, pH 8.0, cut into small pieces
and homogenized in 20 ml of this buffer for 2 minutes. 1 mg
crystallized trypsin per intestine was added and the homogenates
incubated at 37°C for 2 hours. Following the digestion, the
material was poured through 300 and 180 micron sieves, rinsed with
saline and centrifuged at 1000 rpm for 2 minutes. The saline wash
was repeated 4 times to obtain a clean pellet of schistosome eggs.
At this point the eggs can be stored in Tyrodes solution at $+2^{\circ}$C
for up to 2 weeks prior to hatching as described below. The eggs
were placed in dechlorinated water under a strong light for 1 hr;

the miracidia which hatched during this period were then used
to infect the snails.

2. Preparation of the various parasite stages

Cercariae were collected as described in Section 1. Approx-
imately 1 mg/ml sodium benzylpenicillin and 1000 units/ml
streptomycin were added dropwise to the collection water which
caused the cercariae to concentrate in the upper third, while the
snail faeces and debris settled to the bottom. The concentrated
cercariae were then processed aseptically in a positive flow
sterile hood in the following ways:

Cercariae. An aliquot of the concentrated cercarial suspen-
sion was diluted with sterile, dechlorinated water and kept at room
temperature until use.

Mechanical schistosomula (MS). Cercariae were immobilized by
chilling in an ice-bath for 10 minutes and further concentrated
by centrifuging for 15 seconds at 1000 rev/min in a bench model
centrifuge. The supernatant fluid was discarded and the pellet
gently resuspended and washed once in cold dechlorinated water. The
cercarial pellet was resuspended in 2 ml cold water and shaken
vigorously for 60 seconds on a Vortex Jr mixer. Five ml of Hanks
basal salt solution containing 20 mM Hepes buffer((4-2-hydroxyethyl)-
piperazine-ethanosulphonic acid), pH 7.3 (Hanks) were immediately
added and the suspension was shaken for an additional 60 seconds
to break the tails from the bodies as described by Gazzinelli et al.,
(1973), Howells et al., (1974) and Brink, McLaren and Smithers (1977).

38

One to two ml aliquots of the suspension were then layered onto
30 ml aliquots of Hanks in sterile conical centrifuge tubes and
the bodies were allowed to sediment for 10 minutes at 22°C. After
a second sedimentation, the cercarial bodies were layered onto
Earle's saline containing 0.5% lactalbumin hydrolysate, 100 µg/ml
sodium benzyl penicillin and 100 units/ml streptomycin (ELac).
This third sedimentation contained cercarial bodies with a 1-4%
contamination of cercarial tails. Approximately 2000-3000 bodies/
ml were then incubated at 37°C for various times in ELac, pH 7.4.

The supernatants which contained 40% bodies/60% tails were
concentrated by centrifuging for 15 seconds at 1000 rpm. The pellets
were gently resuspended in Hanks and 3 ml aliquots were layered
onto 20 ml onto 20 ml of a 6% Ficol/11.25% sodium metrizoate solution mixed
just prior to use, and centrifuged for 3 minutes at 3000 rpm. The
tails, which concentrated at the interface, and the bodies, which
formed a pellet, were removed and washed 3 times with ELac or Hanks.
These preparations were then processed in various ways depending on
the nature of the experiments. Alternatively, 20 ml continuous
gradients of 10 to 30% Metrizamide dissolved in Hanks were used to
separate the tails from the bodes; with these gradients the bodies
and tails were collected as two separate bands at approximately the
25 and 17% Metrizamide levels. These fractions were collected and
processed as described above.

Rat serum schistosomula (RS). Cercariae were incubated at 37°C
at a concentration of ≈ 1000 organisms/ml in a medium of 50% fresh

rat serum in Eagle's balanced saline plus 20 mM Hepes, pH 7.4, as described by Eveland and Morse (1975).

Skin-penetrated schistosomula (SS). Cercariae were allowed to penetrate through isolated mouse abdominal skins according to the procedure described by Clegg and Smithers (1972). Adult male Parkes mice, weighing 30 g, were killed with chloroform and the fur clipped from the abdominal skin. An area of skin approximately 2 cm^2 was excised and the dermal tissue removed by virorous rubbing with gauze pads soaked in Hanks. The thinned skin was mounted in a glass penetration apparatus as described by Clegg and Smithers (1972). The lower tube was filled with Elac and the prepared skin was placed dermal side down over the Elac to form an air free junction. The upper tube was clamped in position over the skin and 2-4 ml of dechlorinated water were added to this tube. The penetration apparatus was placed in a 37°C water bath so that only the lower tube was warmed. The water in the upper tube was replaced by 2-3 ml cercarial suspension (≈2000-4000 cerc/2 ml). The schistosomula were recovered from the Elac in the lower tube at various times and washed in sterile Elac prior to further use. The ages of the schistosomula in the various experiments were measured from the time the cercariae were placed in the upper tube.

3. Technical Procedures

Gland staining. Purpurin (1,2,4-trihydroxy-anthraquinone) was used to detect the calcium-rich contents of the preacetabular glands of cercariae and schistosomula. Suspensions of the

organisms were mixed with an equal volume of a saturated solution of purpurin in 95% ethanol. After 2-3 min, the organisms were washed once with 50% ethanol and mounted on slides.

Ultrastructural studies. The various schistosomula were collected at intervals after the preparative procedures and processed for electron microscopy (Brink et al., 1977). The specimens were immediately placed in ice-cold 2% glutaraldehyde in sodium cacodylate buffer, pH 7.4 for 2 hours (Lewis and Shute, 1966). The schistosomula were then post-fixed in 1% osmium tetroxide in Millonigs buffer (Millonig, 1961) at pH 7.4 for 1 hour at 4°C, washed x 3 in distilled water and treated with an 0.5% aqueous solution of uranyl acetate containing 45 mg/ml sucrose (de Harven, 1967) pH 5.0, for 1 hour at 4°C. After dehydration in alcohol the schistosomula were embedded in Araldite and sectioned using an LKB ultramicrotome and a diamond knife. Sections were collected on naked 200 mesh copper grids and stained with both uranyl acetate (Gibbons and Grimstone, 1960) and lead citrate (Venable and Coggeshall, 1965) before examination in a Philips 300 electron microscope.

Viability in vitro (Culture). Suspensions of the different schistosomula preparations were adjusted to ≈200 organisms/0.1 ml and transferred to Leighton culture tubes containing 2 ml of Elac or RPMI-1640 supplemented with 50%(vol/vol) human serum of various blood types. The tubes were gassed with 8% CO_2/air (British Oxygen Company) to pH 7.4, sealed and incubated at 37°C.

Larger quantities of organisms (2000-5000/5-10 ml) were cultured
in Falcon plastic culture flasks under the standard conditions.

Modifications to the culture procedure included addition of
2% washed homologous erythrocytes, change of medium every 4th
day and substitution of heat-inactivated foetal calf serum for the
human serum. The age of the schistosomula was measured from the
first day of cultivation.

Viability in vivo (Infectivity). The infectivity of the various
schistosomula (MS, RS and SS) was tested against that of a
standard cercarial infection. Groups of 12 female CBA mice were
exposed to cercariae or injected intravenously with schistosomula
in 0.2 ml Elac containing 1% heat inactivated CBA serum.

Two mice from each group were killed 5 days post-infection
(or injection) and the lungs were examined for viable schisto-
somula as described in Chapter 4, Materials - Preparation of Lung
Forms.

Six weeks after infection (or injection) the hepatic portal
systems were perfused as described in Chapter 4, Materials -
Recovery of Adult Worms, to determine the number of male and fe-
male worms. The livers were examined for granuloma and additional
adult worms.

RESULTS

Preparation of schistosomula

The preparation times and yields of schistosomula were compared
for each of the three methods. The skin penetration technique was

42

the slowest because of the preparation of the mouse skins, and
only approximately 20-30% of the original cercariae were recovered
as schistosomula. The yields of RS and MS were higher (between
50-70%). Of these two procedures, the preparation of MS was more
time-consuming because of the three sedimentations necessary to
separate bodies from tails.

In preparing MS, it had been demonstrated previously that the
cercarial bodies produced after vortex treatment and sedimentation
in cold Hanks were contaminated with less than 5% cercarial tails.
(Ramalho-Pinto et al., 1974). Here, we show that sedimentation in
Hanks at room temperature followed by a third sedimentation in Elac
resulted in a similar or slightly lower (3-5%) contamination with
tails. However, both the appearance and the surface development of the
resulting schistosomula closely resembled those of the skin forms while
the schistosomula produced by the original method had a granular
appearance and were delayed in their subsequent ultrastructural develop-
ment. See Plate 2 for comparative organisms.

A problem inherent in both procedures was the loss of a con-
siderable number of bodies in the tail-rich supernatants. Both
the Ficoll and Metrizamide separation techniques yielded pure pre-
parations of tails and bodies.

Ultrastructural studies. The morphology and developmental
changes in the tegument of MS and RS were compared with those
typically attributed to SS as described by Hockley and McLaren
(1973). The gross ultrastructural morphology of MS and RS was

43

PLATE 2.   Mechanical schistosomula after the 3rd sedimentation.

A. Original method: Hanks, 4°C.   X 500

B. Modified method: ELac, 22°C.   X 500

44

similar to that of SS, both immediately after the preparative techniques and throughout the experiments. However, the pre-acetabular glands of MS and RS appeared to be filled with large, dense secretion granules and in some of the 90 minute organisms these granules were being extruded through the anterior gland aperture (see Plate 3c). This observation was confirmed by the purpurin cytochemical staining technique, which showed that the pre-acetabular glands of cercariae, MS and RS were filled with granular material for up to 48 hours (see Plate 3a,b,c). In contrast, most SS have secreted their gland contents within the first 3 hours of penetration (Stirewalt and Kruidenier, 1961).

The developmental changes of the tegument and outer limiting membrane of the artificially prepared schistosomula are described below; the changes appeared to parallel those reported by Hockley and McLaren (1973) for SS (see Tables 1 and 2).

Culture. Clegg and Smithers showed that the development of SS in vitro parallels the in vivo development of schistosomula resulting from a routine cercarial infection. By day 10 the fastest-growing SS reach the easily recognized 'gut-closed' stage and 50-70% attain this degree of development by day 12 (Clegg and Smithers, (1972).

Similar results were obtained in the present study (Table III). The first MS and RS to reach the 'gut-closed' stage were also seen by day 10. After 12 days of culture, however, it was noted that fewer of the MS and RS attained this degree of development (Table 3).
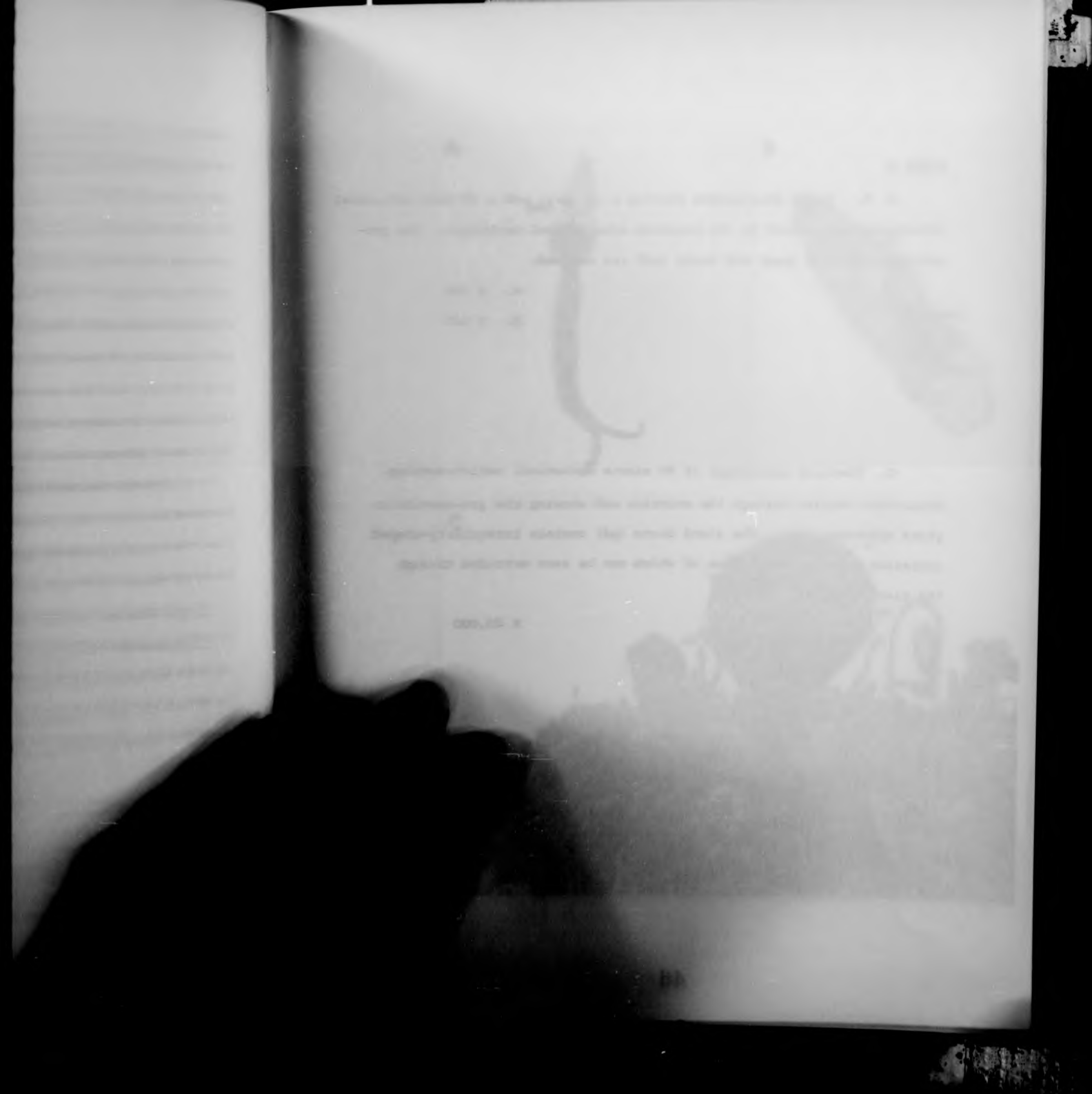
PLATE 3.

A. B. Light micrographs showing a cercaria and a 48 hour mechanical schistosomulum stained by the purpurin cytochemical technique. The pre-acetabular glands (pag) and ducts (gd) are stained.

A. X 200

B. X 525

C. Electron micrograph of 90 minute mechanical schistosomulum. Ultra-thin section through the anterior end showing the pre-acetabular gland apertures (ga). The gland ducts (gd) contain irregularly-shaped secretion granules (sg), some of which can be seen extruded through the gland aperture.

X 23,000

46

and a 48 hour mechanical

technique. The pre-

A.   X 200

B.   X 525

cal schistosomulum.

g the pre-acetabular

n irregularly-shaped

extruded through

X 23,000

Tables 1 and 2 Ultrastructural Studies

Key for Tables 1 and 2.

Glycocalyx - cercarial surface coat of loosely stranded material containing
small fragments of the trilaminate surface membrane. RS surface
coat differs from the typical surface coat in that it is thicker,
more dense and has no organised structure.

Outer Surface Membrane -

1. trilaminate - (a lipid bilayer consisting of two dense
leaflets separated by a less dense layer), - 85nm thick

2. pentalaminate -

3. heptalaminate - now designated "double outer membrane"
(two apposed lipid bilayers) (McLaren and Hockley, 1977).

Cercarial Bodies - spherical tegumental inclusions with a trilaminate
membrane surrounding a peripheral electron-lucent area and
a central dense granular mass, - 120nm dia.

Discoid Bodies - elongated tegumental inclusions with a trilaminate
membrane and granular contents, 1 120 x 20nm.

Surface Microvilli - tegumental projections with trilaminate membrane
forming a shaft which is narrow at the basal end and
expanded distally, - 300 to 2000nm x 50nm along shaft
x 70nm distally.

Membranous Vacuoles - spherical tegumental and subtegumental
inclusions limited by two closely apposed trilaminate
membranes and containing dispersed granular material or
additional fragments of membrane, - 100-150nm dia.

Table 1. Ultrastructural Studies

	Glycocalyx	Outer Surface Membrane	Cercarial Bodies	Discoid Bodies	Surface Microvilli	Membranous Vacuoles
Cercariae	present as as thick coat	trilaminate	present as the most numerous inclusion	present occasionally	not present	not present
Mechanically prepared Schistosomula 30'	present in reduced amounts	trilaminate	present in decreased numbers	few	numerous	present as most numerous inclusion in both tegument and subtegument
60'	reduced or absent	trilaminate with larger multilaminate areas	rare	few	numerous	still present in large numbers
Rat Serum transformed Schistosomula 30'	tegument covered by thick layer of dense amorphous material	trilaminate	present in decreased numbers	few	present	present
60'	amorphous surface coat reduced	trilaminate with multilaminate areas	rare	few	present	still present in large numbers
Skin penetrated Schistosomula 30'	present in reduced amounts	trilaminate	present in decreased numbers	few	numerous	present as most numerous tegumental and subtegumental inclusion
60'	absent	trilaminate with multilaminate areas	very rare	few	absent of rare	still present in large numbers

Table 2. Ultrastructural Studies

	Glycocalyx
Mechanically prepared 90' Schistosomula	absent
120'	absent
Rat Serum transformed 90' Schistosomula	traces of surface mat-erial still present
120'	as with 90'
Skin pene-trated 90' Schistosomula	absent
120'	absent

Outer Surface Membrane	Tegumental Inclusion	Microvilli
large heptalaminate regions	rare cercarial bodies few discoid bodies	present occasionally
entirely heptalaminate	large membranous vacuoles less frequent than in 60' specimen numerous small membranous vacuoles	absent
large heptalaminate regions	rare cercarial bodies few discoid bodies	present occasionally
almost entirely heptalaminate	large membranous vacuoles less frequent than in 60' specimen numerous small membranous vacuoles	absent
large heptalaminate areas	no cercarial bodies rare discoid body	absent
entirely heptalaminate	[1]large membranous vacuoles less frequent than in 60' specimen [2]numerous small membranous vacuoles present	absent

A study comparing schistosomula growth in Elac and in RPMI-1640 suggested that RPMI is a better medium for long term cultivation; furthermore the percentage of serum added to the medium can be reduced to 25% without affecting the schistosomula. Clegg and Smithers (1972) reported that erythrocytes were not essential for initiation of growth but did appear to enhance the growth rate. In the present study cultures have been maintained for varying periods up to 8 weeks without erythrocytes and schistosomula cultured in RPMI supplemented with only 25% serum appeared to develop normally and at the same rate as those cultured with erythrocytes (Plate 4).

Infectivity. Viable larval forms of the three types of schistosomula were recovered from the lungs of mice 5 days after intravenous injection. Perfusion at 6 weeks post-infection yielded adult worms from all groups. The percentage recovery of worms after cercarial exposure or injection of MS, RS or SS did not differ significantly, although there were lower recoveries from the MS or RS injected mice (Table 3).

DISCUSSION

In this study comparisons were made between two types of schistosomula prepared by artificial techniques and schistosomula formed during penetration of isolated skin. The ultrastructure and development of the surfaces, and the viability in vivo and in vitro of the three types of schistosomula were compared. The objective of these studies was to determine the most convenient

47

PLATE 4.

Schistosomula cultured in RPMI-1640 or ELac.

A.   Mechanically prepared schistosomulum (MS) cultured for 21 days in
RPMI-1640 containing 25% human serum (no erythrocytes).

                                        X 94

B.   MS cultured for 21 days in ELac containing 25% human serum
(no erythrocytes).

                                        X 94

C.D.   Schistosomula obtained from mouse lung 5 days after cercarial
infection (LS) and cultured in RPMI-1640 containing 25% human serum (no
erythrocytes).

                              C.   Cultured for 15 days
                                        X 53
                              D.   Cultured for 35 days
                                        X 53

A

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).

man serum

fter cercarial
5% human serum (no

for 15 days

for 35 days

D

B

C

TABLE 3:   Culture and Infectivity of Cercariae and Schistosomula

	Culture (% reaching 'gut-closed' stage by day 12)	Infectivity (% recovered as adult worms from mice)
Cercariae	0	38.8 ± 6
Schistosomula		
SS		
Exp. I	50	44.8 ± 10
II	70	
III	67	
MS		
Exp. I	30	28.3 ± 6
II	50	
III	47	
RS		
Exp. I	25	31.8 ± 10
II	50	
III	51	

*   Mice were exposed to standard cercarial infection or the
    schistosomula were injected I.V.

preparative technique for producing large numbers of 'fully developed' schistosomula as defined by Stirewalt (1974), preferably uncontaminated by host material.

Previous work has shown that the trilaminate surface membrane of the cercaria transforms into a heptalaminate membrane within 3 hours of penetration of host skin in vivo or isolated skin preparations in vitro (Hockley and McLaren, 1973). The present study has demonstrated the formation of a heptalaminate tegumental membrane on both MS and RS in the same time range. In addition, the glycocalyx of MS, like that of SS (Hockley and McLaren, 1973) diminished after a 30 minute incubation and very little remained at 60 min. A similar loss of glycocalyx may have occurred during the transformation of cercariae to RS. However, these organisms develop an amorphous surface coat, unlike the fibrillar structure of the glycocalyx, but more reminiscent of the appearance of the'cercarienhullen reaktion' as described by Kemp (1970). The surface material of RS may therefore be due to an interaction between a component in fresh rat serum and surface material on the schistosomula. See Plates 5, 6 and 7.

McLaren and Hockley (1976) have reported the occurrence of microvilli on the surfaces of both SS and MS between 20 and 90 minutes after transformation; they suggest that the formation and subsequent loss of these microvilli serves to eliminate the cercarial trilaminate membrane and thus make way for the new heptalaminate membrane. This study has confirmed the appearance of microvilli

PLATE 5.

A. Thirty-minute mechanical schistosomulum (MS). The tegument (t)
still retains some reminants of the cercarial glycocalyx (g).

X 58,400

B. Thirty-minute rat serum schistosomulum (RS). The tegument (t)
is covered by a thick layer of dense, amorphous surface material (sm).

X 94,500

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lyx (g).

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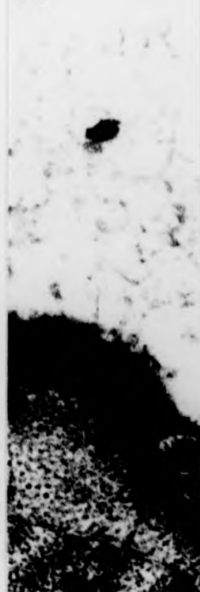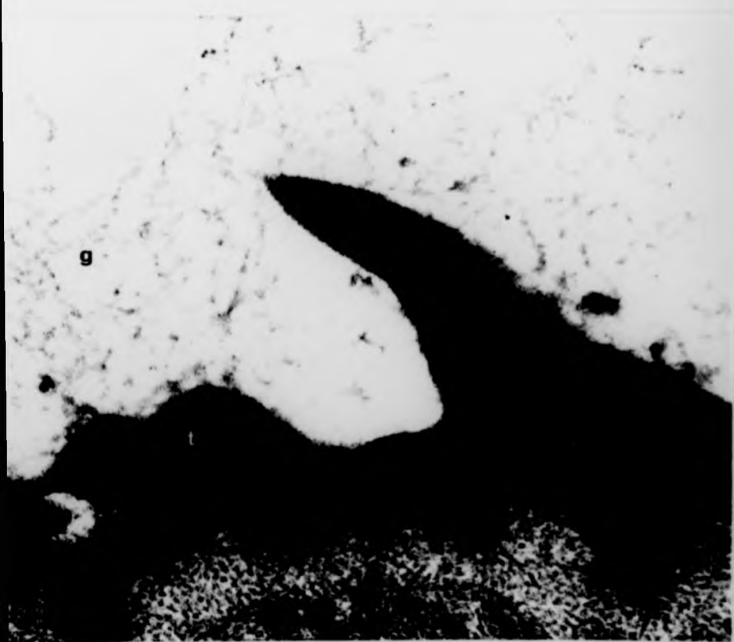
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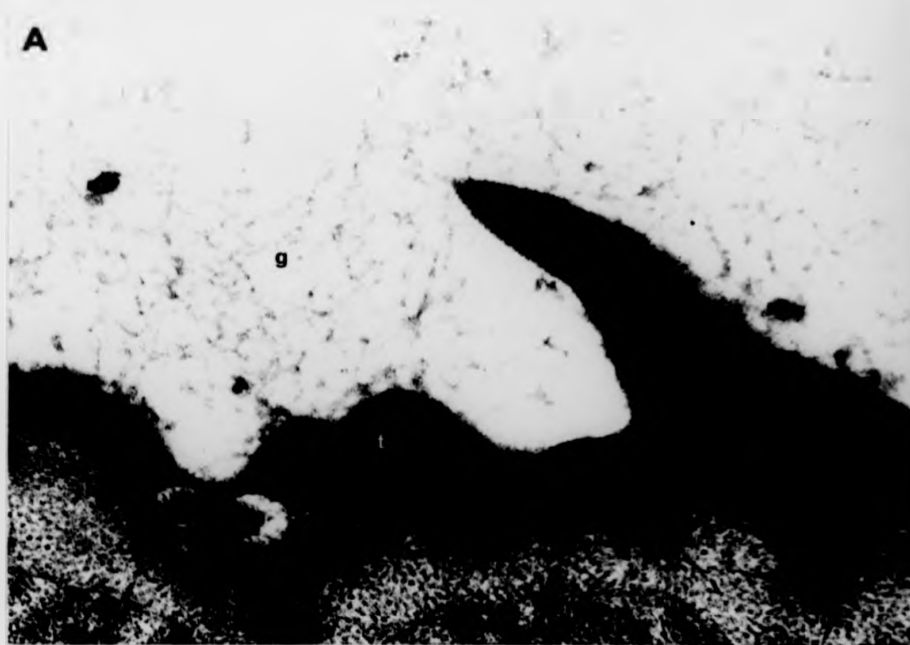
PLATE 6.

A. Thirty-minute mechanically prepared schistosomulum (MS). The
tegument (t) is limited by a typical trilaminate membrane (tm)
to which are still attached some remnants of the cercarial glyco-
calyx (g). Trilaminate structures (ts) are present in the glyco-
calyx.

X 131,300

B. Sixty-minute MS. The tegument (t) and the connections (c)
to the sub-tegumental cells are filled with vacuoles (v). Some
larger vacuoles (lv) are opening to the exterior (ext). The
tegument and the large vacuoles are limited by heptalaminate
membranes (hm).

X 83,760

51

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brane (tm)

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131,300

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3,760

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31,300

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PLATE 7

A. Two-hour skin penetrated schistosomulum showing the typical
heptalaminate outer limiting membrane (hm) of the tegument (t).

X 83,800

B. Two-hour mechanical schistosomulum. The tegument (t) is limited
by a heptalaminate membrane (hm) and contains membraneous bodies (mb).

X 91,000

C. Two-hour rat serum schistosomulum. The tegument (t) is limited by a
heptalaminate membrane (hm) which still bears remnants of the amorphous
surface material (sm).

X 93,000
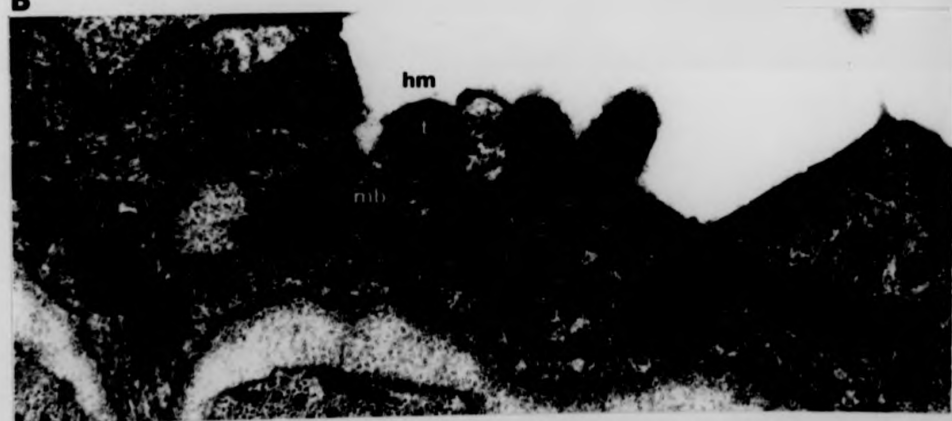
he typical

ument (t).

(t) is limited

ous bodies (mb).

t) is limited by a

of the amorphous

on SS and MS, and has also shown that microvilli develop on RS
even though the surfaces of these schistosomula are covered with
a thick layer of amorphous material (Plate 8).

The cercarial tails of MS appeared normal after the physical
separation technique used to prepare MS; they continued to
move for several hours and ultrastructural studies showed them
to be morphologically normal. In contrast, the tails of the RS,
which were still attached to the bodies quickly became immobile,
wrinkled and granular. Many RS tails remained attached to the
bodies for up to 2½ hours. An ultrastructural study of the tail/
body junction at 2 hours revealed that the attachment was limited
to a small region of tegumental and muscular tissue and that all
the other tail tissue had completely degenerated (Plate 9).

The purpurin staining technique confirmed the electron
microscopic observations that the pre-acetabular glands of MS
and RS had retained their contents during in vitro conversion.
One of the criteria set forth for a schistosomulum is that both
the pre and post-acetabular glands must have released their secretions.
This criterion was suggested because gland secretion had been
observed to occur when cercariae penetrate skin in vivo (Stirewalt,
1963) and in vitro (Jensen, Stirewalt and Walters, 1965). Much
speculation remains as to the function of the secretions and,
as with tail separation, the release of gland secretions has been
considered necessary for cercaria-schistosomula transformation.
The retained glandular secretions of fully developed MS and RS

PLATE 8:

A. Thirty-minute mechanically prepared schistosomulum (MS) showing short microvilli with bulbous distal tips. Both the tegument (t) and the microvilli are limited by a continuous trilaminate membrane (tm). Vestiges of the cercarial glycocalyx (g) are seen.

X 126, OOO

B. Sixty-minute MS showing long microvilli with beaded appearance. Glycocalyx (g), trilaminate membrane (tm) and tegument (t).

X 55,600

um (MS) showing

egument (t) and

membrane (tm).

aded appearance.

(t).
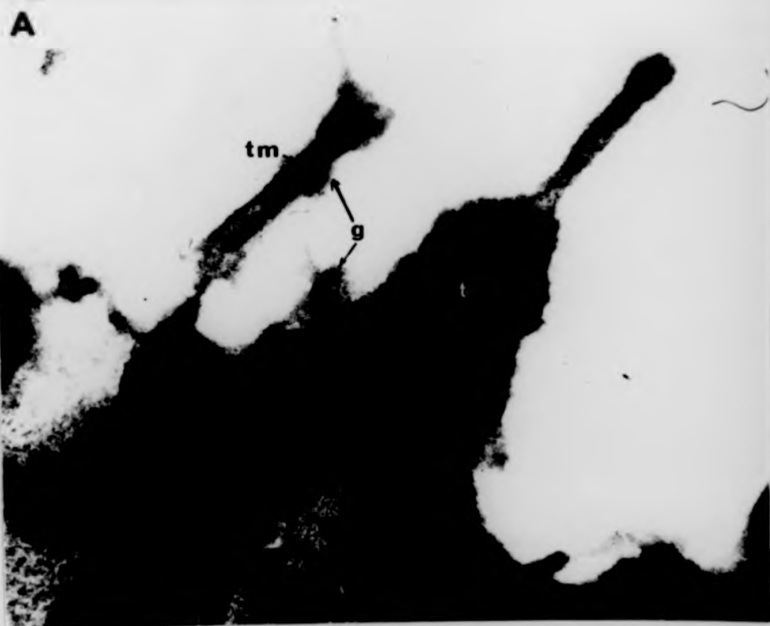
PLATE 9

A.   Two-hour rat serum schistosomulum in which the tail (tl) is still
attached to the body (b). The tail is higly degenerate and has no
tegument or surface material, whereas the body is morphologically normal and
has both tegument (t) and surface material (sm). The region of attach-
ment is minimal (arrows); it is limited to tegument and muscular tissue.

X 7,980

B. Enlargement of the region of attachment.

X 14,850

PLATE 9

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X 14,850

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B. Enlargement of the region of attachment.

X 14,850

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nd muscular   tissue.

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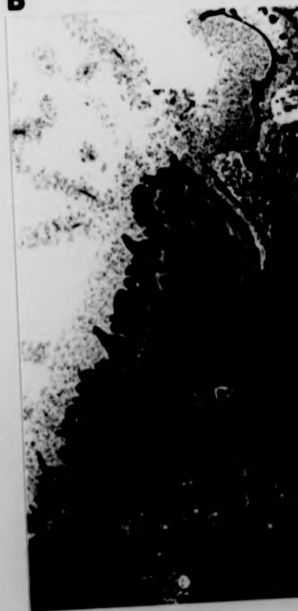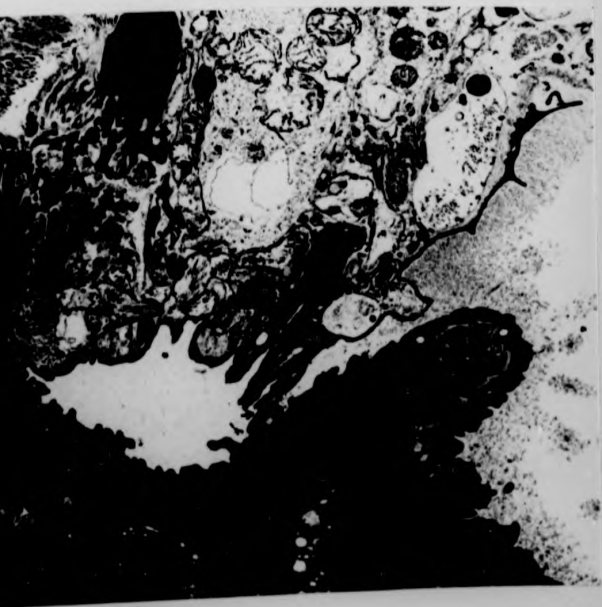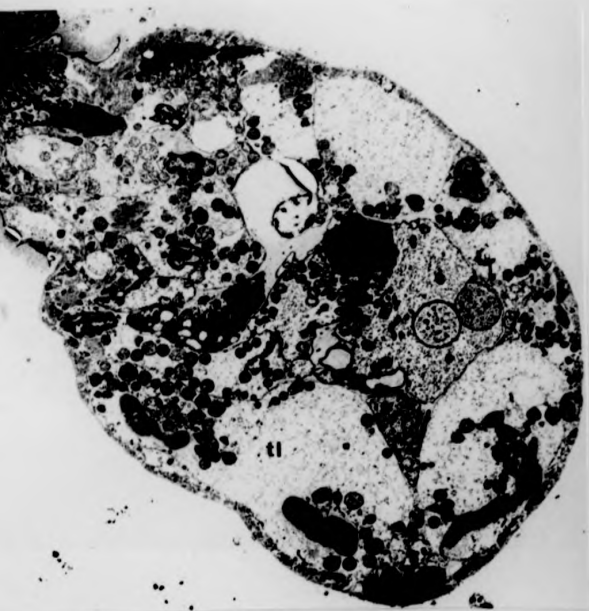
suggest that this is unlikely and that gland evacuation is there-
fore not a necessary criterion for defining a schistosomulum.

Clegg (1965) considered that the ability to develop in vitro
is an important criterion for the successful transformation from
cercaria to schistosomulum. He has demonstrated this feature
for SS. When these forms are cultured, the 'gut-closed' stage is
first reached on day 10 and 50-70% of the schistosomula go on to
attain this degree of development by day 12. The in vitro
development of MS and RS is almost identical with both forms; the
first organisms reach the 'gut-closed' stage on day 10 but by
day 12 only 25-50% of the schistosomula reach this stage. This
reduction in growth index may be explained, in part, by the
exclusion of the skin-penetrating stage. Many cercariae die
as they attempt to penetrate intact mouse skin (Clegg and
Smithers, 1968), a fact which may also partly explain the low
yields obtained when preparing SS. It is possible that the
death of the 'weaker' cercariae as they penetrated the isolated
skins eliminated many of the schistosomula which were incapable of
further development in vitro. However the increased growth
rate and normal development observed with schistosomula cultured
in RPMI-1640 suggested that this was better medium for MS. Further-
more if the observation that erythrocytes are not necessary is
confirmed by ultrastructural studies of the schistosomula, then
establishing a defined culture system would be more likely, and
subsequent biochemical analysis of the schistosomula surfaces

could be pursued more fully.

All three types of schistosomula developed to maturity
when injected intravenously into mice; there was no significant
difference in the percentage of adult worms recovered from the
three forms, although the recoveries from MS and RS were lower.

The results of these studies indicate that the two main
criteria of transformation from cercaria to schistosomulum should
be (1) the development of a heptalaminate surface membrane and
(2) the capability of growing in vitro, at least to the 'gut-closed'
stage. The transformation of cercariae after penetration of
isolated skin (SS) by mechanical tail breakage (MS) or by incuba-
tion in fresh rat serum (RS) resulted in the formation of
schistosomula which met both these important requirements. The
only difference observed between the artificially produced
schistosomula and those produced after skin penetration was the
amorphous surface coat which surrounded RS but which was absent
from MS and SS. It is believed that this material is derived
at least in part from the rat serum and its presence on the RS
surface limits the usefulness of these organisms for biochemical
and immunological studies. In contrast, MS are uncontaminated
by host material and can be readily produced in large numbers;
thus these organisms were considered to be the most convenient and
logical material for immunochemical and physiological studies
and to serve as the reference organism for all future comparisons.

CHAPTER 3: INVESTIGATIONS OF ANTIBODY EVOLUTION IN S.MANSONI

INFECTED MICE

INTRODUCTION

The role of humoral antibody in acquired immunity to schistosomes has not been completely characterized. Passive transfer experiments have shown that antibody is involved (Perez, 1974; Sher, Smithers and Mackenzie, 1975; Phillips et al., 1975), while in vitro culture experiments have suggested that cells, either macrophages (Capron et al., 1975) or eosinophils (Butterworth et al., 1977; Mackenzie et al., 1977), are activated in an antibody dependent reaction against the surfaces of the young schistosomula. However, the characteristics of these antibodies and their development during an infection have not been completely clarified.

Hypergammaglobulinemia is a well documented feature of many protozoal and helminth infections in humans and experimental animals (Smithers, 1967; Cohen, 1974), but only a small proportion of the elevated serum immunoglobulin has demonstrable specificity for parasite antigens (Cohen, 1974). Hypergammaglobulinemia was first noted in murine S.mansoni infections by Evans and Stirewalt (1957) and has been re-examined recently by Sher, McIntyre and von Lichtenberg (1977). They infected mice with 30-40 cercariae and studied the levels of immunoglobulin classes and subclasses at bi-weekly intervals during the course of infection. IgG_{2a} and IgG_{2b} concentrations increased during the first four weeks, decreased during the next ten week period and there was a sub-

58

sequent increase of IgG_{2a} between the fourteenth and twenty-second week. The levels of IgG_1, IgM and IgA began to increase between the sixth and eighth weeks of infection, reaching peak values by the twelvth to fourteenth weeks. These immunoglobulin levels were measured by single radial immunodiffusion (Mancini, Cerbonara and Heremans, 1965) and therefore represent total immunoglobulin, rather than specific antischistosome antibodies. However, the specificity of the fourteenth week serum was checked by absorbing the serum with homogenates of either adult schistosomes or eggs; these absorptions removed 8.2% and 30.7% of the IgG, respectively, but none of the IgM or other immunoglobulins.

The question of whether or not the immunoglobulins elevated during a S.mansoni infection represent antibodies directed against antigens important to the survival of the challenge schistosomula is crucial to the understanding of acquired resistance. Smithers and Terry (1976) suggested that the targets of the immune response were the early schistosomula which were killed or damaged before they acquired their host-like disguise. Studies have shown that within a relatively short time early schistosomula are able to acquire host material in vivo or in vitro and subsequently do not bind anti-schistosome antibodies (McLaren et al., 1975; Goldring et al., 1977). Similarly, Sher (1977) has used his schistosomula lung model to demonstrate that schistosomula lose their susceptibility to immune humoral attack after 30 hours, either in the host or in culture.

59

Since mechanically prepared schistosomula (MS) have had no contact with host materials, they should be an excellent source of unmodified parasite surface antigens. Therefore, the next logical step was to use these organisms to detect and characterize surface specific antibodies in serum from S.mansoni infected mice. Initially, an indirect fluorescent antibody technique was employed; later, radio-labelled Protein A and immunoglobulins were used to quantitate the concentrations of murine antibodies binding specifically and non-specifically to the surfaces of MS.

Experiments were designed to answer the following questions concerning the evolution of anti-schistosomula antibodies, and the absorption of host immunoglobulins by the schistosomula:

1. When do anti-schistosomular antibodies first appear following a standard cercarial infection? In what order do specific immuno-globulin classes and subclasses appear?

2. How does the number of cercariae in the initial infection affect the evolution of the antibodies?

3. Are there differences between mouse strains in their immuno-logical response to S.mansoni infections?

4. Do the lymphoid cells of normal and infected mice differ in the expression of surface immunoglobulins?

5. What is the mechanism of immunoglobulin binding to the schistosomula surface; do schistosomula possess Fc receptors?

60

MATERIALS AND METHODS

Mechanical schistosomula. Cercarial bodies were separated
mechanically from the tails and incubated for 2 hours in ELac as
described in Chapter 2: Materials and Methods. These organisms
are referred to as "2 hr MS".

Lymphoid cells. CBA mice were killed with CO_2 and the lymphoid
organs collected and teased apart immediately in cold PBS-4 mM
EDTA-2% Ovalbumin (PBS-EO). Cells from Peyer's patches and lymph
nodes were passed through a glass wool column to remove aggregated
cells prior to 2 washes in cold PBS-EO by centrifugation at 1200
rpm for 5 minutes. The cells were counted in a Coulter Counter and
adjusted to 5×10^7 cells/ml prior to use in immunofluorescence (IF)
or ^{125}I-labelled Protein A (^{125}I-Pro A) experiments. Only cell
preparations which contained greater than 92% viable cells, as
tested by eosin exclusion, were used. Cells from the following
lymphoid organs were studied: spleen, thymus, Peyer's patches,
peripheral lymph nodes, mesenteric lymph nodes and bone marrow.

1. Preparation of sera and immunofluorescent reagents

Mouse sera. The normal and infected sera used throughout these
studies were collected from groups of female CBA, and female or male
Parkes mice infected with 30-40 cercariae, 150 cercariae or 500
cercariae per mouse. Blood was obtained from the tail veins of
5-10 mice at weekly intervals and the sera were pooled according
to group, heat inactivated for 30 minutes at $56^{\circ}C$ and stored at
$-20^{\circ}C$ in small aliquots. Large groups of animals were infected

with the same batch of cercariae so that an individual mouse was
bled only once in any two week interval. The serum was des-
ignated by the number of weeks after the initial infection.
For example, pooled serum collected from CBA mice one week after
infection was labelled "1 wk CBA sera". Mice from each group
were killed at week 6 and the number of schistosomes determined;
all other mice were similarly examined at the end of the
experiment.

   Rabbit and goat antisera. The reagents used in the immuno-
fluorescence tests were generously supplied by Dr R.M.E. Parkhouse
or prepared in Dr Parkhouse's laboratory by the author as described
in Table 4. The reagents included antisera produced in rabbits
or goats.

   The preparation of specific rabbit anti-mouse Ig, and goat
anti-rabbit Ig antisera are described by Parkhouse, Hunter and
Abney (1976). In brief, the specific antisera were raised
against mouse myeloma proteins of different classes and subclasses.
For example, the anti-mouse μ chain serum was raised against purified
MOPC 104E, IgM λ myeloma protein and absorbed with 7 S mouse Ig
coupled to Sepharose 4B and found to be non-reactive in a radio-
immunoassay against the other myeloma proteins. The anti-Fab (k)
antiserum precipitates all the proteins including IgM. Specificities
were then checked by immuno-precipitation with a panel of ^{125}I-labelled

Table 4. The reagents included antisera produced in rabbits or goats:

Specificity	Immunogen
All Ig classes (k)	Mouse Myeloma Protein Ajd PC5 (Fab fragments)
IgG_1 (k)	" " " MOPC 21
IgG_{2a} (k)	" " " Ajd PC5
IgG_{2b} (k)	" " " MOPC 195
IgG_3 (k)	" " " FLOPC 21
IgM (k)	" " " TEPC 183
IgM (1)	" " " MOPC 104e
IgA (1_2)	" " " MOPC 315
k*	" " " MOPC 41
1*	" " " RPC 20
IgD**	Lymphocyte membrane immunoglobulin
α_2-macroglobulin	Mouse α_2-macroglobulin
Normal mouse serum	Serum from Parkes mice
Mouse erythrocytes	Erythrocytes from CBA mice
Rabbit IgG	Ammonium sulphate cut of rabbit serum

*   Urinary light chains

** Lymphocyte membrane immunoglobulin prepared by Abney et al., (1976)

The fluorochrome conjugated antisera included:

Fluorescein Iso Thio Cyanate (FITC)

or

Tetra methyl Rhodamine Iso Thio Cyanate (TRITC)

conjugated to:

Rabbit anti Mouse Fab (k).....⟨FITC-Rab αMo Fab (k) or
                                TRITC-Rab αMo Fab (k)
    or
Goat anti Rabbit IgG ....⟨FITC-GaRIgG or
                               TRITC-GaRIgG

mouse immunoglobulins (described below) and by immunodiffusion against mouse myeloma proteins.

Absorption of sera and antisera. Sera and antisera were absorbed with the following cells and cell membranes, as described below.

Absorbants	Normal Rabbit serum	Rabbit α Normal Mouse serum	Rabbit α Mo Fab (k)	Rabbit α Mo IgM	Rabbit α Mo IgA	Rabbit α Mo IgD	Normal Mouse Serum	4 wk CBA sera (30-40 cerc)	11 wk CBA sera (30-40cerc)	16 wk CBA sera (30-40 cerc)
Mouse kidney cell membrane (MKCM)	✓	✓	✓	✓	✓					
Guinea pig erythrocytes (Gp rbcs)	✓	✓	✓	✓	✓					
MKCM followed by Gp rbcs (MK Gp)	✓	✓	✓	✓	✓					
Adult CBA mouse spleen cells (Spl)	✓	✓				✓				
Mouse thymocytes (Thy)	✓	✓	✓	✓	✓	✓				
Foetal CBA liver cells (Liv)	✓	✓				✓				
Human A + Erythrocytes (A + rbcs)	✓	✓	✓	✓	✓		✓	✓	✓	✓
Human B + Erythrocytes (B + rbcs)	✓	✓	✓	✓	✓		✓	✓	✓	✓
Human O + Erythrocytes (O + rbcs)	✓	✓	✓	✓	✓		✓	✓	✓	✓

The absorbants, cells or cell membranes, were processed from the organs of CBA mice or from heparinized human blood. The cells were washed by centrifugation 3 times in cold Hanks containing 0.2% BSA or PBS-EO. The sera were absorbed with the cells or cell membranes for 30 minutes at 4°C at least 2 times, or more if preliminary checks were still positive.

Preparation of rabbit or goat immunoglobulin conjugates. The conjugated rabbit and goat IgG were prepared by the method of Cebra and Goldstein (1965), which consisted of an ammonium sulphate cut of the serum followed by fractionation on a DEAE-52 column and conjugation by dialysis of the Ig against the fluorochrome (see Methods below). The conjugates were titrated to determine the optimal dilutions for the various reactions, aliquoted and frozen at -70°C. Protein concentrations were estimated from the optical density of the solution (OD) at 280 nm using an extinction coefficient of 15 for a 1% solution (Steiner and Lowey, 1966).

Ammonium Sulphate Precipitation of Ig Serum - 1 volume

Phosphate Buffered Saline 0.01M, pH 7.5 (PBS) - 1 volume

      stir on ice and add gradually

Ammonium sulphate 3.2 M - 2 volumes

      stir for 1 hour on ice

      wash x 2 by centrifugation with

Ammonium sulphate 1.6 M

      dissolve ppt in

PBS - 2 volumes

     reprecipitate with

Ammonium sulphate 3.2 M - 2 volumes

     centrifuge, redissolve ppt in

PBS - ½ serum volume

     dialyse extensively against

PBS

     centrifuge, measure OD 280

Preparation of Ig fractions

To

10 ml DEAE-52 column, equilibrated with PBS

     apply

     Ig sample, 50-70 OD_{280} units/10 ml column

     elute with

PBS

     pool fractions of the peak, measure volume and OD_{280} =

     <u>Pool I</u>

     elute with

PBS, 0.01 M, pH 7.5 - 0.05M NaCl

     pool fractions of the peak and measure as above =

     <u>Pool II</u>

     elute with

PBS, 0.01 M, pH 7.5 - 0.1 M NaCl

     pool fractions and measure as above = <u>Pool III</u>

     elute with

PBS, 0.01M, pH 7.5 - 0.2 M NaCl

        pool fractions and measure as above = <u>Pool IV</u>

Dialyse pools I to IV against 0.9 % NaCl

        concentrate to 15 OD_{280} units/ml ($\simeq$ 10 mg/ml)

Conjugation by addition of fluorochrome

<u>Fluorescein Derivative</u>

Adjust Ig solution to $\simeq$ 15 OD_{280} units/ml ($\simeq$ mg protein/ml)

        place in ice bath with magnetic stirrer and pH electrodes

Add Fluorescein Iso Thio Cyanate (FITC) 10 mg/ml

        in 0.9% NaCl at a concentration of 12.5 µg FITC/

        1 mg protein/ml

Maintain pH at 8.7-9.5 with 0.1 M $NaHCO_3$

        stir over ice for 2 hours and then O/N at $4^{\circ}C$

Add solution to Sephadex G-25 column

        equilibrated with PBS (vol. of G-25 = 20 x sample volume)

        pool fractions of 1st peak

        dialyse against PBS

        centrifuge and measure volume and OD_{280}

Apply to DEAE-52 column as described above

        collect Pools I to IV; measure volumes

        and OD_{280}, OD_{320}, OD_{492}, and OD_{495}

<u>Rhodamine Derivative</u>

Exactly as above except:

Tetra methyl Rhodamine Iso Thio Cyanate (TRITC)

        at 10 mg/ml in 0.9% NaCl added

        at a concentration of 30 µg TRITC/mg protein/ml

Sephadex G-50 column equilibrated with PBS

measure volumes and OD_{280} and OD_{515}

Conjugation by Dialysis against Fluorochrome (Raff, 1970)

As above except:

FITC solution prepared at a concentration of 0.125 mg/ml and

10 x the volume of the protein solution

Protein solution dialysed against FITC O/N at $4^{\circ}C$

Protein/FITC solution then dialysed against 4 changes of

PBS for 48 hrs at $4^{\circ}C$.

Preparation of Mouse or Rat Ig and Fluorochrome Conjugation

As above except:

Dialyse protein against

FITC 125 mg/ml

TRITC 30 mg/ml

and check that pH of fluorochrome is between

8.5 to 8.7

Complete as described above

Calculation of Coupling ratio

FITC Derivative

using OD_{280}, 320, 492, and 495

correct $OD_{492} = OD_{492} - \dfrac{OD\ 320}{2}$

FITC concentration of conjugate $= \dfrac{OD\ 492\ (corrected) \times dilution}{0.2}$

Estimation of protein of conjugate $= OD_{280} - (0.35 \times OD_{495}) \times$

$0.76 \times dilution$

Fluor:Protein ratio = 0.41 x $\frac{\text{FITC concentration ug/ml}}{\text{Protein concentration mg/ml}}$

TRITC Derivative

Take OD at 280 and 515

Fluor:Protein ratio = $OD_{280}:OD_{515}$

Standard immunofluorescence procedure (IF). The schistosomes or lymphoid cells were washed 3 times by centrifugation in ELac containing 20 mM Hepes, pH 2.5 and 0.2% BSA (ELac/BSA), and appropriate numbers added in 100 μl aliquots to 1.5 ml plastic tubes. 25 μl of the appropriate serum dilutions were added and the tubes incubated with occasional mixing for 20 minutes at room temperature or in an ice bath. The organisms or cells were washed by centrifugation 3 times with ELac/BSA and resuspended in a volume of ≈ 100 μl. 25 μl of the second antiserum (or dilution), or the developing conjugated antiserum, was added and processed as above. Depending on the procedure, the schistosomes or cells, after washing, were either incubated with a third antiserum and processed as above, or mounted on 2 x 3 inch slides with coverslips for fluorescence light microscopical examination. Fluorescence observations were made using Leitz Orthoplan with x 7.5 and x 25 objectives with x 6.3 eyepiece.

Figure 3 demonstrates the 4 systems used for immunofluorescence or radioimmunoassays.

Only living, motile schistosomula were assessed; with these organisms the fluorescence was confined to the surface membranes. Dead or damaged schistosomula were stained throughout the organism, probably due to non-specific absorption or internalization of the fluorochrome conjugated antisera by a faulty and therefore permeable

69

Figure 3   Detection of Mouse Immunoglobulin Binding to 2 hr
Mechanically prepared Schistosomula (MS)

System I ^{125}I- or FITC- GαRab IgG

                 RabαMo proteins
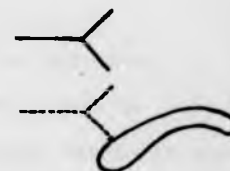
System II ^{125}I- or FITC- GαRab IgG

                 RabαMo Ig class and subclass

                 Moα Schistosome Serum
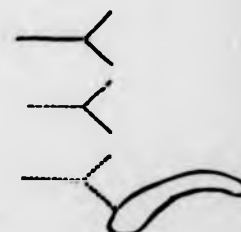
System III ^{125}I- Mo Myeloma proteins

                                                         or
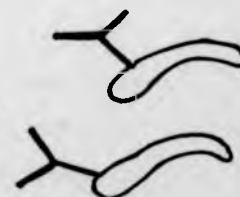
System IV ^{125}I-Protein A

                  RabαMo Ig class and subclass

70

membrane.

The intensity and distribution of fluorescence for any given
population of organisms was estimated visually, graded from + to ++++ and
described. A +++++ was used to designate total sloughing within 10 minutes.
Key: - autofluorescence or injested material only

$\pm$ very weak fluorescence with irregular distribution

+ weak but uniform fluorescence

++ moderate intensity, uniform fluorescence

+++ as above except evidence of sloughing

++++ brilliant fluorescence with much evidence of

sloughing;   some organisms may appear negative but trailing

fluorescent material

Restaining of sloughing schistosomula. 2 hr MS were reacted
with a 13 week CBA serum followed by Rab α Mo Fab (k) and then
stained with TRITC-Goat α Rab IgG. The reactions and washes
performed either at $4^{o}C$ or $22^{o}C$. At timed intervals ≈ 20 schistosomula
were observed for sloughing and then restained with either 13 week
CBA serum followed by FITC-Rab α Mo Fab or by FITC Rab α Mo Fab
alone. The fluorochromes were observed at the appropriate wave
lengths and distribution of fluorescence recorded.

Photography of fluorescent organisms. Ektachrome EH 135 colour
film was used with exposure times of between 15 and 120 seconds.
The final magnifications are noted on the Plates.

Preparation of affinity chromatography columns. The following
proteins were coupled to Sepharose 2B or 4B (Pharmacia) following its

activation with cyanogen bromide as described by Porath, Axen and
Ernback (1967).

Mouse α_2-Macroglobulin (from Dr R.M.E. Parkhouse)

α-Fab (k) (Dr R.M.E. Parkhouse)

α-mouse IgA myeloma protein (Dr R.M.E. Parkhouse)

α-mouse IgD myeloma protein (Dr R.M.E. Parkhouse)

Protein A (Sigma)

Lentil Lectin (from Dr M. Crumpton)

Con A (Pharmacia)

200 mg Sepharose suspended in 10 ml water was placed in a small
beaker with magnetic stirrer and pH electrode in ice bath.

10 ml CNBr (50 mg/ml) added with constant stirring.

2 N NaOH added dropwise to maintain pH at 11.0 - 11.5 for 5 minutes
or until pH was steady.

Sepharose washed on a sintered glass funnel with cold water followed
by cold 0.1 M $NaHCO_3$.

Sepharose placed in universal bottle and concentrated protein
solution (≈ 10 mg) added in ≈ 5 ml 0.1 M $NaHCO_3$ and rotated end-
over end for 2 hours at R.T. or O/N at $4^{\circ}C$.

"Coupled" Sepharose-protein washed as above

"Coupled" Sepharose placed in universal and rotated with 2.5 ml 1M
ethanolamine pH 8.0 for 1½ hours at R.T.

"Coupled" Sepharose put into small column and washed alternatively
with 0.1 M acetate, pH 4.0 - 1 M NaCl and 0.1 M Borate, pH 8.0 - 1 M
NaCl for 3 cycles of 100 ml of each buffer. Followed by a PBS wash

O/N at 4°C column stored with PBS/Azide toluene at 4°C.

^{125}I-labelling of immunoglobulins, Fab fragments and myeloma proteins. Prior to iodination, the preparations were desalted and adjusted to pH 7.4 by passing through a Sephadex G-25 column equilibrated with PBS, pH 7.4 or by dialyzing against PBS, pH 7.4 overnight at 4°C. The protein concentration was adjusted to approximately 3-4 mg/ml and 75 µl of the solution added to 100 µCi ^{125}I-sodium iodide (Amersham NMS-30) and 10 µl chloramine-T (2 mg/ml 0.15 M sodium phosphate buffer, pH 7.4). The mixture was allowed to react for 2 minutes at room temperature with constant mixing and the reaction was then terminated with 25 µl sodium metabisulphite (2 mg/ml 0.15 M sodium phosphate buffer, pH 7.4). 50 µl KI (10 mg/ml, 0.15 M sodium phosphate buffer, pH 7.4) was added and the solution was put through a Sephadex G-25 column to remove the free iodine. The fractions containing the iodinated proteins were pooled and the percentage of TCA precipitable counts determined.

Binding of ^{125}I-myeloma proteins to schistosomula. The 2 hr MS were incubated directly with ^{125}I-myeloma proteins diluted to appropriate concentrations determined previously by titration in the IF procedure. The amount of radioactivity was measured before and after 2 washes with ELac-BSA. In the competition experiment, the schistosomula were first incubated with NMS or IMS and then with the myeloma proteins. Results are expressed as counts per minute (cpm) of bound ^{125}I activity/1500 schistosomula.

^{125}I-labelling of staphylococcus protein A (^{125}I-ProA). Radioiodination of ProA was performed by the chloramine-T method (Hunter

73

and Greenwood, 1962), as described by Dorval, Welsh and Wigzell (1974).

50 µl of ProA (1 mg/ml in 0.15 M PBS, pH 7.5) and 5 µl of chloramine-T (0.8 mg/ml in 0.15 M PBS, pH 7.5) were added to 1 mCi ^{125}I-labelled sodium iodide (Amersham IMS-30). After 1 minute at 23°C with constant mixing, the reaction was terminated with the addition of 5 µl of sodium metabisulphite (1 mg/ml in 0.15 M PBS, pH 7.5). 250 µl of 5% ovalbumin in sodium phosphate - 4 mM EDTA buffer, pH 7.1 (PBS-E) was added to the mixture which was then passed through a Sephadex G-25 column equilibrated with PBS-E to remove the free iodine. The fractions containing the ProA were collected, the volume adjusted to 1 ml with sodium phosphate - 5% ovalbumin - 100 mM KI - 4 mM EDTA. Aliquots were frozen at -20°C and were thawed immediately prior to use.

TCA precipitation of ^{125}I-ProA preparations

5 ml of ^{125}I-ProA was diluted to 100 ml with PBS; 10 ml aliquots were added to 3 tubes, each containing 100 ml 10% NRS, and the amount of radioactivity was measured. The tubes were filled with 10% TCA, mixed and centrifuged 3000 rpm for 10 minutes. The precipitated protein pellet was dissolved in 0.5 ml 0.15 N NaOH and reprecipitated with 10% TCA. After centrifugation the supernatant was discarded, the tubes dried and the TCA precipitable radioactivity measured. The range of % TCA Precipitable activity for the 11 preparations of ^{125}I-ProA labelled during the course of these experiments was 85-92%.

Radioimmunoassay of bound immunoglobulin. Estimation of the mouse class and subclass immunoglobulins using ^{125}I-ProA or ^{125}I-goat and rabbit Ig followed the protocol for IF (see Materials and Methods: Standard Immunofluorescence Procedure) except that PBS-EO was sub-

74

stituted for the ELac-BSA as a washing and diluting buffer and ^{125}I-ProA or ^{125}I GLR IgG replaced the fluorochrome labelled goat or rabbit antisera. Radioactivity was measured on a gamma counter before and after the final incubation and washes and the percent recovery calculated. Extensive titrations of the ^{125}I protein A and the various antisera were performed to ensure that the ^{125}I-ProA was always in excess; the appropriate controls were used to determine background and non-specific binding of the reagents.

Preparation of F(ab)$_2$

Sera: Normal Rabbit Sera Pool (NRS)

Rabbit anti-Mouse erythrocytes (Rab α Mo rbcs)

Goat anti rabbit IgG (Goat α Rab IgG)

Fractionating of serum IgGs.

5 ml of serum was applied to a 10 ml Protein A-Sepharose Cl-4B column (for preparation see Material and Methods; Affinity Columns) equilibrated with PBS. The column was washed and the fractions of the first protein peak were collected and pooled (this peak should contain everything except the IgGs which bind to Protein A). The IgG's were eluted with Tris/glycine buffer, 0.1 M, pH 2.8 and the fractions collected, pooled and concentrated by ultrafiltration. The concentrated pools were desalted by passing through a Sephadex G-25 medium column equilibrated with 0.1 M Na acetate buffer, pH 4.2. The volume and OD$_{280}$ of the IgG preparation were measured to give an approximate protein concentration and sodium azide was added as a preservative.

<u>Fc digestion by pepsin</u>. A volume of pepsin (1 mg/ml) equivalent
to 2% of the protein concentration was added and the solution incubated
18 hrs at 37°C. An additional volume of pepsin (1 mg/ml) equivalent
to 1% of the protein concentration was added and the incubation
continued for a further 3 hr at 37°C. The samples were simultaneously
concentrated and dialysed against PBS, pH 7.4 in a millipore ultra-
thimble apparatus. The volume and OD_{280} were measured and approximate
protein concentration calculated.

Iodination of the $F(ab)_2$ fragments was by the chloramine T pro-
cedure as described for iodination of myeloma proteins.

The reaction of 2 hr MS and ^{125}I-$F(ab)_2$ followed the protocol
described in the Standard IF procedure section. Radioactivity was
measured using a Wallac Gamma Counter.

<u>Reduction and alkylation of IgM and IgA</u>. Myeloma proteins IgM
and IgA, purified from the serum of CBA mice inoculated with MOPC 104E
(IgM producing) or MOPC 315 (IgA producing) tumours, were iodinated
by the chloramine T method and then reduced and alkylated by the
method of Rowe and Fahey (1965). 20 µl of the purified protein
were incubated for 2 hours at room temperature with 5 µl of 250 mM
1, 4-dithiothreitol (DTT) in 2 M Tris buffer, pH 8.0 which was pre-
pared immediately prior to use. The reaction was completed by the
addition of 5 µl of freshly prepared 125 mM iodoacetemide in
2 M Tris, pH 8.0. 70 µl of cold ELac-0.2% BSA was added to give a
final dilution of 1:5 of the reduced myeloma proteins.

<u>Reaction with 2 hr M.S</u>. 2 hr MS were washed x 3 with ELac - 0.2%
BSA and then incubated with the ^{125}I-myeloma proteins at room tempera-

76

<u>Fc digestion by pepsin</u>. A volume of pepsin (1 mg/ml) equivalent to 2% of the protein concentration was added and the solution incubated 18 hrs at 37°C. An additional volume of pepsin (1 mg/ml) equivalent to 1% of the protein concentration was added and the incubation continued for a further 3 hr at 37°C. The samples were simultaneously concentrated and dialysed against PBS, pH 7.4 in a millipore ultra-thimble apparatus. The volume and OD_{280} were measured and approximate protein concentration calculated.

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<u>Reaction with 2 hr M.S</u>. 2 hr MS were washed x 3 with ELac - 0.2% BSA and then incubated with the ^{125}I-myeloma proteins at room tempera-

76

Fc digestion by pepsin. A volume of pepsin (1 mg/ml) equivalent to 2% of the protein concentration was added and the solution incubated 18 hrs at 37°C. An additional volume of pepsin (1 mg/ml) equivalent to 1% of the protein concentration was added and the incubation continued for a further 3 hr at 37°C. The samples were simultaneously concentrated and dialysed against PBS, pH 7.4 in a millipore ultra-thimble apparatus. The volume and OD_{280} were measured and approximate protein concentration calculated.

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Reaction with 2 hr M.S. 2 hr MS were washed x 3 with ELac - 0.2% BSA and then incubated with the ^{125}I-myeloma proteins at room tempera-

ture for 20 minutes.   Radioactivity was measured before and after
2 washes with ELac-BSA.   The controls are listed in the Results
Section.

RESULTS

1.   Immunofluorescence Studies

A.   Indirect procedure: Double layer.   In this test (System I
(Materials and Methods)) 2 hr MS were incubated with antisera taken
from mice at different times after infection, washed and then
reincubated with a commercially available FITC labelled rabbit
anti-mouse whole immunoglobulin antiserum (Rab α Mo Ig).   Using this
technique, binding of anti-schistosome antibody was first observed
3 to 4 weeks after infection of CBA mice with 30-40 cercariae.   When
Parkes mice were used in the same test, antibodies were first
detected earlier, at 2 weeks after infection with 30-40 cercariae.
The 2 hr MS did not appear to absorb immunoglobulins non-specifically
from normal mouse serum or from the conjugated Rab α Mo Ig antisera
as checked with the appropriate controls.

With pooled serum from both strains of infected mice, schisto-
somula exhibited a weak, patchy surface fluorescence.   This stain-
ing was easily distinguishable from the slight autofluorescence
observed on schistosomula incubated in either normal mouse sera
followed by FITC-conjugated antisera, or in the antiserum alone.
However, the intensity and uniformity of distribution of the specific
fluorescence was increased if the schistosomula were formalin-
fixed prior to incubation with the infected mouse serum.

B. Indirect test: Triple Layer. The use of a triple layer
Indirect fluorescence test (System II (Materials and Methods)) offers
several advantages: 1) the sensitivity of the procedure is increased,
2) the number of specific fluorochrome conjugated antisera is de-
creased and 3) semi-quantitative assessment of the specific mouse
immunoglobulin levels is possible. Therefore this procedure was
used to determine the evolution and distribution of the classes
and subclasses of antibody specific for antigens on the schistosomula
surface.

In this series of experiments living schistosomula were first
reacted with sera from normal mice or from mice at different times
after cercarial infections of 30-40, 150 or 500 cercariae. This
was followed by incubation with specific rabbit antisera directed
against the various classes and subclasses of mouse immunoglobulin,
and finally by reaction with a fluorochrome-conjugated goat anti-
rabbit immunoglobulin antiserum. The results are presented in
Figures 4 and 5.

The rabbit anti-mouse Fab (k) antiserum served as a reference
for the level of total immunoglobulin bindings to the schistosomula.
With this fluorescence procedure, an increase in specific immuno-
globulin is first detected at 10 days, gradually increases to a
peak level at = 70 days and remains near this level throughout the
rest of the experiment. The late appearance between 3-6 weeks
and gradual increase in the levels of IgM and IgA should be noted.
Low levels of IgD were observed after the 4th week but were not
significantly increased during the later stages of infection.

78

Figure 4. Evolution of schistosomula-specific immunoglobulin
in sera from infected mice: total, IgM, IgA and
IgD

2 hr MS were reacted first with mouse sera followed by Rab
anti Mo immunoglobulin class-specific antisera and finally with FITC-
Goat anti Rab IgG

CBA or Parkes   { Normal Rabbit Serum
mice sera   Rab anti-Mo Fab (k)
                   Rab anti-Mo IgM   }   FITC-Goat anti Rab IgG
                   Rab anti-Mo IgA
                   Rab anti-Mo IgD

Fluorescence Key

+++++   surface fluorescence totally sloughed within 5-10 minutes

++++   moderate→ bright surface fluorescence, uniform distribution prior
         to sloughing

+++   weak→moderate surface fluorescence, uniform distribution prior
         to sloughing

++   weak→moderate surface fluorescence, uniform distribution, no
         sloughing

+   weak but uniform outline

±   weak, patchy and irregular distribution of fluorescence

-        negative, only bluish autofluorescence visible.

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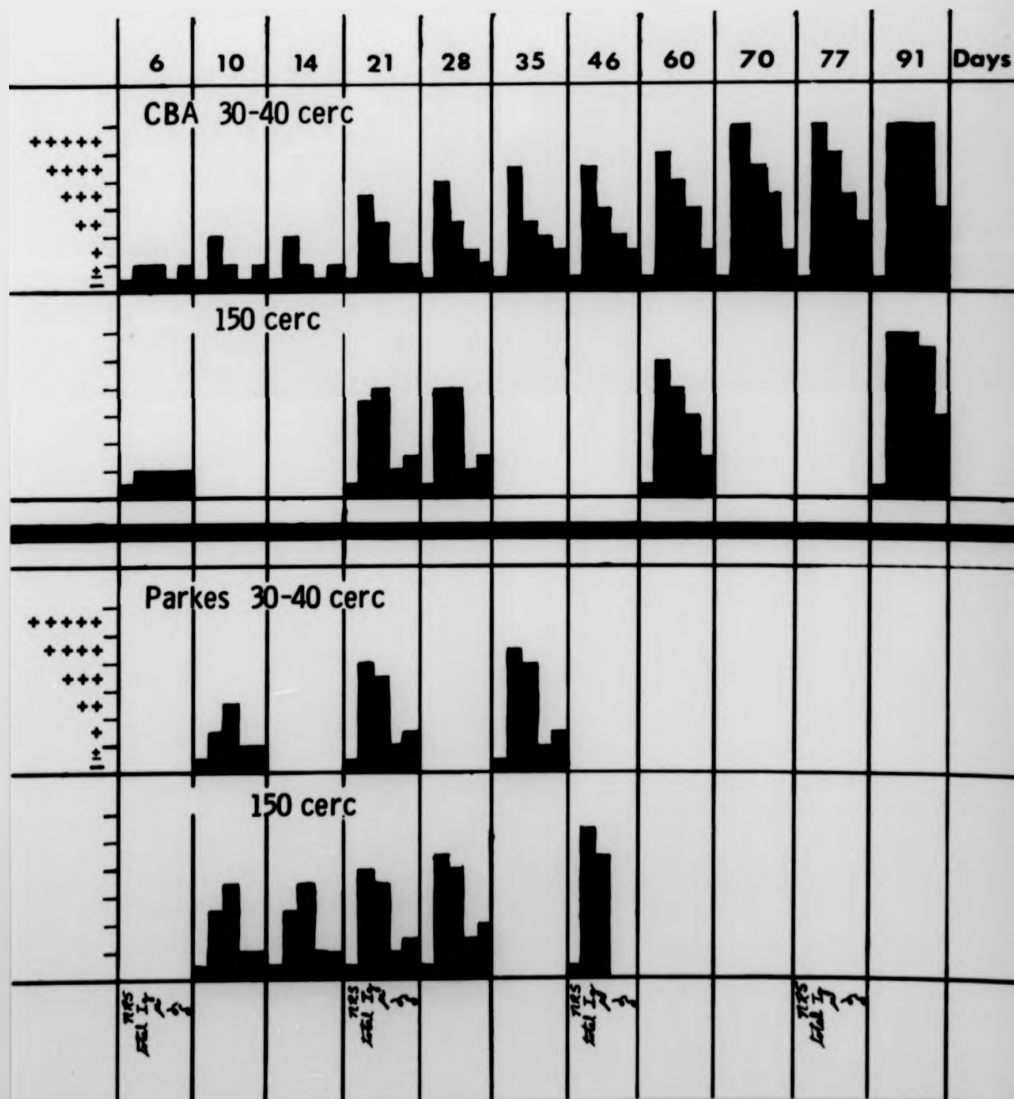
Figure 5. Evolution of schistosomula-specific immunoglobulins in sera from infected mice: IgG_1, IgG_{2a}, IgG_{2b} and IgG_3

2 hr MS were reacted first with mouse sera followed by Rab anti Mo immunoglobulin class-specific antisera and finally with FITC-Goat anti Rab IgG

CBA or Parkes mice sera

$$\left\{ \begin{array}{l} \text{Normal Rabbit Serum} \\ \text{Rab anti Mo } IgG_1 \\ \text{Rab anti Mo } IgG_{2a} \\ \text{Rab anti Mo } IgG_{2b} \\ \text{Rab anti Mo } IgG_3 \end{array} \right\}$$

FITC-Goat α Rab IgG

Fluorescence Key

+++++   surface fluorescence totally sloughed within 5-10 minutes

++++   moderate→bright surface fluorescence, uniform distribution prior to sloughing

+++   weak→moderate surface fluorescence, uniform distribution prior to sloughing

++   weak→moderate surface fluorescence, uniform distribution, no sloughing

+   weak but uniform outline

±   weak, patchy and irregular distribution of fluorescence

−   negative, only bluish autofluorescence visible.

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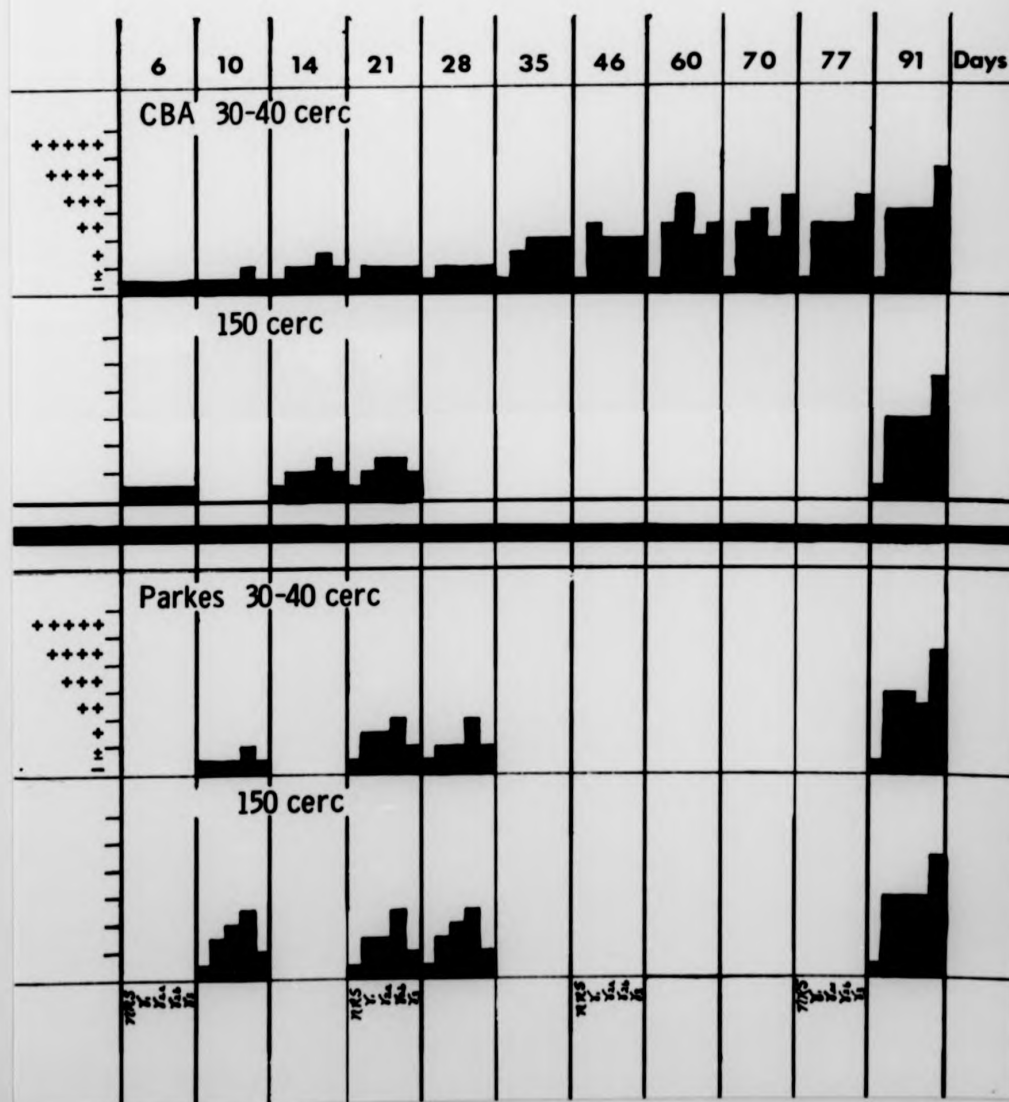
The IgG subclasses began to increase after the fifth week by varying degrees; IgG$_3$ appeared to have the highest and most consistent specific binding levels of all the subclasses.

C. Sloughing of fluorescent surface material. An important aspect of immunofluorescence using living schistosomes as opposed to fixed organisms or frozen sections is the ability of the living organism to shed or slough off the fluorescing surface material. Thus the possibility of false negative results must be considered because positive organisms appear negative after the surface coat is sloughed off (Plate 10). The use of living organisms, however, was considered necessary for these studies because the results obtained would be more relevant to the in vivo interaction of host antibodies and parasite surfaces than those obtained using non-living organisms. Therefore attempts were made to prevent or minimize sloughing without adversely affecting or modifying the surfaces of the organisms.

A procedure was established in which all reagents, incubations and centrifugations were maintained at 4°C and the organisms were observed for fluorescence immediately after the last wash. The importance of the modified procedure was emphasized by the fact that positive schistosomula were observed to begin sloughing their surfaces as soon as they reached ambient temperature. Sloughing was not observed with formalin fixed schistosomula or with frozen sections.

Plate 10 Immunofluorescence

       Light micrographs of 2 hr. mechanically prepared schisto-
somula showing fluorescent staining. Organism (A) with an intact
fluorescent coat (c); organism (B) in which the fluorescent coat
is being 'sloughed'. Note the fluorescence exhibited by the
cercarial tails (t).

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Furthermore, two experiments performed simultaneously, one at $22^{\circ}C$ and one at $4^{\circ}C$ gave significantly different results and it is assumed that the reduced intensity of fluorescence observed with the $22^{\circ}C$ organisms represented a loss of material by sloughing during the incubations.

The possibility that the sloughing observed with the 3 layer procedure represented an artifact due to the conformation of the antibody attachment was re-examined with the 2-layer system. 2 hr MS were reacted with 13 week CBA serum followed by FITC-Rab α Mo Fab (k) or FITC-Rab α Mo IgM. Fluorescence with the double layer technique was not as intense as with the triple layer procedure and only slight to moderate sloughing occurred with the anti Mo IgM; however, using anti Fab (k) antiserum, which detects all the mouse immunoglobulins, the intensity of fluorescence and the amount of sloughing was similar to that observed with the triple layer procedure.

Attempts to restain schistosomula which had sloughed their fluorescent surface material following the triple layer procedure performed at $4^{\circ}C$ or at $22^{\circ}C$, were unsuccessful. Fluorescence was observed only on fragments of the retained surface material regardless of whether 13 wk CBA sera followed by FITC Rab α Mo Fab (k) or FITC-Rab α Mo Fab (k) alone were used (Plate 11).

Ultrastructurally the sloughing schistosomula appeared normal and possessed intact teguments and heptalaminate membranes. The sloughed material had an amorphous appearance and contained no

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) following sloughing.

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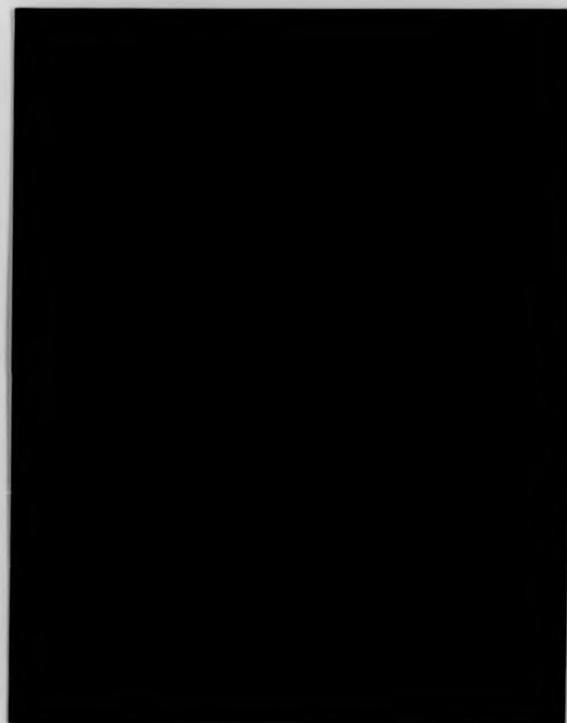
) following sloughing.
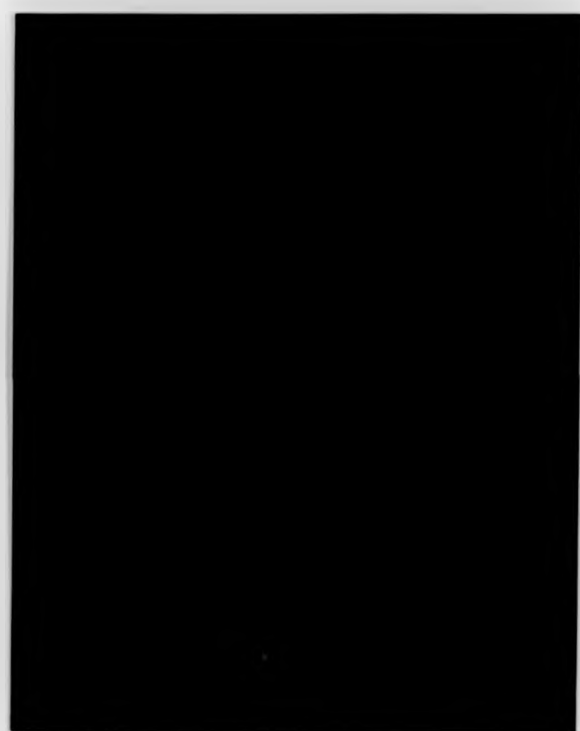
fragments of membrane or recognisable tegumental inclusions.
However detached microvilli were frequently observed which is
indicative of possible membrane damage (Plate 12).

2. Radioimmunoassays

A. ^{125}I-Protein A technique. Staphylococcal protein A (Pro A)
isolated from the cell walls of Staphylococcus aureus strain Cowan I
has a high specific affinity for the Fc region of most mammalian
IgG molecules (Kronwell et al., 1970) and has recently been used
as an immunological probe in the analysis of cell surface markers
(Dorval, Welsh and Wizzell, 1974; 1975; Ghetie et al., 1974).
Therefore, the use of ^{125}I-labelled Pro A seemed to be the logical
solution to the problem of quantitating the amount of immunoglobulin
bound specifically to the surfaces of the schistosomula at different
times after infection.

In this set of experiments ^{125}I-Pro A was substituted for the
FITC-goat anti Rab IgG in the triple layer technique described
above (System II. Materials and Methods). Careful study with the
appropriate controls suggests that ^{125}I-Pro A does not bind in
significant quantities to the surface of the schistosomula or
directly to the surface-bound mouse immunoglobulins. Several
interesting observations were made (Figures 6A   and 6B). Be-
cause the sensitivity of this method is high, non-specific absorption
of immunoglobulin onto the surfaces of the schistosomula was non
detected with normal mouse serum and normal rabbit serum. This back-
ground "noise" could not be absorbed out. The absorption procedures

PLATE 12

Sloughing of Fluorescent Surface Coat

Electron micrographs of 2 hour mechanically prepared schistosomula to show the amorphous nature of the fluorescent surface coat (c). The tegument (t) is normal in appearance.

A.   Fluorescent coat intact.   X 50,000

B.   Lifting of fluorescent coat.   X 50,000

C.   Sloughing of fluorescent coat. X 55,800

D.   Fluorescent coat totally sloughed.   X 44,500

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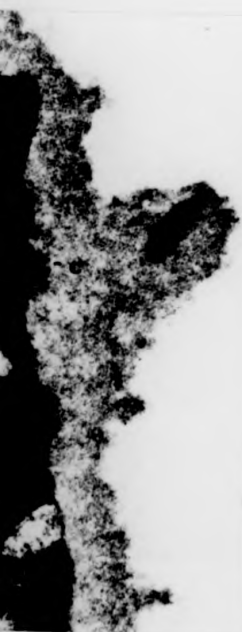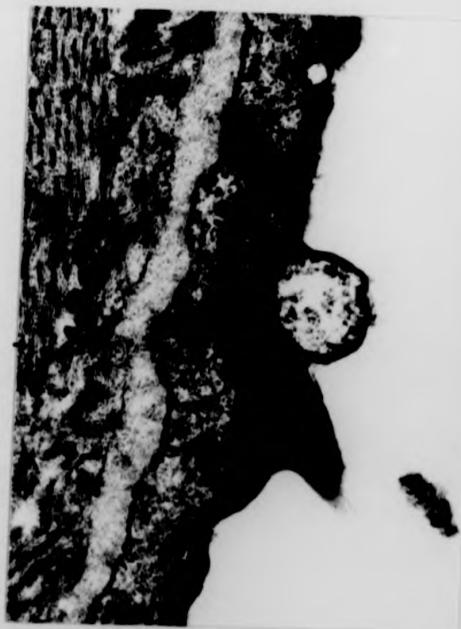
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chistosomula

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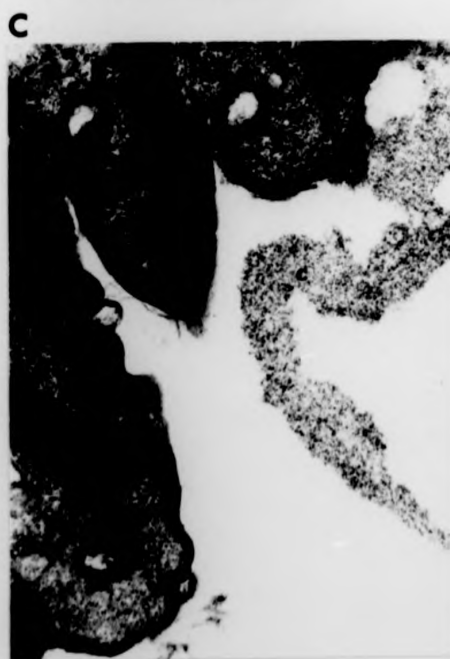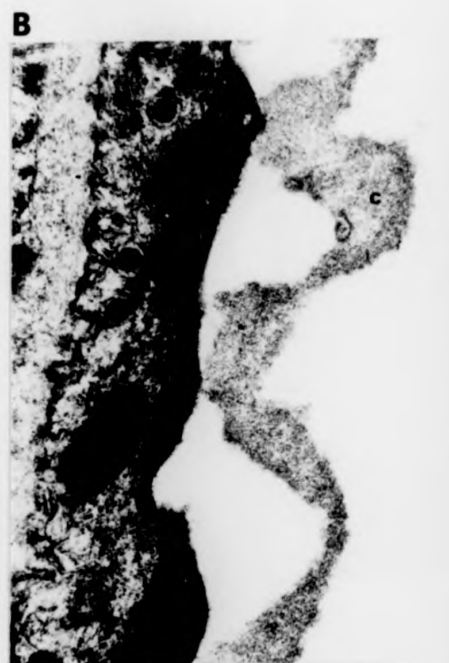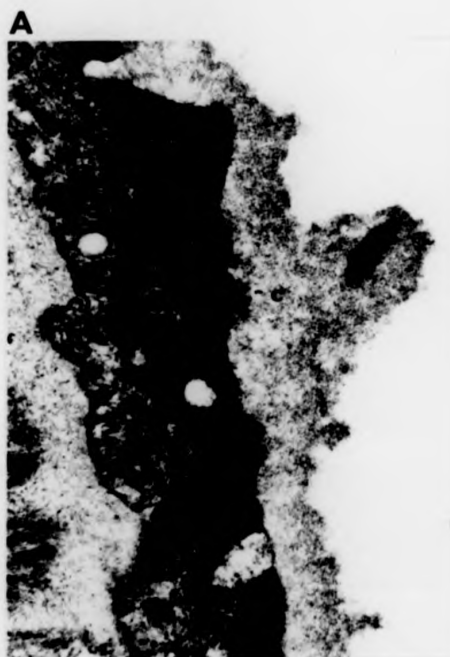
Figure 6.   <u>Radioimmunoassay.   Evolution of schistosomula-specific immuno-</u>
<u>globulins in sera from infected mice.</u>

A.   Assay of total surface-specific immunoglobulin (total Ig), IgM (μ),
IgA (α) and IgD (δ).   Normal rabbit serum (NRS) served as control
for the rabbit antiserum.

B.   Assay of surface-specific IgG (γ_1), IgG_{2a} (γ_{2a}), IgG_{2b} (γ_{2b}) and
IgG_3 (γ_3).   Normal rabbit serum (NRS) served as a control for the
rabbit antisera.

2 hr MS were reacted first with mouse sera followed by Rab anti Mo
immunoglobulin class and subclass-specific antisera and finally with ^{125}I-
Protein A.

The results are expressed as ^{125}I counts bound/1500 schistosomula
(cmp x 10^{-4}).

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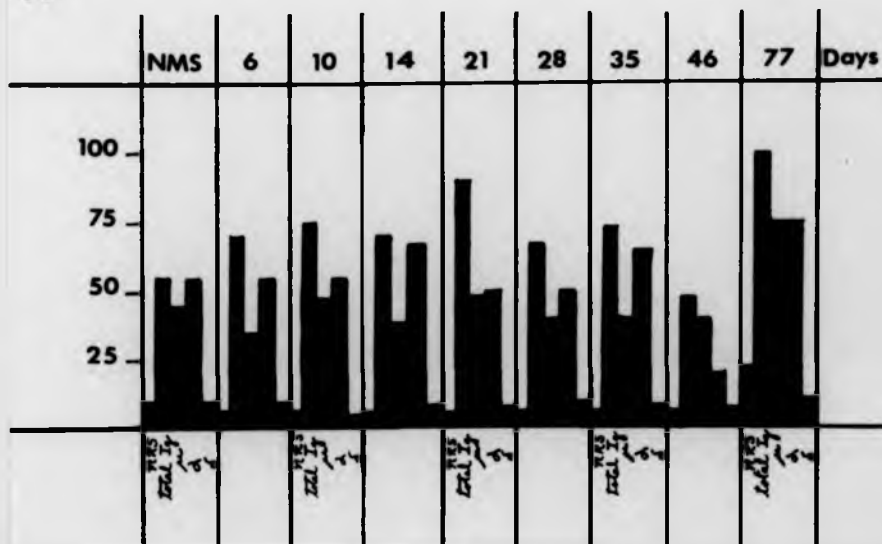
are described separately in the following sections.

The increases of total antischistosome immunoglobulins (evaluated with the Rab anti Mo Fab (k) antiserum) and the IgM and IgA levels detected by radioimmunoassay with ^{125}I-Pro A parallel those observed with immunofluorescence except that a transient decrease of the levels was observed at approximately day 45. Note the high background levels with IgA.

There appears to be little significant difference in the IgG subclass levels with this assay; however at 11 weeks the levels of IgA_{2a} are slightly higher than those for IgG_3 or the other IgG subclasses.

The IgD levels tended to be similar or only slightly higher than the background levels observed with the normal rabbit serum control. This would suggest that the ^{125}I-Pro A is detecting immunoglobulins from NRS and the anti IgD serum which are binding non-specifically to the schistosomula surface.

B. $\underline{^{125}\text{I-Goat anti Rab IgG}}$. An attempt was made to eliminate the significant background levels of non-specific binding detected with the ^{125}I-Pro A assay by substituting ^{125}I-goat anti Rab IgG for the ^{125}I-Pro A. The use of procedure did not result in a significant reduction of the non-specific binding. The results, however, do confirm those using ^{125}I-Pro A for the evolution of antibody classes and subclasses during S.mansoni infections. Table 7 represents the results of two ^{125}I-goat anti Rab IgG experiments which used the identical sera used in a ^{125}I-Pro A experiment

- (Figure 6).

    C. <u>Absorption of normal rabbit sera and rabbit anti-mouse</u>
<u>immunoglobulin antisera</u>. Quantitation of the mouse immunoglobulin
levels during the course of a <u>S.mansoni</u> infection was improved by
the ^{125}I-Pro A technique. However, when the immunoglobulin levels
were low, as in the early stages of infection or as observed with
certain immunoglobulin subclasses which did not increase significantly
during the infection, the levels of non-specific binding with normal
rabbit sera and other controls interferred with the interpretation
of the results. Therefore, attempts were made to determine the
nature of the non-specific binding. Two aspects were considered
1) that the rabbit sera contained antibodies which cross-reacted
with antigenic determinants on the parasite surface or 2) that the
schistosomula passively adsorbed rabbit immunoglobulins. The
second aspect is discussed in a later section of this Chapter.

    The first approach, consisted of absorbing the various
rabbit sera and antisera with cells and cell membranes (Materials
and Methods and Table 8). The later stage schistosomes are known
to possess on their surfaces antigens which are similar to human
blood group antigens (Dean, 1974; Goldring, 1976) and Forssman-like
determinants (Damian, 1967). Since normal sera is known to contain
natural antibodies (IgM and IgA) against blood groups and inter-
species antibodies (for Review see Mourant, 1954); the various anti-
sera were absorbed with human A and B erythrocytes and guinea pig
erythrocytes to remove any natural antibodies or anti-Forssman

89

TABLE 7. <u>Radioimmunoassay: evolution of schistosomula specific immunoglobulins in sera from infected mice.</u>

^{125}I counts bound x 10^4/1500 schistosomula

	NRS	Total Ig	μ	γ_1	γ_{2a}	γ_{2b}	γ_3	α	δ
NMS	7	45	27	5	13	10	7	32	5
Days after infection	5	51	32	5	11	12	5	44	5
14	8	51	37	7	17	8	12	57	4
21	4	70	42	6	22	9	10	43	4
28	5	60	38	4	19	9	6	43	6
35	7	70	32	7	17	7	6	62	5
46	9	47	30	6	7	5	5	18	5
77	27	95	72	12	30	17	22	67	7

90

Table 8. Absorptions of NMS, NRS, RabαMo Fab (k), RabαMo IgM, RabαIgA and RabαMoIgD - detected with ^{125}I-Pro A

% Reduction of Counts Bound to Schistosomula

Absorptions	Normal Mouse Serum	Normal Rabbit Serum	RabαMo Fab (k)	RabαMo IgM	RabαMo IgD	RabαMo IgA
Human A2 cells	Parkes Norm. 7.2%	nil	nil	nil		17.3%
	Parkes Immune 36.9%					
	CBA Norm. 7.8%	nil	nil	nil		10.35%
Human B cells	Parkes Norm. 8.0%	nil	2.6%	1.48%		nil
	Parkes Immune 21.6%					
	CBA Norm. 4.1%	nil	nil	7.75%		nil
Thymocytes			1.96%	1.92%	23.33%	

% Reduction of Counts Bound to Spleen Cells

Absorptions	Normal Mouse Serum	Normal Rabbit Serum	RabαMo Fab (k)	RabαMo IgM	RabαMo IgD	RabαMo IgA
Thymocytes Exp I			8.33%	2.36%	38.60%	
Exp II			7.40%	1.90%	36.80%	
No kidney cell membranes		nil	nil	nil	nil	nil
No kidney followed by guinea pig cells		nil	nil	nil	nil	nil

antibodies respectively. The results (Table 8) show a significant
reduction in binding of IgA to schistosome surfaces following
absorption with human A cells but not with guinea pig cells. All
other absorptions resulted in little or no reduction of binding.

In a similar experiments, NRS Rab α Mo Fab (k), Rab α Mo IgM
and Rab α IgD were absorbed with thymocytes and mouse kidney
cell membranes in an attempt to remove antibodies formed against
additional mouse determinants which might have been present in the
mouse immunoglobulins used for immunization of the rabbits. The
results, presented in Table 8   demonstrate that a significant
reduction of binding occurred only when the Rab α Mo IgD antiserum
was absorbed with thymocytes. All absorptions with kidney cell
membrane produced no significant reduction in binding.

An additional check on the specificity of the Rab α Mo IgD
antiserum was made by absorptions with foetal mouse liver cells,
which do not possess surface IgD, and adult mouse spleen cells,
a small percentage of which do exhibit surface IgD. When the antisera
were checked against cells from spleen or Peyers Patches by the
direct immunofluorescence technique, the reactivity for IgD was
completely removed by absorption with spleen cells but not with
foetal liver. Similar results observed when schistosomula and
immune mouse serum followed by Rab α Mo Fab and FITC G α Rat IgG
were used in the triple layer immunofluorescence system. However,
when ^{125}I-G α Rab IgG was substituted for the fluorescein labelled
antisera it was obvious that a percentage of the reactivity could
not be absorbed out and most likely represented non-specific binding

of immunoglobulin to the schistosomula which was then detected by the radio-labelled antiserum (Table 9).

III. Investigations of Non-Specific Binding

A. ^{125}I-Myeloma Proteins. The results of the radioimmuno-assays demonstrated significant levels of non-specific binding of sera to the schistosomular surfaces which could not be absorbed out. In order to determine what serum factors were involved in this non-specific binding a panel of fractionated and radio-labelled myeloma proteins was used in several experiments. The first experiment compared the binding of the myeloma proteins to schistosomula which had been incubated first in either buffer, or in normal or immune mouse serum. The results of the competition experiments (Table 10) suggest that several reactions take place, 1) γ_1 and γ_{2a} do not appear to bind non-specifically; 2) immunoglobulins in immune serum compete more successfully than those in normal serum for IgM binding sites; while 3) normal and immune serum compete equivalently for the binding sites of the other myeloma proteins. The binding of the ^{125}I-labelled light chains (k and l) should be noted.

1. Reduction and alkylation. Myeloma proteins MOPC 104e and MOPC 315, which are IgM λ and IgA λ_2 respectively, were reduced and alkylated to determine if reduction to monomeric conformation would affect the non-specific binding to the schistosomula surface. In two separate experiments, significant increases in binding were noted when IgM was reduced, from the pentamer to the monomer form, while reduction and alkylation of IgA appeared to decrease the non-specific binding of the protein to the schistosomula surfaces (Table 10).

93

Table 9.   Absorption of RabαMo IgD with Mouse Spleen and Foetal Liver Cells

Antisera	IF (Degree of fluorescence with FITC-GαRIgG)	RI (counts bound with ^{125}I-GαRIgG[*])
		$\underline{X\ 10^{-3}}$
Buffer only	−	2.4
Normal Rabbit Serum (NRS)	±	9.8
Normal Mouse Serum (NMS)	−	5.6
NMS + RabαIgD, untreated	±	36.0
NMS + RabαIgD, abs f. liver	±	32.2
NMS + RabαIgD, abs adult spl	±	28.4
13 wk CBA Serum (IMS)	±	16.2
IMS + RabαMoIgD, untreated	++	87.1
IMS + RabαMoIgD, abs f. liver	+	58.0
IMS + RabαMoIgD, abs adult spl	±	27.5

[*]1. 8.24×10^5 counts ^{125}I-GαRIgG added. Known to be in excess based on previous titrations with untreated RabαMoIgD and 2 hr MS.

2. Counts bound are an average of triplicate tubes containing = 250 schistosomula, with a 92% viability.

Table 10 Non-Specific Binding of ^{125}I-Myeloma Proteins

^{125}I-Myeloma Protein	% Recoverable counts		
	Buffer + ^{125}I-MP	NMS + ^{125}I-MP	IMS + ^{125}I-MP
MOPC 104 ($\mu\lambda$)	2.45	1.48	0.90
	2.36	2.08	0.84
TEPC 183 (μk)	3.63	2.41	1.30
MOPC 41 (k)	10.12	3.28	3.90
RPC 20 (λ)	5.51	1.13	1.36
MOPC 315 ($\alpha\lambda_2$)	5.60	1.30	1.02
MOPC 21 (γ_1k)	0.89	0.99	0.82
Adj PC5 (γ_{2a}k)	1.37	1.25	1.38
MOPC 195 (γ_{2b}k)	3.10	0.84	0.68
FLOPC 21 (γ_3k)	4.82	3.05	3.77

Reduction and alkylation

	Buffer + ^{125}I-MP	NMS + ^{125}I-MP	IMS + ^{125}I-MP
^{125}I-MOPC 104 untreated	2.50	1.94	0.87
" Reduc + Alkylat. Exp I	5.52		
" " Exp II	8.22		
^{125}I-MOPC 315, untreated	4.30	1.03	1.82
" Reduc + Alkylat. Exp I	2.90		
" " Exp II	1.89		

B. Studies using F(ab)$_2$ fragments. The results of the radio-
labelled myeloma protein binding experiments suggested the possibility
that schistosomula might possess receptors for the Fc portion of
immunoglobulins and thus could passively absorb immunoglobulins onto
their surfaces. To investigate this possibility, three sources of
IgG were selected: 1) Normal rabbit serum which would contain
immunoglobulin without specificity for schistosome antigens, 2) Goat
anti rabbit IgG antiserum which would contain IgG antibodies but
directed against rabbit immunoglobulin and 3) Rabbit anti mouse ery-
throcyte antiserum which would contain IgG antibodies directed against
host antigen material but not against 2 hr MS surface determinants.
The IgGs were isolated from the sera by fractionation on Protein A
columns, the Fc fragment was digested with Pepsin and the remaining
F(ab)$_2$ fragments were iodinated as described in Materials and Methods.
The ^{125}I-F(ab)$_2$ fragments were then incubated with 2 hr MS and
the percentage of counts bound to the schistosomula calculated and
compared to the percentage of counts bound of ^{125}I goat anti Rab IgG
(the whole molecule). The results are reported in Table 11.

Detection of mouse α_2-macroglobulin

Damian (1973) reported that adult worms collected from either mice
or monkeys possess mouse α_2-macroglobulin antigenic determinants on
their surfaces. Since the monkey worms had never been exposed to
mouse material, it was assumed that the worms were capable of synthesiz-
ing the mouse α_2-macroglobulin or a cross reactive determinant.
Since schistosome stages other than the adult had not been studied,
it seemed of interest to know when this determinant first appeared.

96

Table 11 ^{125}I-F(ab)$_2$ binding to 2 hr MS

Labelled antisera	Counts Recovered (%)
^{125}I-GαRab IgG	3.90
^{125}I-GαRab Ig-F(ab)$_2$	2.50
^{125}I-NRS-F(ab)$_2$	2.35
^{125}I-RαMo rbcs-F(ab)$_2$	3.60
NRS + ^{125}I-GαRab Ig-F(ab)$_2$	1.40
RαMo rcbs + ^{125}I-GαRab Ig-F(ab)$_2$	1.70
Rα Schisto + ^{125}I-GαRab Ig-F(ab)$_2$	2.30

2 hr MS were either incubated directly with Rab α Mo α_2-
macroglobulin antisera or in normal or immune mouse serum followed
by Rab α Mo α_2-macroglobulin. FITC-Goat α Rab IgG was then used to
detect the rabbit antiserum. Table 12 gives the results of the
α_2-macroglobulin studies which suggest that the surfaces of 2 hr MS
contain an antigenic determinant which is recognized by the Rab α Mo-α_2
macroglobulin antisera. Incubation of schistosomula in immune mouse
serum significantly increases the binding of the rabbit antiserum
while incubation in normal serum does not. The activity of the
rabbit anti α_2 macroglobulin antiserum can be absorbed out on an
α_2-macroglobulin-Sepharose column (see Table 12).

These results have been confirmed by a radioimmunoassay in which
^{125}I-Protein A replaced the FITC-Goat α Rab IgG and by a competition
experiment using a radio-labelled α_2-macroglobulin and normal and
immune serum. However, it was noted ^{125}I-α_2-macroglobulin can be adsorbed
by the living schistosomula and that this adsorption is inhibited
by prior incubation in mouse serum.

V. Comparison of lymphoid cell surface immunoglobulin from normal

a   and infected mice.

A comparative study was made of the surface immunoglobulins on
cells from the various lymphoid organs in normal and infected mice.
The rabbit anti mouse immunoglobulin class and subclass antisera were
used with the ^{125}I-Pro-A procedure. Cells were processed as
described in Materials and Methods, titres of all the antisera and
the ^{125}I-Pro A were made with normal spleen cells and the % re-
coverable counts was estimated for 5×10^7 cells/ml from each lym-
phoid organ. The levels of total immunoglobulin were increased

Table 12. Mo α_2-macroglobulin detection by System I and II Immunofluorescence of Mechanical Schistosomula, living or formalin fixed

Antisera	Description of Fluorescence	
	Living Schistosomula	Formalin Fixed Schistosomula
Buffer + FITC-GαR IgG	-	-
NRS + FITC-GαR IgG	-	- $\pm$
RαMo α_2-m + FITC-GαR IgG	+, sloughing	+, no sloughing
RαMo α_2-m abs* + FITC-GαR IgG	- $\pm$	- $\pm$
RαMo α_2-m eluted* + FITC-GαR IgG	+, sloughing	+, no sloughing
Buffer + Buffer + FITC-GαR IgG	-	-
NMS + Buffer + FITC-GαR IgG	-	-
NMS + NRS + FITC-GαR IgG	-	- $\pm$
NMS + RαMo α_2-m + FITC-GαR IgG	+, sloughing	+, no sloughing
IMS + Buffer + FITC-GαR IgG	-	-
IMS + NRS + FITC-GαR IgG	$\pm$	$\pm$
IMS + RαMo α_2-m + FITC-GαR IgG	++, sloughing	++, no sloughing
IMS + RαMo α_2-m abs*+ FITC-GαR IgG	$\pm$ +	$\pm$ +
IMS + RαMo α_2-m eluted*+ FITC-GαR IgG	++, sloughing	++, no sloughing

* Affinity Chromatography technique

Moα_2-macroglobulin-Sepharose column

RαMo α_2-macroglobulin antisera through column x 2

1st peak - No α-α_2-m   "RαMo α_2-m abs"

   eluted with 1M Acetic Acid

2nd peak - purified α-α_2-m   "RabαMo α_2-m eluted"

NMS = Normal CBA sera

IMS = Sera from CBA mice infected c̄ 30-40 cercariae for 13 weeks

significantly.   However, only slight increases were observed in any of the individual classes and subclasses;   IgM represented the most notable increase (Table 13).

DISCUSSION

Summary

In this section, sera from mice infected with S.mansoni have been shown to contain immunoglobulins which bind to the surface of 2 hr MS. However, the levels of antibody detected and/or the specificity of their binding depended on a number of factors, including 1) the strain of mice infected, 2) the numbers of cercariae used in the initial infection, 3) whether the MS were incubated under conditions where sloughing of the external coat could occur, 4) the relative sensitivities of the direct and indirect immunogluorescence and radioimmunoassay techniques used to measure immunoglobulin interaction with the schistosome surface and 5) the class and subclass of immunoglobulin studied. These factors are discussed in detail below.

I. Differences between strains of mice

Using indirect immunofluorescence techniques, immunoglobulin binding of the surface of MS was first detected 2-3 weeks after infection of CBA mice with 30-40 cercariae. Using Parkes mice, infected at the same level, immunoglobulin binding was detected earlier, after 10 days. Increased levels of total immunoglobulin occurred earlier when larger numbers of cercariae were used for infection of both mouse strains. Significant increases in the

Table 13.   Levels of Cell-Bound Immunoglobulin in Spleens of Normal
or 14 wk infected CBA mice determined by ^{125}I-Pro A binding
to Rab anti Mouse immunoglobulin antisera

	% Recovery			
	Normal		**Infected**	
Buffer + cells	0.70		0.60	
NRS	3.6		5.57	
RabαMo Fab (k)	1:5	1:10	1:5	1:10
	11.5%	9.7%	17.9%	15.8%
RabαMo IgM	4.86		5.95	
RabαMo IgA	4.6		4.1	
RabαMo IgD	5.7		5.9	
RabαMo IgG$_1$	4.3		3.9	
RabαMo IgG2a	3.2		3.3	
RabαMo IgG2b	3.6		3.4	
RabαMo IgG3	4.2		3.9	

levels of immunoglobulins could not be detected in the sera of
CBA mice infected with 30-40 cercariae before week 5. After this
time, the levels of IgM, IgA and the IgG subclasses increased
gradually during the remaining weeks of the experiment.

Mouse strain differences. It is known that the capacity of an
animal to mount an immune response is influenced by hereditary
factors (reviewed by McDewitt and Benacerraf, 1969; Green, 1974).
Specific immune responses are controlled by immune response (Ir)
genes linked to the histocompatibility gene complex; the Ir genes
are expressed in T-cells which have a carrier recognition function
in humoral immune responses.

By selective breeding, Biozzi et al., (1970) developed two
lines of mice, known as low responders and high responders,which
differed in the levels of antibody they produced. Although
selection was based on response to sheep erythrocytes, the two
lines also differed in the magnitude of their humoral antibody
response to a number of unrelated antigens.

Two recent reports have suggested that CBA mice may be low
responders to certain antigens. Firstly, Minga, Segre and Segre
(1975) studied the immune response of eleven mouse strains in
relation to the numbers and avidity of anti-DNP antibody plaques
they produced; the response of CBA mice was significantly lower
than that of 2/3 of the other mice strains studied. Secondly,
Warr and James (1975) used a similar technique to study the
adjuvant effect of Corynebacterium parvum on the production of

classes and subclasses of antibody specific for sheep erythrocytes. CBA mice exhibited the lowest IgM response of the 3 strains of inbred mice studied.

The present comparative study of antibody development in Parkes and CBA mice suggests that with similar levels of S.mansoni infections CBA mice produce lower levels of specific antibody later in the course of a schistosome infection than do Parkes mice.   Further studies are necessary to determine the significance of this finding particularly since CBA mice are frequently used for studying immunity to S.mansoni infection.

II.   Immunofluorescence techniques

Since immunofluorescence techniques were first introduced and standardized (Coons et al., 1942; Coons and Kaplan, 1950), they have become one of the most useful and widespread immunological procedures available. The dye most frequently used for antibody labelling is fluorescein iso thio cyanate (FITC), introduced by Riggs et al., (1958). This highly fluorescent compound can be covalently bound to immunoglobulins or other proteins. The resulting conjugates have a high fluorescent yield and emit at a wavelength to which the human eye is very sensitive and which is substantially different from the background fluorescence of most tissues. Because of the availability and widespread and defined use of this technique direct and indirect fluorescence techniques were chosen for the initial studies of antibody development during a schistosome infection and to demonstrate the interaction of these antibodies with the living schistosomula surface.

As noted in previous studies, increased intensity and
uniformity of fluorescence were obtained when schistosomula
were formalin fixed prior to incubation with antiserum. Several
factors could contribute to these differences in surface fluorescence
of living and fixed organisms: 1) formalin fixation causes
contraction of the schistosomula, which might artificially bring
the surface antigens closer together and thus cause the resulting
fluorescence to appear brighter and more uniform 2) the fixation
procedure prevented the sloughing of the fluorescent surfaces
which occurs with living organisms so that the entire original
fluorescent material is retained and 3) formalin may make the
antigenic sites more readily available for antibody binding. The
differences in the intensity of fluorescence between living and
fixed organisms probably result from a combination of these
factors but there is no evidence at present to distinguish
between the possibilities. Whatever the mechanism, the use of
formalin fixed whole schistosomes or acetone treated frozen
sections as substrates in immunofluorescence procedures enables
antibodies to be detected at much higher serum dilutions than
when living organisms are used (Kein-Truong, Sarasin and Ambroise-
Thomas, 1970; personal observations).

Although formalin fixation allows more precise quantitation
and consistancy in measuring levels of immunoglobulin, the use of
living schistosomula permits observation of the dynamic interaction
of antibodies and the schistosomula surface. For this reason it
was felt that this method is more representative of the actual

in vivo interaction of antibodies and schistosomula and thus has more relevance to the questions being asked (see Introduction). Living organisms were therefore used in all subsequent experiments.

The study of living worms by immunofluorescence was given greater sensitivity and flexibility by substituting a triple layer procedure for the original double layer method. In this way, the levels in the infected serum of the various classes and sub-classes of immunoglobulin could be semi-quantitated by using, in the second layer, specific rabbit antisera directed against the different classes and subclasses of mouse Ig (see Methods). A fluorescent goat anti-rabbit IgG antiserum was used in the third layer.

The initial experiments used these specific rabbit anti-mouse class and subclass antisera to study differences in the development of specific anti-schistosome immunoglobulins in the sera of CBA and Parkes mice which had been infected with varying numbers of cercariae (30-40, 150 or 500 cercariae/mouse) (Figures 4 and 5).

The use of increasing numbers of cercariae for infecting the two strains of mice was correlated with the appearance of sig-nificantly higher levels of immunoglobulin earlier in the course of the infection (Figures 4 and 5). This phenomenon was also observed by Phillips et al., (1975) when they infected rats with varying cercarial numbers.

105

III. Radioimmunoassays

The results obtained with the triple layer immunofluorescence technique were, for the most part, confirmed and extended by the use of the ^{125}I-goat α Rab IgG and the ^{125}I-Protein A radioimmuno-assays. Several interesting observations were made with these radioimmunoassays by using as the second layer the specific rabbit anti-mouse immunoglobulin class and subclass antisera as described above. Firstly, there was a late appearance and gradual increase in both IgM and IgA levels which persisted to the end of the experiment. Secondly, there were variable but relatively low increases in the levels of the IgG subclasses. Finally, low levels of IgD were detected in the sera from infected mice

A. **IgM response.** IgM has frequently been reported as the first detectable antibody following exposure to antigen (Uhr, 1964; reviewed by Lawton, Kincade and Cooper, 1975). However, these observations must be considered with caution because methods such as haemagglutination and complement fixation are more sensitive in detecting IgM than IgG (Rowe, 1975; Coombs and Smith, 1975). At present, it is generally accepted that the initial appearance of IgG and IgM is dependent on the antigens involved and that detectable levels of either one may precede the other. IgM antibodies tend to be catabolized more rapidly than IgG (Rowe, 1975), so that the gradually increasing level of IgM observed throughout the S.mansoni infection suggests the presence of con-tinued antigenic stimulus. Possible sources of antigens include

106

surface membrane lost as a result of normal membrane turnover

(Kusel et al., 1975), and eggs which are first produced about

week 6 in the infection and have been shown to contain antigens

which cross react with adult worm antigens (Capron, 1965) and

therefore possibly with schistosomula antigens. Another source

of antigenic stimulus may be metabolic products released in

relatively large quantities during egg laying by the sexually

mature worms.

B. IgG$_1$ response

The mouse IgG$_1$ subclass has been shown to be more T-cell

dependent than any of the other immunoglobulin (Torrigiani,

1972; Dresser and Phillips, 1973) and an IgG$_1$ response is markedly

enhanced (Torrigiani, 1972; Dresser, 1972; Dresser and Phillips,

1973) by adjuvants postulated to act on T-cells (Dresser and

Phillips, 1973). Schistosomula have been shown to activate the

helper T-cell response in mice and rats as measured by plaquing

tests (Ramalho-Pinto, De Souza and Playfair, 1976; Ramalho-Pinto

et al., 1976a). Other antigenic preparations such as formalin-

fixed cercariae, miracidia, egg, adult worm surface membrane and

adult worm culture fluid all share common carrier components with

the schistosomula (Ramalho-Pinto et al., 1976b). Therefore the

late but significant increase in total IgG$_1$ levels (Sher, 1977)

could be related to the massive helper T-cell activity components

of the later S.mansoni infections. IgG$_1$ from myeloma proteins

does not appear to bind non-specifically to schistosomula, nor

do the levels of specific IgG$_1$ reach those of the other IgG sub-

classes as measured by Protein A radioimmunoassay.

It is also of interest that the serum immunoglobulin levels at the peak of a S.mansoni infection (approximately week 12) are higher (Sher, 1977) than those levels resulting from myeloma protein plasmocytomas (Potter, 1967). This could represent continued antigenic stimulus as the different stages of the parasite develop and the resulting antigens are available to the host immune system.

Mouse IgG_1 and IgE are both homocytotrophic (Reaginic) antibodies (Nuzzenweig, Merryman and Benacerref, 1964) and attached by Fc receptors to mast cells and basophils. If other similarities can be implied then the increased levels could be due to nonspecific immunopotentiation as seen with IgE levels in rats infected with the helminth Nippostrongylus brasiliensis (Jarrett and Bazin, 1974; Jarrett and Ferguson, 1974). The fact that only a small portion (8-30%) of the elevated immunoglobulin level is specifically absorbed parasite antigens, adult and egg homogenates (Sher, 1977) would tend to support the hypothesis of non-specific immunopotentiation.

The level of mouse IgG_1 has been reported to be increased with several helminth infections including Ascaris suum or Trichinella spiralis (Crandall and Crandall, 1971; Crandall and Crandall, 1974) or with murine Heligmosomoides polygyrus infections (Crandall, Crandall and Franco, 1974). IgG_1 has been implicated

as the immunoglobulin responsible for passive transfer of immunity
in S mansoni infection (Perez, 1974).   Sher (1977) has reported a
significant increase in total mouse IgG_1 levels in murine S.mansoni
infections during the later stages of infection. He has suggested
that this increase might result from the immunopathology of granuloma
formation. Antibodies of the IgM and IgG_1 class and subclass
respectively have been reported in the granulomas of mice infected
for 8 weeks (Houba, personal communication); therefore, the decreased
immunoglobulin levels observed with the ^{125}I-Pro A radioimmunoassay
at approximately 45 days may represent sequestering of the antibodies
in the newly formed granulomas with a subsequent but temporary
lower level in the serum. However, the synthesis of the antibodies
is subsequently increased and the serum immunoglobulin levels
return to elevated levels by day 70.

C. Detection of IgD binding.   The detection of IgD in serum
from infected mice is interesting because this immunoglobulin,
which has not been shown to have specific antibody properties in
humans, is not present in detectable amounts in normal mouse serum
(Abney and Parkhouse, 1974), nor has a mouse myeloma producing IgD
been found. The IgD used to raise the Rab anti Mo IgD for the
fluorescence assays was isolated from spleen cell membranes
(Abney and Parkhouse, 1974). Although most of the activity in the
antiserum could be absorbed out with adult spleen cells but not
with foetal liver cells, which suggests that the antiserum was
specific for mouse IgD and not simply mouse determinants, the background
levels as measured with control sera remained high. This could not

be accounted for by the antiserum controls. So far, no function has been determined for the IgD as part of the immune response against schistosomula.

D. Non-specific binding of immunoglobulins. The significant levels of non-specific binding observed with the normal rabbit sera and other control sera were studied from several aspects. The first studies showed that the Rab α Mo Ig antisera were specific for mouse immunoglobulin and contained little or no absorbable cross-reactive antibodies. This was not unexpected because of the exacting nature of the production of the antisera and the fact that they were shown to be specific by immunodiffusion, radioimmuno-assay and other tests prior to their use in this study (Parkhouse, personal communication). However several antigens have been observed on the surfaces of schistosomes which could possibly react with natural antibodies in rabbit serum, for example, bacterial antigens, human blood group antigens and certain serum factors so the absorptions were justified.

The second set of experiments were carried out to determine if there was non-specific adsorption of immunoglobulins by the schistosomula surface. Kemp (1976) has demonstrated that adult schistosomes have immunoglobulins adsorbed onto their surfaces and in a recent paper Kemp et al., (1977) showed that immunoglobulins directed at unrelated but specific immunogens could be adsorbed non-specifically on to the adult worm surface; IgG_{2b} being in the highest concentration of the non-specifically adsorbed immuno-globulins.

110

Studies using ^{125}I-myeloma proteins and the appropriate
competition experiments with normal and immune sera have shown
non-specific adsorption by the schistosomula surface. The amount
of adsorption and the degree to which either type of sera inhibited
adsorption varies with the myeloma protein. However, schisto-
somula were shown to bind non-specifically IgG$_{2b}$ of the IgG
subclasses and IgM and IgA. Further studies using electron dense
labelling and cross competition experiments with various myeloma
proteins might clarify the nature of this binding.

IV. Sloughing of surface material

In the course of these studies, sloughing of the fluorescesing
material from the surfaces of living 2-3 hr schistosomula occurred
when the organisms were incubated at 37°C or 22°C. Ultrastructurally
the tegument of the sloughing organisms appeared intact; but
microvilli, which at this developmental stage are associated with
surface damage, were present in the sloughing material.

The use of living organisms in the immunofluorescence studies
and the subsequent observation of sloughing or shedding of the
fluorescent surfaces extend the observation that schistosomes
are capable of membrane turnover. Increased membrane turnover
rates and surface damage have been observed when schistosomes
are incubated for extended periods in sera from immune or immunized
animals (Kusel and Mackenzie, 1972; Perez and Terry, 1973;
McLaren, Clegg and Smithers, 1975). The use of fluorescein-conjugated
antisera in combination with radiolabelled antisera would offer a
method for observing this phenomenon and might make possible
characterization of the antibody class or subclass and the antigen

involved in this reaction.

The sloughing of surface fluorescence with living organisms must be considered in any comparison of immunoglobulin levels or of various sera. Sloughing is definitely increased at higher antibody concentrations and with antisera against total immunoglobulin levels (Rat α Mo Fab(k). Sloughing was not observed to the same extent with IgA as with the other immunoglobulin classes; although the serum concentrations of IgA were higher than for the four IgG subclasses and almost as high as for IgM.

Considered together, non-specific binding and sloughing of immunoglobulins by schistosomula surfaces suggest the adaptive nature of the schistosome and its ability to survive against the host immune response. Further studies of these phenomenon could possibly extend the knowledge of the evasion mechanism.

V. **Mouse α_2-macroglobulin**

Mouse α_2-macroglobulin or an antigenically similar determinant was detected by a direct fluorescence proceudre on the surfaces of 2 hr MS which had never been exposed to mouse tissue or sera. This observation was confirmed and extended by the use of the specific Rab anti Mo α_2 macroglobulin antiserum in a radioimmuno-assay. These findings suggest two possibilities: firstly Damian's observation (Damian, Green and Hubbard, 1973) that adult schistosomes synthesize mouse α_2-macroglobulin can be extended to the early schistosomula stage and would thus represent confirmation of synthesis rather than acquisition of the mouse α_2-macroglobulin

112

because 2 hr MS have had no contact with mouse serum or tissue. Secondly the Rab anti Mo α_2 macroglobulin antiserum could have bound non-specifically to the surfaces or the antisera recognized cross-reactive CHO groupings between the α_2 macroglobulin molecule and other immunoglobulins or surface components. However the absorption studies would suggest that the antisera was specific for mouse α_2-macroglobulin molecules.

VI. Cell surface immunoglobulins

Studies of surface immunoglobulins on rat lymphoid cells by Williams (1975) have shown increased levels of IgG_2 on spleen and cervical lymph node cells of rats kept in routine animal houses as opposed to rats kept under sterile conditions. Also few IgA positive cells are found in the spleens of rats. Thus Williams (1975) suggests that the IgA and IgG_2 positive cells occur only after a recent antigenic stimulus and the cell bound IgM levels are usually identical to those for total immunoglobulin (Williams, 1975; Strober, 1975). The immune status of an animal as well as the source of lymphocytes can have a large effect on the result in studies in the class of cell surface immunoglobulin. Also anomalous binding of antibody can occur unless $F(ab)_2$ antibody fragments are used (Jendenius and Williams, 1974; Winchester et al., 1975).

In retrospect, the comparative studies of lymphoid cells in normal and infected mice would have been more informative if germ-free animals had been employed and if $F(ab)_2$ fragments had been used for the study. However a significant difference in the level total cell bound immunoglobulin and IgM were noted.

113

CHAPTER 4: IMMUNOLOGICAL INVESTIGATION OF THE SCHISTOSOME

SURFACE COMPONENTS AT DIFFERENT STAGES OF DEVELOPMENT

Introduction

The concept of concomitant immunity in schistosomiasis (Smithers
and Terry, 1969) implies that the host immune response is effective
against the schistosomula of a challenge infection. This hypothesis
is supported by lung recovery experiments; the numbers of challenge
schistosomula recovered from the lungs of immune animals is reduced
when compared to those recovered from normal animals (Perez, Clegg
and Smithers, 1974; Sher, Mackenzie and Smithers, 1974; Smith and
Clegg, 1976).

The concept of concomitant immunity also suggests that the
young schistosomula develop a mechanism (or mechanisms) for evad-
ing the host immune response. Although the mechanisms of evasion
remain theoretical, it is becoming increasingly evident that they
are effective soon after the schistosomula are established within the
host. For example, passive transfer of immunity with serum from
infected animals cannot be demonstrated if the serum is administered
3-4 days after a challenge infection, although earlier serum
administration is effective (Sher, Smithers and Mackenzie, 1975;
Phillips, Reid and Sadun, 1977). Also, in contrast to newly trans-
formed schistosomula, schistosomula which have been maintained in vivo
or in vitro for 24 to 44 hours are not destroyed in the lungs when
injected intravenously into immune mice (Sher, 1977), and lethal
antibody which kills young schistosomula in vitro has no effect
on schistosomula which have been maintained in culture for 4 days or

have been recovered from the lungs of mice 4 days after an infection (Clegg and Smithers, 1972). Furthermore, the antibody mediated adherence of eosinophils, demonstrated with newly transformed schistosomula, does not occur when schistosomula recovered from the lungs are used as the target (Ramalho-Pinto, personal communication).

This loss of susceptibility to the effects of the immune response suggests that changes may occur in the surface of the developing schistosomula either as a result of modification by extrinsic factors such as the acquisition of host antigens or immunoglobulins which mask parasite antigens, or as a result of intrinsic changes in the structure, chemical composition or turnover of the parasite surface.

Therefore a comparative study of the immunology and biochemistry of the surfaces of schistosomes at different stages of development was undertaken. The stages chosen for study were mechanically prepared schistosomula (MS), which served as reference organisms, schistosomula collected 3 hours after penetration of isolated mouse skin (SS) or after a 5 day incubation in a medium containing human serum and erythrocytes (CS), schistosomula recovered from mouse lungs 5 days after a cercarial infection (LS) and adult males and females perfused from the hepatic portal system of mice 6 weeks after infection. In addition, MS and SS were studied after in vitro cultivation in various media for different periods of time.

This Chapter describes the interaction of the surfaces of these various schistosome stages with:

1) total anti-schistosome immunoglobulin and IgM present in the
   sera of mice collected at different times after a cercarial
   infection

2) specific antisera against a number of mouse antigenic determinants

3) specific antibody against mouse α_2-macroglobulin and

4) specific antisera against human blood group determinants and
   IgG and IgM

The results of the biochemical studies are presented in
Chapter 5.

Materials and Methods

I. Preparation of schistosomula stages

    A. Cercariae.   Described in Chapter 2.

    B. Mechanical schistosomula (MS). Described in Chapter 2.

    C. Skin penetrated schistosomula (SS). Described in Chapter 2.

    D. Cultured schistosomula (CS). The basic culture procedure
is described in Chapter 2. 5 Day CS used in the initial experiments
were SS cultured for 5 days in either an A or B homologous system;
the media was changed at Day 3.   Later experimental modifications
of media and incubation are noted in the Results section of this
Chapter.

    E. Lung schistosomula (LS). (Plate 13). CBA mice infected with 1000
cercariae 5 days previously were killed by chloroform and the body
cavity opened immediately to expose the heart and lungs. The lungs
were perfused by injecting 10 ml Hanks containing 10 units Heparin/ml,
pH 7.4 into the right ventricle which caused the lungs to expand
and become white due to the removal of the blood from the capillaries.
The lungs were dissected out, washed with 5 ml heparinized Hanks,
placed in a sterile universal bottle and cut into 1-2 m^3 pieces with

PLATE 13. <u>Extension of lung schistosomulum</u>

Schistosomulum collected from the lungs of a CBA mouse infected
for 5 days.

X 365

Lung schistosomula continue this form of expansion during the first
48-72 hours in culture. This expansion process was not observed with
the other schistosomula (MS, SS or CS) at any stage of cultivation.

a CBA mouse infected

xpansion during the first

was not observed with

tage of cultivation.

a CBA mouse infected

expansion during the first

was not observed with

stage of cultivation.
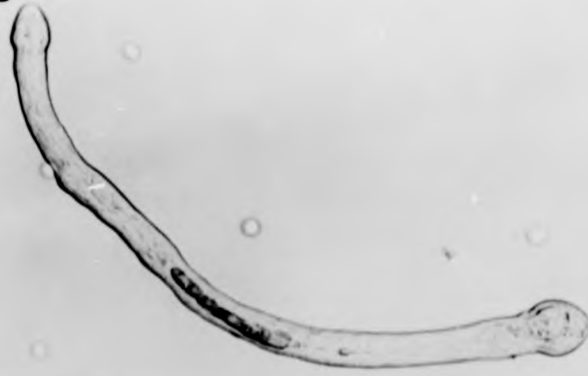
finely pointed scissors. The pieces of lung were covered with
10 ml of heparinized RPMI or Hanks, and incubated at $37^{o}C$ for
3 hours. After this time the contents of the universal were gently
mixed by swirling and filtered through two stainless steel sieves
(mesh size 36/in followed by 60/in). The filtered suspension was
centrifuged for 3 minutes at 800 rpm, the supernatant fluid
carefully removed, and the pellet resuspended and washed as above
two additional times. Schistosomula suspensions obtained by this
procedure were not noticeably contaminated by erythrocytes or
pieces of lung. The organisms were either used immediately or
incubated in fresh RPMI or ELac at $37^{o}C$ for a defined period of time.

F. <u>Adult worms</u>. Adult <u>S.mansoni</u> worms were obtained from the
hepatic portal systems of infected mice by the perfusion procedure
described by Smithers and Terry (1965). Mice were killed with 0.2 ml
Nembutal injected intraperitoneally, dipped in warm water to wet
the hair and then skinned to expose the thorax and abdomen. The
thoracic and abdominal cavities were opened and the rib cage
removed. The mice were then attached by means of spring clips to a
vertical sheet of perspex. The hepatic portal vein was opened and
heparinized - Hanks was then forced into the left ventricle of the
heart. Worms which were expelled through the opening in the vein
were recovered on a 100 mesh stainless steel wire screen suspended
below the animal. Well-perfused mice had pale-coloured kidneys and
liver after approximately 1 minute of perfusion.

The worms were collected by inverting the wire screen in a large gridded petri dish which contained ELac and then either counted macroscopically or on a Nikon stereoscope, or transferred to universal bottles which contained ELac, 37°C. The worms were washed 3 times by sedimentation through ELac, 37°C and either used immediately or incubated for the times noted in the various experiments.

II. Sera and antisera

A. Serological analysis of the human blood used for culture procedures. The serological typing of the blood was done by Mr I. Thompson, Army Blood Supply Depot, Aldershot.

	ABO	Rhesus	Minor anigen grouping								
			M	N	S	P_1	Lu^a	K	Duffy	Le^b	Kidd
(LW)	A_1	R_1R_1	+_	+	-	+	-	-	+_	+.	+_
(KG)	A_1	R_2R_2	+	-	+	+	-	-	+	+	+
(LB)	B	R_1r	+	+	+	+	-	-	-	+	+

B. Monospecific human antisera. Commercially available human bood group typing sera are prepared by hyper-immunizing human male volunteers with human B erythrocytes or a A blood group-like substance isolated from pig stomach. The IgG fractions of the immunoglobulins raised against these two immunogens are then purified and used for the typing of erythrocytes.

All antisera were either centrifuged at 14,000 rpm for 10 minutes or millipore filtered prior to use.

Human anti-A (Hu α A)
Human anti-B (Hu α B)   Ortho Pharmaceutical Company

FITC-Rabbit anti Human IgM (Rab α Hu IgM)

Wellcome Research Laboratories

FITC-Rabbit anti Human IgG (Rab α Hu IgG)

C. Rabbit anti-mouse antisera. All antisera were heat inactivated
at $56^{\circ}C$ for 30 minutes and centrifuged at 14,000 rpm for 10' or
millipore filtered prior to use.

Rabbit anti-mouse erythrocyte antiserum (Rab α Mo rbc)
(supplied by Dr D.W. Dresser, National Institute for Medical
Research, Mill Hill)

Rabbit anti-mouse whole serum antiserum (Rab α NMS)
(supplied by Dr D.W. Dresser, National Institute for Medical
Research, Mill Hill).

Rabbit anti-mouse whole immunoglobulin (Fab(k)) antiserum
(Rab α Mo Fab(k). Described in Chapter 3.

Rabbit anti-mouse immunoglobulin class and subclass specific
antisera (Rab α Mo IgM, Rab α Mo IgA etc.). Described in
Chapter 3.

Rabbit anti-mouse α_2-macroglobulin antiserum (Rab α Mo α_2-macro-
globulin antiserum (Rab α Mo α_2-macroglobulin). Described in
Chapter 3.

Rabbit anti-mouse C3 antiserum (Rab α Mo C3)
Gift of Dr J.H. Humphrey (National Institute for Medical Research,
Mill Hill).

D. <u>Mouse anti-schistosome antisera</u>.   All antisera were heat
inactivated at 56°C for 30 minutes and centrifuged at 14,000 rpm
for 10 minutes or Millipore filtered prior to use.

6 Day CBA, 150 cerc. serum

4 Wk CBA, 150 cerc. serum

7 Wk CBA, 150 cerc. serum

13 Wk CBA, 150 cerc. serum

Described in Chapter 3.

III. <u>Immunofluorescence procedure</u>

Described in Chapter 3. For the experiments in this Chapter
the degree of fluorescence was recorded as follows:

<u>intensity</u>

++++   very bright intensity

+++   moderate to bright intensity

++   moderate intensity

+_   weak to moderate intensity

±   weak intensity, the occasional organism within

a group under observation was negative

−   autofluorescence or ingested material only

<u>Distribution of surface fluorescence</u>

uniform − unbroken outline or complete fluorescing surface

patchy − moderate sized areas of fluorescence on the surface

pin point − very small areas or dots of fluorescence, frequently

seen near the tubercles.

<u>Loss of surface fluorescence</u>

sloughing − large sections of the fluorescing surfaces were

lost to the medium as the organisms moved

shedding - very small fragments of fluorescing material

were lost to the medium as the organisms moved.

Only living motile organisms were assessed for fluorescence.

IV. Radioimmunoassay: ^{125}I-Goat α Rab IgG.

Described in Chapter 3.

V. Mixed agglutination

The principle of the mixed agglutination test (Coombs, Bedford, and Rouillard, 1956) is the formation of mixed aggregates of two cell types by antibody reacting with similar antigenic determinants on the surfaces of the different cells. In this study, schistosomula were aggregated with human erythrocytes by an antibody directed at common surface determinants as described by Dean (1974).

The various schistosome stages were washed 3 times in ELac-O.2% BSA by centrifugation at 1000 rpm for 30 seconds and then incubated at 22°C for 20 minutes with antiserum to either human A or B blood group antigens. The organisms were then washed 3 times in ΓLac-O.2% BSA and 50 μl of a 2% suspension of washed human A or B type erythrocytes were added. The cells and schistosomes were sedimented by slow centrifugation at 700 rpm for 30 seconds and then incubated at room temperature for 15 minutes. The pellet of schistosomula and cells was gently resuspended and poured onto a 2 in x 3 in microscope slide. A Nikon stereoscope was used to scan for attachment of the erythrocytes to the surface of the schistosomes (positive reaction). Questionable positives and all negative reactions were

examined further with a Zeiss inverted microscope.

Results

I. Immunofluorescence

A. Detection of anti-schistosome specific total immunoglobulin
and IgM. The triple—layer, indirect immunofluorescence procedure
described in Chapter 3 was used to study the surface reactions of
the various schistosome stages with sera from CBA mice infected
with 150 cercariae for 6 days, 4, 7 and 13 weeks. Following
incubation in mouse serum the schistosomes were reacted with either
Rab α Mo Fab(k), which detects total mouse immunoglobulin, or with
Rab α Mo IgM, and then washed and incubated with FITC-Goat α Rab IgG.
The results are recorded in Table 14.

Several significant differences between the various schisto-
some stages were noted in the degree and distribution of the mouse
immunoglobulin binding, as detected by the appearance of the surface
fluorescence.

1) With the 6 day serum, cercariae, MS and SS exhibited a weak,
patchy fluorescence without sloughing, while the LS, CS and adults
showed no fluorescence at all.

2) With later sera, there was a marked increase in the intensity
of fluorescence exhibited by cercariae and MS. SS, on the other hand,
showed only a limited increase and LS, CS and adults exhibited even
less.

3) With the later sera cercariae exhibited no sloughing or
shedding, MS showed extensive sloughing, while shedding was observed

Table 14 Binding of total immunoglobulin and IgM from infected mouse serum to the surface of various schistosome stages as detected by immunofluorescence

	6 Day CBA, 150 cerc	4 wk CBA 150 cerc	7 wk CBA 150 cerc	13 wk CBA 150 cerc
Cercariae	total* and IgM $\pm$ patchy no sloughing	total and IgM +++ uniform no sloughing	total and IgM +++ uniform no sloughing	total and IgM ++++ uniform no sloughing
2 hr MS	total and IgM $\pm$ patchy no sloughing	total and IgM ++ uniform some sloughing	total ++ IgM + patchy slight sloughing with total	total and IgM +++ uniform prior to excessive sloughing
3 hr SS	total and IgM $\pm$ patchy no sloughing	total and IgM + patchy no sloughing	total and IgM + patchy to uniform slight shedding	total and IgM ++ patchy shedding
5 Day LS	total and IgM -	total and IgM $\pm$ patchy or pinpoint no sloughing	total and IgM $\pm$ + patchy or pinpoint slight shedding	total and IgM + patchy or pinpoint shedding
5 Day CS	total and IgM -	total and IgM $\pm$ + patchy or pinpoint no sloughing	total and IgM $\pm$ + patchy or pinpoint slight shedding	total and IgM + patchy or pinpoint shedding
Adults	total and IgM -	total and IgM $\pm$ patchy or pinpoint slight shedding	total and IgM $\pm$ + patchy or pinpoint slight shedding	total and IgM + large patches of fluorescence** excessive shedding

FOOTNOTE

* total immunoglobulin as detected by RabαMo Fab (k)

** most fluorescence noted on dorsal side of males, frequently associated with the base of the tubercules

with the other stages.

As shown in Table 14 and described above, there was significantly
more binding of immunoglobulins to the surfaces of cercariae and
MS than to the surface of the other schistosome stages: this result
suggested that surface changes might occur during early schistosome
development. Since all stages, with the exception of the cercariae
and MS, had been exposed to host material (SS, during the penetration
of mouse skin, CS, during culture in human serum, and LS and the
adult schistosomes, during growth in mice) the possibility of
acquired host antigens interferring with anti-schistosome immuno-
globulin binding was considered. In order to test this possibility,
the following experiments were performed.

B. Effect of pre-incubation of schistosomes on the binding of
immunoglobulins. This experiment was designed to determine whether
surface material, which might prevent antibody binding, was lost
during pre-incubation in a serum-free medium, leading to an increase
in the binding of anti-parasite immunoglobulin. The various
schistosome stages were pre-incubated for 30 or 180 minutes in
ELac-0.2% BSA and then reacted with immune mouse serum (13 wk, CBA,
150 cercariae) followed by Rab α Mo Fab(k) and FITC-Goat α Rab IgG.
No change in the intensity of surface fluorescence was noted for MS,
CS or SS after either pre-incubation period. However, the intensity
of fluorescence was slightly increased on LS and adult surfaces
after the 180 minute pre-incubation.

C. Detection of mouse antigenic determinants on schistosome
surfaces. The various schistosome stages were tested before and
after pre-incubation for the presence on their surfaces of mouse
antigens using specific rabbit antisera against mouse erythrocyte
components (Rab α Mo rbc), normal mouse serum (Rab α NMS) or total
mouse immunoglobulin (Rab α Mo Fab(k). The bound rabbit immuno-
globulin was detected with FITC-Goat anti-Rab IgG as described
previously. The results are presented in Table 15.

With all three antisera, cercariae, MS and most SS exhibited a
weak, patchy fluorescence similar to the fluorescence observed when
these organisms were reacted with serum from CBA mice collected 6
days after infection (see Table 14); however a few SS (less than
10%) were positive when reacted with Rab α Mo rbc antiserum and less
than 2% were positive with the Rab α Mo Fab(k) antiserum. CS
exhibited a weak but uniform fluorescence when reacted with Rab α Mo Fab(k)
antiserum and were negative with the other antisera.

The adults and LS exhibited positive reactions with all the
antisera and the greatest intensity of fluorescence was observed
with the Rab α Mo rbc antiserum. The fluorescence was more pronounced
on the adult worms. In general, the dorsal surface of the males
was more uniformly covered with fluorescing material, while the
females showed large patches of fluorescing material on an other-
wise negative surface.

No significant decrease in the intensity of the surface
fluorescence was noted with any of the schistosomes when they were

126

Table 15   **Binding of Rabbit antisera directed at Mouse**

antigenic determinants to the surface of various

Schistosome stages as detected by immunofluorescence

	RabαMo RBC + FITC-Goat αRab IgG	Rabα MNS + FITC-Goat αRab IgG	RabαMo Fab (k) + FITC-Goat αRAB IgG
Cercariae	±	±	±
2 hr MS	±	±	±
3 hr SS	±; few were +	±	±, rare + organism
5 Day LS	++, no sloughing	± no sloughing	± weak, uniform no sloughing
5 Day CS	-	-	±, weak, uniform
Adult	++, some shedding	++, some shedding	+, no shedding observed

incubated in ELac-0.2% BSA for 3 hours prior to the immunofluorescence reactions.

D. <u>Detection of mouse α_2-macroglobulin</u>. Several reports have demonstrated the presence of mouse α_2-macroglobulin on the surfaces of adult schistosomes (Damian <u>et al</u>., 1973; Damian, 1974; Kemp and Damian, 1974) and evidence suggests (Damian, 1974) that this determinant is synthesized by the schistosomes. Since the schistosome stages in the present study had been exposed to host tissue and sera for varying amounts of time, they were compared for the presence of mouse α_2-macroglobulin. 3 methods were chosen:
1) immunofluorescence using specific Rab α Mo α_2-macroglobulin antiserum and FITC-Goat α Rab IgG
2) radioimmunoassay where the fluorescein conjugated goat antisera was replaced with ^{125}I-Goat α Rab IgG in order to increase the sensitivity and to quantitate the reaction
3) the direct binding of ^{125}I-mouse α_2-macroglobulin to the surfaces of the various schistosome stages before and after 20 minutes incubation in normal and immune mouse serum.

This procedure not only measured the relative amount of α_2-macroglobulin initially present on the various schistosome stages but also showed the extent to which α_2-macroglobulin and other serum components could compete with the binding of anti-α_2-macroglobulin antiserum.

Table 16 contains the results of these studies and shows that using both immunofluorescence and radioimmunoassay techniques

Table 16   **Mouse α_2-macroglobulin on schistosomes of various stages**

	RabαMo α_2-macro + FITC-Goat α Rab IgG	RabαMo α_2-macro + ^{125}I-Goat α Rab IgG	^{125}I-Mo α_2-macroglobulin
Cercariae	±	N.D.	N.D.
2 hr MS	+ sloughing	+	+ binding was decreased $\bar{c}$ both NMS and 13 wk CBA, 150 cerc serum
3 hr SS	+ slight shedding	+	+ binding was decreased $\bar{c}$ both NMS and 13 wk serum less binding than MS
5 Day LS	+ slight shedding	+	+ binding was decreased $\bar{c}$ both NMS and 13 wk serum less binding than $\bar{c}$ MS
5 Day CS	-	-	- no binding
Adult	+	+	+ as for LS

$Mo\alpha_2$-m can be detected on MS, SS, LS and adult schistosomes but not on cercariae and CS. Of the three positive organisms, MS appeared to bind slightly less of the antiserum than either the adult schistosomes or LS which appeared to be equally positive for this determinant. In contrast, MS bound more ^{125}I-Mo α_2-macroglobulin than did SS, LS and adult schistosomes which bound similar amounts of the radioactivity. Pre-incubation with both normal and immune serum decreased the amount of ^{125}I-Mo α_2-macroglobulin bound to the various schistosome stages.

E. Detection of human A and B blood group-like determinants on the surface of schistosomes. Goldring (1976) demonstrated human blood group-like determinants on the surfaces of approximately 30% of 3 hr SS, which had not been exposed to human material. In view of this finding, it was of interest to know if cercariae or younger SS also express these determinants. Schistosomula were prepared as described in Chapter 3 by allowing cercariae to penetrate isolated mouse skin, and were harvested at 15 minute intervals from the ELac in the collection vessel below the mouse skin. When these schistosomula were examined by the mixed agglutination procedure (Table 17), no reaction was observed for the first 45 minutes, but by 60 minutes positive organisms were seen and the numbers gradually increased until at 3 hours approximately 30% were positive. The numbers of erythrocytes which bound to the positive schistosomula also increased with time and at 3 hours large areas, or even the entire organism, was covered with erythrocytes. The re-

130

Table 17   Expression of human A and B blood-group-like determinants by schistosomula at intervals after cercarial penetration of isolated mouse skin

Time	Anti A (% positive)	Anti B (% positive)
0 minutes (Cercariae)	0	0
15 "	0	0
30 "	0	0
45 "	0	0
60 "	3.8	13.0
90 "	19.1	22.0
120 "	26.4	29.1
180 "	25.7	35.0
24 hours	26.9	34.0
48 hours	27.5	32.6

action appeared slightly stronger, i.e. more cells were bound to each organism, when the Hu B antiserum rather than the Hu A antiserum was used as the aggregating antibody. As shown in Table 17 there was no increase in the percentage of SS which bound erythrocytes after 24 or 48 hours incubation in ELac or in ELac medium containing 50% human serum (A or B). (Plate 14)

In the next set of experiments, the other schistosome stages were tested with the Hu A or B antiserum and A, B or O erythrocytes in all combinations. Only homologous reactions that is, Hu A antiserum and A cells (A system) or the B antiserum and B cells (B system) gave positive results (Table 18). Cercariae and MS were negative for all reactions even when these organisms were incubated in ELac alone in ELac containing aqueous extracts of mouse skin for 3 or 18 hours, or in an ELac medium containing 50% human serum (A or B) for 24, 48 or 72 hours. 100% of CS were positive with either the A or the B system depending on the blood group of the serum and cells used for 5 day of cultures. 1-3% of LS bound small numbers of cells using the A system but using the B system 75-90% of the LS were positive and the majority of these were either covered in or were obviously sloughing off large fragments of aggregated cells. No bound cells were observed on the adult schistosomes reacted in the A system but some cells were bound to adult males and females when the B system was used.

To check the findings of the mixed agglutination reaction and to observe more accurately the location of the Hu blood group-

132

<u>Plate 14</u>   <u>Mixed Agglutination Reaction</u>

A. Mechanically prepared schistosomulum, cultured for 4 days in ELac medium containing 50% human B serum, showing positive mixed agglutination reaction (B system).

x 484

B. Three hour skin-penetrated schistosomulum showing positive mixed agglutination reaction (B system).

x 800

ltured for 4 days in

tive mixed agglutination

n showing positive

A
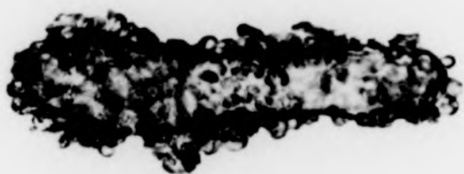
B

like determinants, FITC-Rab-Hu IgG was substituted for the Hu
erythrocytes. The results, presented in Table 18 , confirm the
observations of the mixed agglutination experiments. When positive
organisms were compared, the intensity of fluorescence was weaker
with the Hu α A reaction than with the Hu α B reaction. The only
exception to this was CS which had been cultured in A serum.
Differences in the distribution of fluorescence were not noted.

F. Acquisition of human A and B blood-group determinants:
Mixed agglutination following culture. In view of the finding,
described above, that 100% of CS cultured in human A or B serum react
positively with either anti human A or B blood group substances
respectively, it was decided to study in more detail the expression
of determinants after incubation of MS and SS for various times in
medium containing human blood components. The different culture
conditions and the results are shown in Table 19..

11 ELac containing 50% human B serum + 2% B erythrocytes.
The media was not changed during the 11 days of the experiment.
At 48 hrs, 30% of SS were still positive in both the A and B mixed agglu-
tination tests, while all the MS were negative. By 5 days, approx-
imately 100% of the SS were positive for the B system and
5-10% weakly positive for the A system, while 30-40% of MS were
now weakly positive in the B system. On days 7 and 11, 100% of
SS were positive for B blood group determinants and all were negative
for the A system, while 60-80% and 90-98% of MS were positive for B
determinants on days 7 and 11 respectively. It should be noted that

Table 18 <u>Detection of Human A and B blood-group-like determinants on the surface of various schistosome stages</u>

	Mixed Agglutination (% positive)		FITC-α Hu IgG	
	α A sera + A cells	α B sera + B cells	α A sera	α B sera
Cercariae	0	0	ND	ND
2 hr MS	0	0	-	-
3 hr SS	$\approx$ 30%	$\approx$ 30% binding stronger $\bar{c}$ α B than α A	$\pm$;+ some organism with uniform fluorescences others neg	$\pm$;++ uniform fluorescence on positive organism no sloughing
5 Day LS	1-3%, occasional organism had few cells attached but not in an even distribution	75-90%, most were completely covered and sloughing	$\pm$	++ very patchy sloughing
5 Day CS	100% All organisms had some cells attached	100% All organisms had some cells attached; more binding of cells than with A system	+++ patchy	+++ patchy sloughing
Adults	0	$\pm$ few cells on both males and females	-	$\pm$;+ patchy

Table 19

			O Time	2 Days	5 Days	7 Days	11 Days
25% Serum, no cells	A	FITC-α Hu IgM (1)	-				
		FITC-α Hu IgG (2)	-				
		BαA + FITC-α Hu IgG (A)	-				
	B	FITC-α Hu IgM (1)	-			+ ++, patchy	+, patchy
		FITC-α Hu IgG (2)	-			++, patchy	+.. ++, patchy sloughing
		Hu α B + FITC-α Hu IgG (B)	-			++, patchy sloughing	++, patchy sloughing
50% Serum, no cells	A	(1)	-			+++, very patchy	
		(2)	-	++, uniform		+++, very patchy	
		(A)	-	++, uniform or slightly patchy		+++, very patchy	
	B	(1)	-			+++, very patchy	+++, very patchy
		(2)	-	++, uniform	++, uniform	+++, very patchy	+++, very patchy
		(B)	-	++, uniform or patchy sloughing	++, uniform or patchy sloughing	+++, very patchy	+++, very patchy

136

2 hr NS

A   FITC-α   -

50% Serum

		Col1	Col2	Col3	Col4	Col5
	(A)		slightly patchy		+++, very patchy	
B	(1)	-			+++, very patchy	+++, very patchy
	(2)	-	++, uniform	++, uniform	+++, very patchy	+++, very patchy
	(B)	-	++, uniform or patchy sloughing	++, uniform or patchy sloughing	+++, very patchy	+++, very patchy

25% Serum, no cells

			Col1	Col2	Col3	Col4
A	FITC-α Hu IgM	(1)	-			
	FITC-α Hu IgG	(2)	-			
	Hu α A + FITC-α Hu IgG	(A)	± +, uniform many organisms are neg.			
B	FITC-α Hu IgM	(1)	-		+, patchy	
	FITC-α Hu IgG		-		++, patchy	
	Hu α B + FITC-α Hu IgG	(2)	± ++, uniform many organisms are neg.		++, patchy	

50% Serum, no cells

		Col1	Col2	Col3	Col4	Col5
A	(1)	-			++, patchy	
	(2)	-	± +		++, patchy	
	(A)	± +, uniform many organisms are neg.	+ ++ some organisms still neg.		++, very patchy	
B	(1)	-			+++, very patchy	+++, very patchy
	(2)	-	± +		+++, very patchy	+++, very patchy
	(3)	± ++, uniform many organisms are neg.	+ ++ some organisms still neg.		+++, very patchy	+++, very patchy

MS did not express the A determinant at any time and SS did not express it after the 5th day.

2) ELac containing 50% human B serum, without cells. Culture in medium containing 50% serum without erythrocytes did not appear to significantly reduce the expression of the B determinant by SS. However, no MS expressed this determinant until day 7 when 20-30% were positive, and only 50% were positive at 11 days. In the mixed agglutination test, the number of cells binding to each schistosomula was decreased slightly with SS and to an even greater extent with MS.

3) ELac containing 25% human B serum, without cells. When cultured in 25% serum, MS were negative throughout the 11 days of the experiment and only 50% of the SS were positive at day 7. Unfortunately, later stages of SS were not tested.

G. Acquisition of human antigenic determinants: Immuno-fluorescence following culture. The culture conditions described for the previous experiment were used: ELac containing 50% human serum and 2% erythrocytes, ELac containing 50% human serum and ELac containing 25% human serum. To detect the presence of human determinants, SS and MS were reacted either directly with FITC conjugated Rab Hu IgM or IgG or first with the Hu   A or Hu   B antisera and then with the FITC conjugated Rab Hu IgG. Two major observations were made with these procedures (Table 19):

138

1) MS and SS both appear to adsorb IgM and IgG from the human sera. MS bound significantly more of these immunoglobulins than SS as detected by the intensity of the resulting fluorescence.

2) This adsorption of human IgG by SS and MS during culture in the higher serum concentration made it very difficult or impossible to interpret the results of the experiment in which binding of specific Hu α A or Hu α B antisera were used to detect A or B blood group determinants.

DISCUSSION

Binding of anti-schistosome antibodies

The investigation into the binding of anti-schistosome anti-bodies to the schistosome surface begun in Chapter 3 has been extended, in this section, to the later developmental stages of the parasite. Essentially similar. results were obtained whether total immunoglobulin or IgM was measured from CBA mice infected with 150 cercariae for 6 days, or for 4, 7 or 13 weeks, although more binding was always observed with the later sera. When the three early schistosome stages were cercariae bound higher levels of.the immunoglobulins than MS and SS bound slightly less. Most important, however, were the very low levels of immuno-globulin binding which were observed with LS, CS and adult males and females. The significant differences in surface binding among the various stages, suggests some modification of surface composition in the later stages which interferes with the binding of the anti-parasite immunoglobulins.

139

McLaren et al., (1975) suggested that the failure of lung schistosomula to bind antibody from immune serum were correlated with the acquisition of "host antigens", while lack of host antigens on 3 hr SS was associated with binding of antibody from the immune serum. In other words, they proposed that when host antigen is present, antischistosome antibody does not bind and conversely when host antigen is absent, anti-schistosome antibody does bind. The present study suggests that this may be an oversimplification of the complex interaction which occurs in vivo between the schistosome surfaces and host antigens and anti-schistosome antibodies. In contrast to the work of McLaren et al., (1975) the present study has detected low levels of anti-schistosome antibody binding to the older forms. This is almost certainly due to the highly specific antisera and the increased sensitivity of the triple-layer immunofluorescence technique used in this study.

In addition, the absence of bound antibody noted by McLaren et al., (1975) and Goldring et al., (1976) may be due to the incubation conditions used in their techniques, which have since been observed to cause sloughing of surface material into the incubation medium (Brink, McLaren and Smithers, 1977; Kemp, 1977). This hypothesis was tested by pre-incubating the various schistosome stages in serum-free ELac-0.2% BSA at 37°C prior to reaction with immune serum. When LS and the adults were incubated for 180 minutes, the intensity of the fluorescent reaction appeared to be slightly increased when compared to that of organisms which had not been pre-incubated, thus indicat-

ing a possible increase in the binding of the anti-schistosome antibody. No change in the intensity of fluorescence was observed if MS, SS or CS were pre-incubated prior to the immunofluorescence reaction.

Acquisition of host determinants

The nature of the host derived substances which might interfere with anti-schistosome antibody binding was explored. Clegg (1972) and Goldring et al., (1976) have suggested that host antigens are blood group specific glycolipids acquired by the schistosomes from erythrocytes or serum of the host. Rabbit antiserum produced against mouse erythrocytes was used to detect the presence of mouse host antigens on the surfaces of the various schistosome stages. LS and adult schistosome bound this antiserum to a significantly greater degree than did the other schistosome stages. However, a few SS within any observed group did bind the antiserum, although not as strongly as LS and the adults. CS did not appear to bind the anti-mouse erythrocyte antiserum. Incubation in ELac-0.2% BSA prior to the immuno-fluorescence reaction did not produce notable changes in the binding of the antiserum to the surfaces of the various schisto-some stages. The results suggest that host erythrocyte determin-ants are present on the surfaces of LS and adult schistosomes and possibly on some SS; furthermore these determinants do not appear to be lost when the schistosomes are incubated in a serum-free medium for 3 hours.

The presence of host antigenic determinants other than blood group substances was tested by the use of specific rabbit antisera produced against normal mouse serum or against mouse Fab(k). Cercariae, MS and SS exhibited weak, patchy surface binding with both antisera while CS exhibited a weak but uniform binding only with the Rab α Mo Fab(k) antiserum. The adult schistosomes and LS reacted positively with both antisera but to a lesser extent than they had with the anti-mouse erythrocyte antiserum. The reactions suggest that host-derived serum components, especially immunoglobulins, are present on the surfaces of LS and adult schistosomes.

When the results of the previously described experiments are examined as a whole, they are compatible with the basic concept of concomitant immunity; namely that the presence of host antigens may be associated with decreased binding of anti-schistosome antibody. In addition, the increased binding of anti-parasite antibody following incubation conditions shown to be conducive to loss of host material also supports this concept, although the specific loss of host erythrocyte determinants was not demonstrated.

These studies also demonstrate the presence of additional host determinants, such as immunoglobulins, which may interfere with the binding of anti-schistosome antibody. Pre-incubation, as described in the present experiments, did not result in detectable loss of these determinants, although loss of surface material by sloughing can be detected with an antigen-antibody

type reaction as in the immunofluorescence procedure and Kemp (1977) has reported loss of non-specific or unrelated host antibodies from adult schistosomes following 4 hour incubations in ELac, 37°C alone. However, both the studies of Kemp (1977) and those described in Chapter 3 have demonstrated that schistosomes rapidly adsorb immunoglobulins non-specifically onto their surfaces and it is possible that loss of the original immunoglobulin during pre-incubation was not detected due to the non-specific adsorption of immunoglobulin in the reaction that followed.

There are several ways in which the apparent interference of host anti-parasite immunoglobulin binding by host material already incorporated onto the surface could be investigated further:

1) by radioactively labelling the surface proteins and glycoproteins (of both host and parasite origin) on the surface of the parasites and then studying the effects of various incubation conditions and antigen-antibody reactions on the loss of labelled material and the ability of the organisms to bind the anti-parasite immunoglobulins. These studies are reported in the next Chapter.

2) by detecting (antigens) lost from the parasite surface during incubation of various times and conditions with immunoprecipitation tests using anti-host antisera. Similar studies have been reported by Kusel in which he demonstrated several proteins of similar molecular weights on parasite surfaces and in the culture medium following 24 hr or longer incubation. However, he did not

attempt to characterize these proteins further nor was he specifically looking for host material.

3) by looking for incorporation by the parasite of radioactively labelled precursors into blood group determinants and other antigens on the parasite surface. Goldring (1976) has attempted to demonstrate the incorporation of glycolipid precursors but was unable to detect synthesis of intermediates or glycolipids.

4) study of the acquisition and loss of blood group determinants using ^{125}I-labelled blood group CHO specific lectins. This will be discussed in the next Chapter.

Presence of Mo α_2-macroglobulin on schistosomes

The immunofluorescence and radioimmunoassay techniques demonstrated the binding of a Rab α Mo α_2-macroglobulin antisera to the surfaces of all the schistosome stages except cercariae and CS. Since MS had not been in contact with mouse tissue or serum, this finding confirms the suggestion by Damian (1974) that schistosomes synthesize either Mo α_2-macroglobulin or an antigenically similar determinant. This observation was discussed in more detail in Chapter 3.

There does not appear to be any increase in $\alpha 2$-m on the surface of schistosomes as the parasite develops from the lung stage to the adult. However, both stages appear slightly more positive than MS. The significance of the lack of binding by CS is not known although the acquisition of human serum components from the culture medium may affect the binding of the Rab anti-mo α_2 macroglobulin antisera to the CS surface. This explanation seems likely since both normal and immune mouse serum was shown to inhibit binding

to positive organisms (Table 16).

Expression of human blood group-like determinants by schistosomes

These studies demonstrate that cercariae and MS do not express human blood group-like determinants even when incubated with aqueous extracts of mouse skin for 3 or 18 hrs or cultured in ELac containing 50% human serum for 24, 48 or 72 hrs. In contrast, schistosomula, collected at intervals after cercarial penetration of isolated mouse skin, begin to express these determinants after 45 minutes. Increases were noted by the mixed agglutination procedure, both in the numbers of organisms binding erythrocytes and in the numbers of erythrocytes bound. These results suggest a difference in the surface composition of MS and SS which may be dependent on the skin penetration process. Some physical or chemical influence during penetration may stimulate SS to either express human blood group-like determinants coded for by the parasite genome or to acquire these determinants in a soluble form from the mouse skin. This implies that during artificial transformation, MS are not influenced to either express or acquire these determinants.

The results of the present experiments provide some support for the de novo synthesis of blood group-like substances by SS and later schistosome stages, although they are not entirely conclusive. The best evidence in favour of synthesis rather than acquisition is that 30% of SS and some LS express an. A-like blood group determinant. Although mice possess an antigen which cross reacts with the human B determinant, they have not been shown to have one which cross-reacts with A, so that presence of A determinant on SS and LS suggests

145

that it was synthesised and not acquired from host material. The
fact that only 30% of SS ever express this antigen may just mean
that the incubation conditions used were not stimulating SS to
synthesize the determinant. The absence of A from most LS and
adults may be a result of masking by host material.

The results of the experiments in which MS and SS were incubated
in ELac containing human serum of different blood groups and show in-
creased expression of the homologous antigen and decreased expression
of the heterologous one do provide good evidence that these
schistosome stages can acquire blood group-like determinants (in
addition to any endogenous synthesis which may be going on in SS).
In addition, there are reports suggesting that schistosomes are
capable of adsorbing and losing IgG (Kemp, 1977), adsorbing
Forssman antigens (Dean and Sell, 1972), and adsorbing an alcoholic
extract of A (Dean, 1974). The fact that the acquired host blood
group determinants are thought to be glycolipid and adsorption of
glycolipids has been demonstrated in several systems(Laine and Hakomori, 1973)
supports the suggestion that determinants are being acquired from the
culture medium.

The expression of human blood group determinants by MS and
SS when cultured in ELac containing 50% serum and 2% erythrocytes
demonstrated differences in the rates at which these organisms
expressed these determinants. The percentages of MS expressing
human A or B antigens was always lower than that of SS, but by day 11
90-98% of MS were positive compared to the SS, which were 100%
positive by day 7 and again on day 11. It should be noted that this

is the time when both types of schistosomes reach the gut-closed stage of development. Again this finding is most easily explained by the assumption that the surface of the early MS has not reached the same developmental level as SS. A study involving culture of MS and SS in ELac containing 25 or 50% human serum without cells suggest that the organisms still express the appropriate blood group determinant, but at a slower rate in the lower concentration of serum and that MS may also be slower than SS in this expression.

A further study involving the culture of MS and SS in ELac containing 25% or 50% human serum without cells demonstrated the acquisition of human IgM and IgG. The acquisition appears to be related to the serum concentration in the incubation medium.

The expression of human blood group-like determinants was studied for the other schistosome stages by both mixed agglutination and radioimmunoassay, the presence of the determinants can be demonstrated on LS and adult males and females reacted with the human B systems. However, the quantities detected on LS are significantly greater than those detected on adults or on LS reacted with the human A system. 100% of CS were positive for both systems after culture in the appropriate blood group serum and cells.

SURFACE AT DIFFERENT STAGES OF DEVELOPMENT

INTRODUCTION

As discussed previously the loss of susceptibility of
developing schistosomula to the host immune response may be correlated
with changes in their surface properties. In Chapter 4, the results
of comparative immunological studies on the surfaces of living schisto-
somes were presented. This chapter investigates biochemical changes
in the surfaces of the schistosomes at different stages of development.

Several experimental approaches were used and are discussed
in detail in the appropriate sections. Briefly, the exposed surface
components of living schistosomes were radioactively labelled by one
of three methods; the lactoperoxidase-catalysed iodination procedure,
the recently developed Bolton and Hunter iodinating reagent, and the
galactose oxidase-tritiated sodium borohydride technique. The labelled
components (proteins, glycoproteins and glycolipids) were then separated
by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and
quantitated by gamma or scintillation counting, or visualized by auto-
radiography or fluorography.

The essential assumption of the lactoperoxidase-catalysed
iodination and the galactose oxidase-tritiated sodium borohydride tech-
niques is that the enzymes, being macromolecules, do not cross the
membrane, so that only "external" surface components are labelled
(Phillips and Morrison, 1971; Hubbard and Cohn, 1975). These surface
components may be an integral part of the membrane with peptide or carbo-
hydrate units exposed externally or extracellular secretions or material
adsorbed onto the membrane from external sources. With the lactoperoxidase
technique accessible tyrosine and possibly histidine residues of surface

148

proteins are labelled (Marchalonis, 1969). This technique has been used to study surface components of intact cells such as erythrocytes (Phillips and Morrison, 1971; Hubbard and Cohn, 1972), lymphocytes (Zimmerman, 1974) and fibroblast monolayers (Nairn, 1976). With the galactose oxidase-tritiated sodium borohydride technique, the galactosyl and N-acetylgalacto-saminyl residues of surface glycoproteins or glycolipids are labelled with tritium (^{3}H). Steck and Dawson (1974) and Gahmberg and Hakomori (1973) have utilized this method for labelling surface glycolipids and glyco-proteins of intact erythrocytes, fibroblasts (Gahmberg and Hakomori, 1975) and lymphocytes (Gahmberg, Häyry and Andersson, 1975).

The Bolton and Hunter reagent has been used to iodinate proteins lacking tyrosine residues and proteins altered by the standard iodination techniques (Bolton and Hunter, 1973). This reagent, an iodinated 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester, conjugates to free amino groups in the protein molecule very rapidly under very mild conditions. The structure of the reagent and the reaction conditions suggested that it could be used as a surface label although, at present, there is no information concerning the extent to which it penetrates cells.

The procedures outlined above were used to compare the surface components of various schistosome stages, i.e. cercariae, mechanically prepared schistosomula (MS), skin penetrated schistosomula (SS), lung schistosomula (LS), cultured schistosomula (CS) and adults. These studies included 1) analysis by one dimensional SDS-PAGE of the total proteins from the schistosome stages and estimation of the molecular weights of proteins unique to certain stages, 2) analysis of components (proteins,

glycoproteins and glycolipids) externally exposed and available for surface labelling by the techniques described above and 3) comparisons of the relative amounts of these components on the surfaces of the various schistosome stages.

I. Preparation of cercariae and schistosome stages.

   Cercariae

   2 hr mechanically prepared schistosomula (MS)

   3 hr skin penetrated schistosomula (SS)

                           Described in Chapter II

   5 day lung schistosomula (LS)

   5 day cultured schistosomula (CS)

   Adult schistosomes perfused from Parkes mice,

       6 weeks after infection with 150 cercariae

                         Described in Chapter IV

II. Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE)

One of the most widely used methods for membrane polypeptide separation is acrylamide gel electrophoresis in sodium dodecyl sulphate (Lenard, 1970).   The basis of SDS-PAGE on either cylindrical gels (Laemmli, 1970) or slab gels (Maizel, 1971) is the same.   The solubilized proteins or polypeptides are separated on the basis of differences in thier molecular size (see Discussion) by electrophoresis through the acrylamide gel (see Molecular Weight Determination Section).

The procedures for both cylindrical gels (Laemmli, 1970) and slab gels (Maizel, 1971) utilize a discontinuous buffer system in which there is a difference in the ionic strength of the electrode buffer (pH 8.3), the stacking gel (pH 6.7) and the resolving gel (pH 8.9).   In addition, glycine is present in the electrode buffer, therefore, during electro-migration the SDS-protein complexes

concentrate between the glycine and chloride ions in the spacer gel and then are seived out according to their molecular size in the resolving gel. Because of the concentration step, the resolution in the resolving gel is higher than if a continuous buffer system had been used.

Stock solutions for SDS-PAGE

1) Acrylamide:bisacrylamide (ABA)

   30 g acrylamide

   0.8 g NN'-methylenebisacrylamide per 100 ml glass distilled water. Prepared fresh every two weeks and stored in the dark at 4°C.

2) SDS 10% w/v SDS in glass distilled water. Prepared every four weeks.

3) Resolving gel buffer (3.0 M Tris/HCl, pH 8.9)

   36.6 g Tris

   48 ml IN hydrochloric acid

   Dilute to 100 ml with glass distilled water and pH 8.9

   Prepared every four weeks and stored at 4°C

4) Spacer gel buffer (0.5 M Tris/HCl, pH 6.7)

   5.98 g Tris

   48 ml of IN hydrochloric acid

   Dilute to 100 ml with glass distilled water and pH 6.7

   Prepared every four weeks and stored at 4°C

5) Ammonium persulphate - 10% w/v ammonium persulphate in glass distilled water.

   Prepared immediately prior to use.

6) Tris - Tris (hydroxymethyl)aminomethane - Sigma.

7) TEMED - NNN'N'-tetramethylethylene-diamine, MW116.2 - Koch-Light Laboratories.

8) 5% SDS Sample Buffer

   10 ml 10% SDS

   1 ml 2-mercaptoethanol

   2 ml glycerol

   2 ml of spacer gel buffer

   Dilute to 20 ml with glass distilled water.

   Aliquot and freeze at $-20^{\circ}C$; do not refreeze aliquot.

9) Bromophenol blue in spacer gel buffer added at 1/10th the SDS sample volume after heating the sample.

10) Electrode Buffer

    15 g Tris

    72 g glycine

    25 ml 10% SDS

    Dilute to 2.5 liters with glass distilled water.

11) Coomassie Brilliant Blue R250   2%

    5 g Coomassie

    1250 Absolute methanol

    1250 ml glass distilled water

    For use, add 25 ml glacial acetic acid to 225 ml 2% solution.

12) Destaining solution.

    25% methanol

    7.5% acetic acid

13) Gel storage solution

   5% methanol

   7½% acetic acid

(14) Phenylmethyl sulphonyl fluoride (PMSF)

   100 mM solution in absolute ethanol added at final concentra-
   tion of 20 mM to protein samples.

Method 1: Cylindrical (disc) gels (Laemmli, 1970)

Preparation of the gels

i) 7.5% resolving gel, pH 8.9

   ABA 30:08 stock   12.5 ml

   Resolving gel buffer   6.2 ml

   TEMED   0.074 ml

   10% SDS   0.5 ml

   Water to 50 ml

   10% Amm. persulphate   0.34 ml

Gels were prepared either in precision bore glass tubes (15 cm
x 0.8 cm ID) or in perspex tubes (12 cm x 0.4 cm ID). One end of the
tube was sealed with a double thickness of parafilm, and either
5 or 2 ml of resolving gel added to the glass or the perspex
tubes respectively. The gels were overlaid with 0.1% SDS and
allowed to polymerize for 30 minutes at 22°C.

ii) 3.0% Spacer gel, pH 6.7

   ABA 30:08 stock   2 ml

   Spacer gel buffer   2.5 ml

   TEMED   0.025 ml

   10% SDS   0.2 ml

   Water to 20 ml

   10% amm. persulphate 0.125 ml

The overlay of 0.1% SDS was carefully removed and either 1.5 ml of spacer gel added to the glass tubes or 0.75 ml added to the perspex tubes. The gels were overlaid with 0.1% SDS and left to polymerize for 45 minutes at $22^{o}C$.

The sample buffer and the electrode buffer were as described in the Solutions Section. Samples of 50 to 400 μl, depending on the sample concentration and the gel size, were layered beneath the electrode buffer onto the spacer gel with either a Hamilton syringe or a micro-capillary tube.

Electrophoresis was at 8 mA per gel tube and was continued until the bromophenol blue dye marker has migrated approximately 10 cm.

The gels were removed from the tubes by carefully forcing 0.1% SDS between the spacer gel and the inside of the tube and thus expelling the gel. The gels were stained in 2% Coomassie (Solution 11 of Solutions Section) for 2-3 hours at $22^{o}C$ and destained for 18 hours at $22^{o}C$ with mixing in several changes of destaining solution (Solution 12 of Solutions Section).

The cylindrical gels were frozen in a mixture of solid CO_2 in absolute ethanol and sliced transversely into 1-2 mm sections on a razor blade gel slicer and the radioactivity measured as described below.

Method 2: Slab gels (Maizel, 1971)

Initially the slab gels were prepared in gel boxes modelled on the commercially available E-C apparatus (E-C Apparatus Corp, Fla, USA). This apparatus consists of a perspex box into which a

second box fits perfectly on 3 sides and forms a 4 mm thick space
along the 4th side.   The bottom of this space is sealed with the
plug gel (see below) over which the resolving gel is polymerized.
The two boxes serve as the buffer reservoirs. A sample slot
former with 8 spaces (8 mm x 15 mm) was inserted into the
spacer gel prior to polymerization to form indentations in this
gel. The samples are then layered beneath the electrode buffer
in these slots and enter the gel through electromigration.

Modifications of this system were made by using the Studier
(1973) gel mold, which consists of 2 glass plates separated by 2 mm
thick perspex spacers, clipped together and sealed with the 10%
plug gel and agarose to prevent leakage. The glass plates contain-
ing the polymerized resolving gel and the spacer gel with its
sample slot former were then mounted with a watertight seal between
the two buffer reservoirs using a perspex stand. The reservoirs
were filled, the slot former removed and samples were processed
as described above. Two further modifications were made:
1) a third glass plate was added, thus permitting two slab gels
to be run under identical conditions and 2) the number of
indentations in the gel was increased to 16 or 22 (4 mm x 15 mm)
thus permitting more samples to be analysed with a higher concentra-
tion of the sample in a smaller area (Figure 7).

Preparation of the gels

    10% Plug gel, pH 8.9

    ABA 30:08 stock   16.5 ml

FIGURE 7:   <u>Samples electrophoresed on 2 gels within one mold:</u>

   <u>modification of the Studier (1963) gel system</u>

   The figure shows the marker proteins and samples electrophoresed on
a modification of the Studier (1973) gel system.   As described in
Materials and Methods, a third glass plate is added thus allowing the
formation of 2 slab gels which are then exposed to identical conditions.
Note that all samples migrate at exactly the same rate on both gels.

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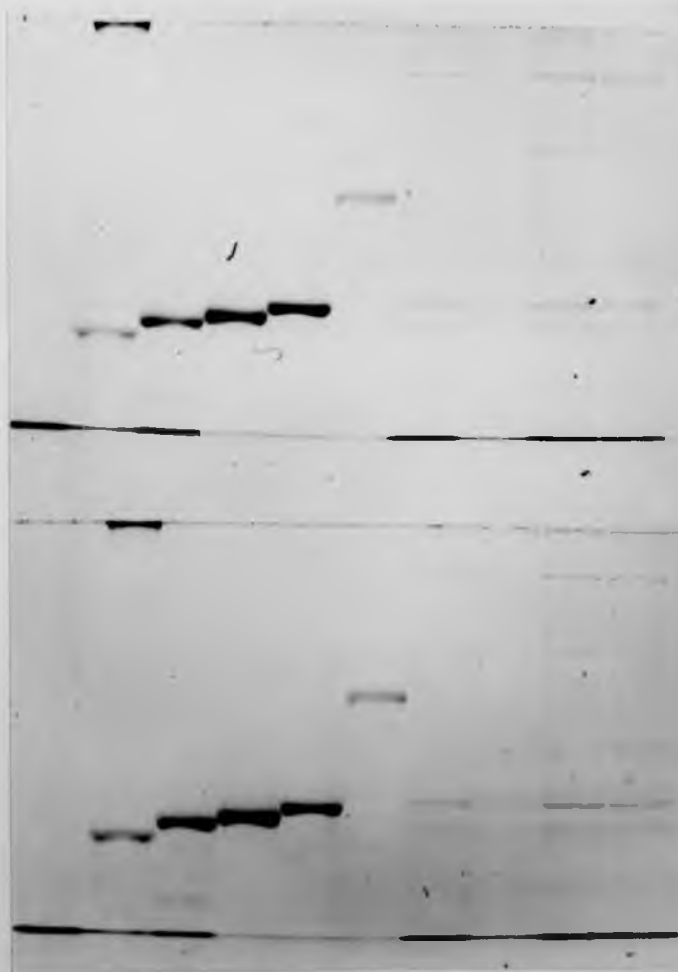
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tical conditions.

on both gels.
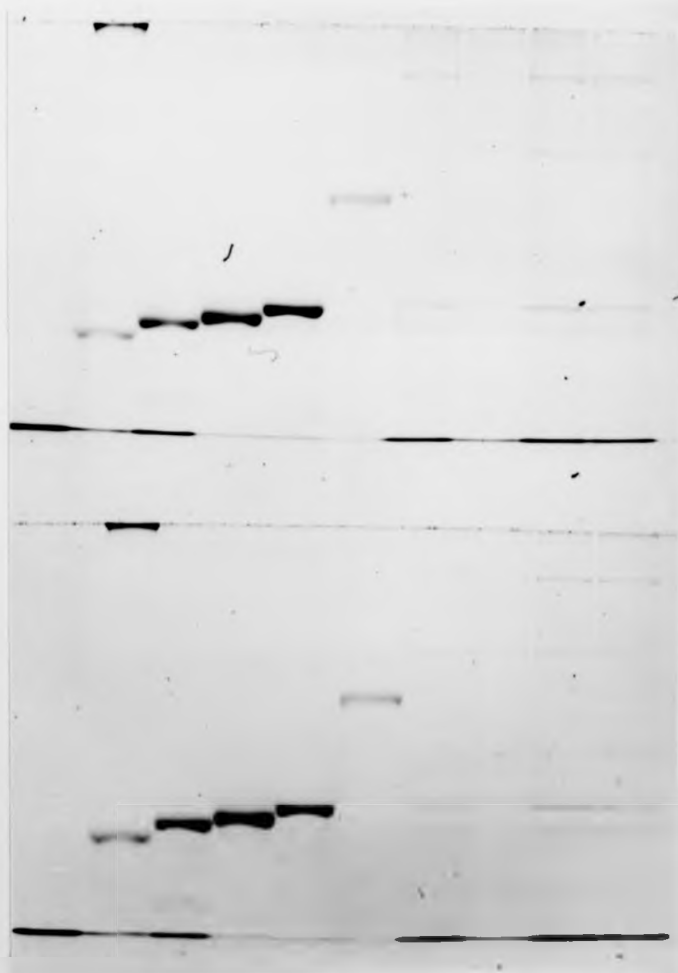
Resolving gel buffer   6.25 ml

TEMED   0.1 ml

10% SDS   0.5 ml

  Water to 50 ml

10% Amm. persulphate   0.5 ml

The plug gel was used to seal the bottom of the gel mold of either the E-C apparatus or the glass plates. A layer ≈ 2 cm deep was poured for each container and the gel allowed to polymerize for 10 minutes.

The resolving and spacer gels were prepared and poured as described for the cylindrical gels. The amount of sample added depended on the size of the indentations but was always the same for any one gel and any unused slots were filled with an equal volume of sample buffer to ensure a homogenous electric field throughout the gel.

The samples on slab gels were electrophoresed at 30 volts through the spacer gel and at 150 volts through the resolving gel to a distance of 10-11.5 cm as judged by the marker dye. After cutting away the plug gel the gels were carefully removed by forcing water between the gel and the gel box or glass plate. The gels were stained and destained as described for the cylindrical gels, except that the staining time was increased to approximately 8 hours.

III. Molecular weight determinations of proteins by Slab Gel-SDS-PAGE

Due to the micellar complexes which are formed with the ionic detergent, sodium dodycyl sulphate (SDS), all proteins migrate as anions in SDS-PAGE. Shapiro, Vinuela and Maizel (1967) and Maizel (1971) have shown that with this system there is an inverse

FIGURE 8: <u>Molecular weight standard curve for SDS-PAGE in 10%</u>
<u>acrylamide</u>

    The following proteins were solubilized in the SDS sample mix and
electrophoresed in a 10% slab gel under standard conditions (Materials and
Methods).   After staining with Coomassie blue the migration of the
proteins relative to the bromophenol blue dye marker was determined.   The
relative mobilities are shown versus the molecular weights of the
proteins The ordinate is logarithmic.

Lactoperoxidase (LPO)	93,000
Bovine serum albumin (BSA)	68,000
Catalase (CAT)	60,000
Ovalbumin (OV)	43,500
Aldolas (ADH)	40,000
Lactic dehydrogenase (LDH)	36,000
Chymotrypsinogen (CHY)	25,700
Cytochrome C (CYT)	11,700

BSA

OV ADH

LDH

200

100

60
50
40

30

.2 .3 .4 .5 .6 .7 .8 .9 1.0

relationship between migration distance and the logarithm of the molecular weight of many proteins. Thus a plot of the relative mobilities of known protein markers will yield a straight line over a molecular range which is characteristic for a defined gel density and the tentative molecular weights of unknown proteins can then be calculated. However, it must be noted that a considerable number of proteins, and especially, certain glycoproteins do not migrate according to this regular pattern and have thus been assigned incorrect molecular weights by SDS-PAGE (Weber and Osborn, 1975; Grefnath and Reynolds, 1974). Based on these observations, the schistosome components noted in these studies have been assigned tentative molecular weights.

The following standard proteins were electrophoresed in 7.5, 10 and 12.5%.SDS gels and calibration curves produced. Figure 8 gives the plot for these proteins in a 10% gel.

Lactoperoxidase (LPO	93,000
Bovine serum albumin (BSA)	68,000
Catalase (CAT)	60,000
Ovalbumin (Ov)	43,500
Aldolase (ADH)	40,000
Lactic dehydrogenase (LDH)	36,000
Chymotrypsinogen (CHY)	25,700
Cytochrome C (CYT)	11,700

The preparation of tadpole tails containing myosin (200,000) and actin (47,000) was a gift from Dr K. Beckingham-Smith.

IV. Iodination techniques

Stock solutions for iodination procedures

Glucose oxidase from Aspergillus niger, Type V

Activity   1400 units/ml aqueous solution with 0.002%

Thimerosal as preservative

1 Unit will oxidase 1.0 μMole glucose to gluconic acid per

minute at pH 4.1 at 35°C.   (Sigma)

Lactoperoxidase from milk (activity 40 units/mg)

(Sigma)

Sodium (^{125}I)iodide   IMS 30   100 mCi/ml

(Radiochemical Centre, Amersham)

Bolton and Hunter Reagent (N-succinimidyl 3-(4-hydroxyl,5-^{125}I-iodophenyl)propionate) supplied as a mono-iodo ester in benzene

containing 0.2% dimethyl formamide.

(Radiochemical Centre, Amersham)

Krebs-Ringer's buffer (KRT) - 12mM NaCl, 4.8mM KCl, 2.6mM CaCl$_2$,

1.2mM MgSO$_4$ and 25mM Tris, adjusted to pH 7.2 with NaOH as described

by Read et al.. (1963)

Phosphate buffered saline (PBS, pH 7.4) - 8.09g NaCl, 0.29g KCl,

0.29g KH$_2$PO$_4$, 1.159g K$_2$HPO$_4$, 0.19g CaCl$_2$, and 0.19g MgCl$_2$ 6 H$_2$O/liter

of H$_2$O.

Phosphate buffered iodide (PBI, pH 7.4) - as for PBS except 8.09g

NaI replaced the NaCl.

Determination of Iodination Conditions

Purity of lactoperoxidase

Two lots of commercially available lactoperoxidase, one
from Sigma and one from Calbiochem, were tested for degree of purity.
The samples were electrophoresed on 10% acrylamide SDS-PAGE, both
samples migrated as double bands with an average molecular weight of
78,000 daltons. The Sigma lactoperoxidase, used for all subsequent
iodinations, was dissolved in 1mg/ml PBS and stored at -20°C in small
aliquots until use.

Optimal concentrations of lactoperoxidase and glucose oxidase

The influence of the concentrations of the two enzymes on the
efficiency of iodination was tested by the checker-board method. Each
reaction mixture contained 2000 MS (94% viability), 200μCi ^{125}I, 0.250ml
PBS, pH 7.4 containing 5mM glucose. The concentrations of lactoperox-
idase were varied from 10 to 80μg/ml while the concentration of glucose
oxidase varied from 0.1 to 1.0 unit/ml. The reaction was maintained
for 10 min at room temperature. The reaction was stopped by the addition
of PBI, pH 7.4, and the schistosomula were immediately sedimented and washed
with PBI followed by PBS. The organisms were then solubilized by boiling
in SDS sample buffer containing 2-mercaptoethanol and processed by SDS-
PAGE.

The lactoperoxidase and glucose oxidase concentrations which
gave the highest efficiency of incorporation were determined to be
40μg/ml of lactoperoxidase and 0.4 units of glucose oxidase. These
concentrations were used for all subsequent iodinations.

Optimal time and temperature

The time and temperature of the reaction were varied from
2 minutes to 20 minutes and the temperature from 4°C to 37°C. For these

experiments, incorporation was determined by TCA precipitation of a
whole schistosomula homogenate made by sonication of the organisms
in PBS, pH 7.4. Increasing the reaction time after the first 5 minutes
did not significantly increase the incorporation of ^{125}I but did decrease
slightly the viability of the organisms. Therefore a time of 10 minutes
was chosen for all subsequent procedures. Extremes in temperature
adversely affected the viability of the schistosomula and therefore,
room temperature was chosen as the reaction temperature.

Effect of omission of reagents on ^{125}I- incorporation

The omission of one of the reagents from the reaction
resulted in a significant (10-fold) decrease in incorporation. However
a low level of incorporation was observed when lactoperoxidase was
omitted, suggesting possible peroxidase on the surface of the schistosomes,
or adsorption of the ^{125}I into surface folds.

Method 1: Lactoperoxidase-catalysed iodination

Living organisms were iodinated essentially by the procedure
of Hubbard and Cohn (1972) as modified by Butters and Hughes (1975).
Iodination was catalysed by lactoperoxidase, and H_2O_2 was generated by
glucose oxidase and glucose. The numbers of organisms and the amounts
of ^{125}I or ^{131}I (carrier-free, Amersham) were varied but the ratios of
the different reagents were maintained as follows:

200μCi carrier-free sodium ^{125}I or ^{131}I iodide in 0.250ml

PBS, pH 7.4 containing 5mM glucose

0.04ml lactoperoxidase, 1mg/ml in PBS, pH 2.4, prepared

immediately before use

0.40 units glucose oxidase, aliquots were frozen and diluted

with PBS immediately before use.

The incubation time was 10 minutes at room temperature with
gentle mixing to keep the organisms suspended in the reaction mixture.

164

The reaction was stopped by the addition of PBI, pH 7.4, (see Solutions Section) the organisms were immediately sedimented and washed x 2 with PBI and then x 2 with PBS or ELac and processed as described in the various experiments.

Method 2: Iodination with the Bolton and Hunter reagent

This reagent, originally developed by Bolton and Hunter (1973), is now available from Amersham and is supplied as a mono-iodo ester in benzene containing 0.2% dimethyl formamide. This procedure is discussed in greater detail in the Results Section of this Chapter.

The iodination procedure consists of evaporating the benzene under gentle vacuum at room temperature immediately prior to adding the schistosomes in $100\mu l$ 0.1M borate buffer, pH 8.5. The reaction mixture was then agitated gently for 5-10 minutes at $22^{o}C$. The reaction was stopped by the addition of a large volume of ELac and the schistosomes were sedimented and washed 4 times with PBS or ELac by centrifugation at 1000 rpm for 30 seconds. The iodinated organisms were then processed as described in the various experiments.

V. Galactose oxidase, Tritiated borohydride labelling of glycoproteins and glycolipids

Stock solutions for GO, ^{3}H-Borohydride procedure

Galactose oxidase of Polyporus circinatus

(Kabi Labs, Stockholm)

Protease activity destroyed by incubating enzyme at $50^{o}C$

for 30 minutes.

Galactose oxidase (Sigma)

Potassium (or sodium) Boro (^{3}H) hydride-specific activity

potassium salt considered more stable and is dissolved

in 0.01M

KOH, aliquoted and stored at $-70^{o}C$.

<u>Trypsin</u> - 0.25% recrystallized trypsin in PBS, pH 7.4

Made fresh.

<u>Pronase</u> - 0.5mg/ml pronase in PBS, pH 7.4

Made fresh.

<u>Neuraminidase</u>

Cercariae and schistosomes were either reacted with 50 units neuraminidase in 1ml PBS pH 7.4 containing 2mM PMSF (a protease inhibitor) for 30 minutes or held in ELac at 37°C. Both groups of organisms were washed 3 times in PBS pH 7.4 by centrifugation and then incubated with 10 units galactose oxidase for 60 minutes at 37°C. The organisms were washed 3 times in PBS, pH 7.4 by centrifugation. The viability of the organisms was checked and then the organisms were either incubated in ELac at 37°C for 3 hours or centrifuged to a pellet and the pellet solubilized by boiling for 3 minutes in SDS sample mix and frozen -20°C until use.

Modifications of the procedure included pre-treatment with trypsin, pronase or PBS for 30 minutes at 37°C.

VI.   <u>Measurement of Radioactivity</u>

(i) <u>Cylindrical gel slices</u>

Gamma: ^{125}I and ^{131}I

This was measured in either an LKB-Wallac 80,000 Gamma Sample Counter or a Packard Autogamma Scintillation Spectrometer Model 5230. Corrections were made for ^{131}I counts recorded in the ^{125}I channel. A Pitman Isotope Assay Calibrator Model 238 was used for an estimation of mCi amounts of γ-radiation.

Beta: ^{3}H

This was measured in a Packard TRICARB liquid Scintillation Spectrometer Model 2450.

A toluene based scintillant containing 10g 2,5-Diphenyloxazole (PPO) and 0.25g 1,4-Di-[2-(5-phenyloxazolyl)]-benzene (POPOP) dissolved in 2.5 lof toluene was used.

(ii) _Autoradiography of slab gels_. After staining for either proteins with Coomassie Blue R250 or for carbohydrates with the Periodic Acid Schiff (PAS) technique, the gels were photographed. Subsequently the gels were dried under vacuum according to the method of Maizel (1971) between polyethylene sheets over a boiling water bath. After drying, autoradiography was performed by exposing Kodak film to the dried gels for periods up to 3 weeks.

Fluorography of the ^{3}H-gels was performed.

VII. _Protein estimation: Lowry et al., (1951)_

Except the samples were dissolved in 0.5M NaOH prior to addition of the alkaline copper reagent. Bovine serum albumin (BSA) was used as the standard.

VIII. _Periodic Acid-Schiff's Stain for Glycoprotein and Glycolipid_
(Zacharius et al., 1969)

Modified Periodic acid-Schiff (PAS) technique

gel fixed in 12.5% TCA for 30 min.

rinsed in deionized water,

immersed in 1% periodic acid, 3% acetic acid for 50 min.

washed x 6 with H_2O and overnight c̄ shaking

check with 0.1N $AgNO_3$ for any remaining periodate

immersed in fuchsin-sulfite solution in the dark for 50 min

immersed in freshly prepared solution of 0.5% K metabisulfite

x 3 10 min changes with 25-50ml/gel

washed with H_2O to clear background (O/N)

stored in 7% acetic acid.

Fuchsin-sulfite solution

        basic fuchsin 8.0g in 2 litres of

            16g K metabisulfite

            21.0ml conc. HCl

            dilute to 2 litres with deionized

        stir for 2 hours at room temperature

        let stand for 2 hours

        add small amount charcoal (Darco G-60) for 15' filter

        colourless reagent stored in cold stable for several months

Control   50-100μg protein (IgG) on tube gel to check staining.

IX. Viability of schistosomes

The viability of the schistosomes was determined by measuring their ability to exclude 0.1% eosin dye and by observing their motility. *In vivo* viability was demonstrated by injecting schistosomula into the tail vein of mice and checking the lungs 3 days later. Adult worms were transferred surgically into the hepatic portal system of hamsters and recovered by perfusion 1-2 weeks later.

X. Isolation of adult schistosome membranes freeze/thaw technique (Kusel, 1972)

Adult worms were perfused from Parkes mice infected with 150 cercariae for 6 weeks and washed extensively in ELac, 37°C and then 3 times in PBS, 37°C. The method for isolating membranes was based on the freeze-thaw procedure of Kusel (1972). The worms, which were either separated by sex or maintained as pairs, were rapidly frozen in solid CO_2/ absolute ethanol and thawed in a 37°C water bath 3 times. The denuded parasites were then collected on a wire gauze (mesh size 60/inch) while the (f/t) membranes were washed through the gauze with PBS containing 20mM PMSF. The washings were centrifuged at 1400 rpm for 10 minutes

to pellet the (f/t) membrane fragments. The fragments were resuspended
and washed by centrifugation in PBS containing 20mM PMSF. The resulting
(f/t) membrane pellet was either processed immediately or solubilized
in SDS sample buffer by heating to 100°C for 3 minutes and then frozen
-20°C.

RESULTS

Separation by SDS-PAGE of the total proteins of the various schistosome stages

These experiments were performed to compare the total proteins of the various developmental stages, and to characterize by molecular weight those proteins unique to a developmental stage. In the first experiment, the E-C gel apparatus with a standard sample slot former for 8 samples was used (see Materials and Methods). Freshly prepared organisms were washed extensively, solublized in the SDS sample buffer by heating to $100^{\circ}C$ for 3 minutes (Materials and Methods) and then either stored at $-20^{\circ}C$ or applied to 7.5% acrylamide slab gels. Solubilization was performed immediately to prevent either degradation of the protein components due to proteolysis, or loss of surface components into the supernatant which might occur if intact organisms were frozen and thawed prior to processing for application to the gels. After electrophoresis the protein bands were visualized with Coomassie Blue (Materials and Methods). Using this method, the number of bands was similar for all the organisms and was in the range of 16 to 20, although 28 to 32 could be detected when larger amounts of parasite material were applied (Figure 9). Since the intensity of staining of protein bands by Coomassie Blue is often proportional to the amounts of protein present (Fairbanks, Stick and Wallach, 1971), these bands were considered to represent the major proteins of the organisms. However, this must be considered semiquantitative, especially with highly glycosylated membrane proteins which show

170

FIGURE 9:   Separation of total proteins of MS and SS by SDS-PAGE

The figure shows a Coomassie blue stained 7.5% acrylamide slab gel.
The samples were solubilized in spacer gel buffer containing 1% (w/v)
SDS and 1% (v/v) 2-mercaptoethanol. Samples of MS were applied to
tracks 2 and 3, samples of SS were applied to tracks 4 and 5,
track 6 contains a collagen marker. Track 7 contains solubilized tadpole
tails; note the two heavily stained bands, myosin (200,000 daltons) and
actin (47,000 daltons). Sample mix buffer was applied to tracks
1 and 8.

SDS-PAGE

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bilized tadpole

0 daltons) and
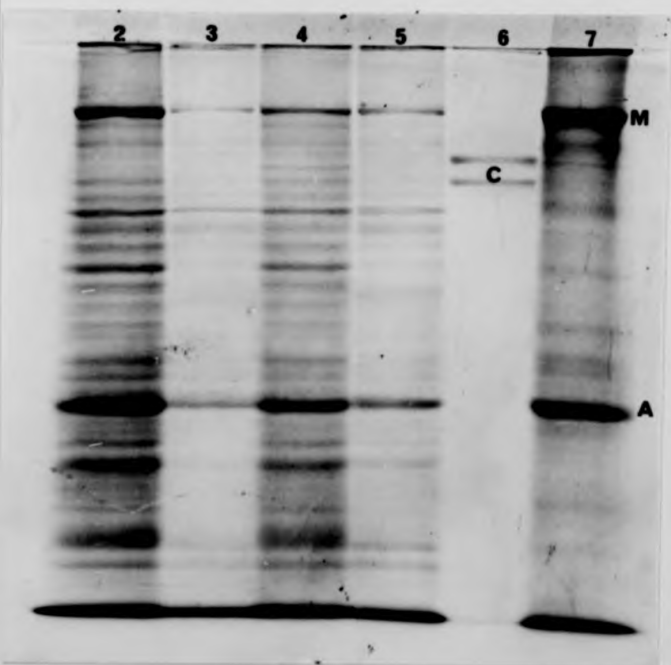
racks
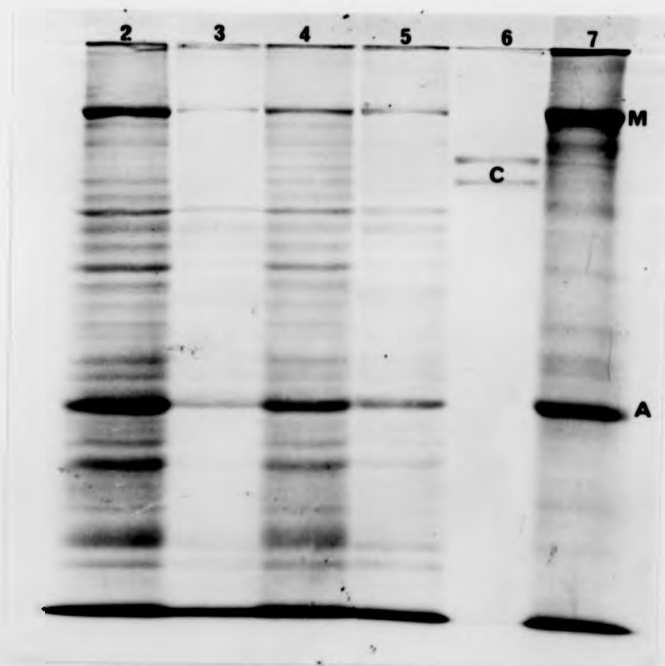
poor staining relative to their protein content (Carraway, Kobylka and Triplett, 1971). Furthermore, it is appreciated that one band may represent more than one protein and with more sensitive techniques such as two dimensional PAGE or isoelectric focusing additional components would be resolved.

In the next experiment the number of samples analyzed was increased from 8 to 16 or 22 (see Materials and Methods). In addition, due to the decreased width of the slots formed, the samples were concentrated in a smaller area. This lead to improved resolution of the separation, so that more bands (in the range of 45-55) were noted with samples of the various schistosome stages. In this case, increasing the size of the samples did not yield more bands and some resolution of the existing bands was lost (Figure 10).

With these methods only qualitative similarities and differences in the major protein bands of the schistosome stages can be given. As shown in Figure 10 , of the bands common to all stages, one migrated at a rate similar to myosin ($\simeq$ 200,000 daltons) and another, similar to actin (47,000 daltons).

Cercariae possessed a unique, high molecular weight protein band ($\simeq$ 160,000 daltons) which migrated slightly faster than the myosin band. This cercarial-specific band was later observed in a sample from cercarial tails but was not observed in a sample containing the same amount of protein from cercarial bodies. 2 hr MS, 3 hr SS, LS and CS did not appear to have any additional major protein bands, although several of the common protein bands

172

FIGURE 10: <u>Separation of total proteins of various schistosome stages</u>

<u>by SDS-PAGE</u>

The figure shows a Coomassie blue stained 10% acrylamide gel. The samples were processed as described in Materials and Methods.

Track 2	-	MS
" 3	-	isolated adult membranes
" 4	-	cercariae
" 5	-	CS in A serum
" 6	-	CS in B serum
" 7	-	LS
" 8	-	adult males
" 9	-	adult females
" 10	-	) TCA ppt. of supernatant
" 11	-	) from MS preparation

Tracks 1 and 8 contain sample mix buffer.

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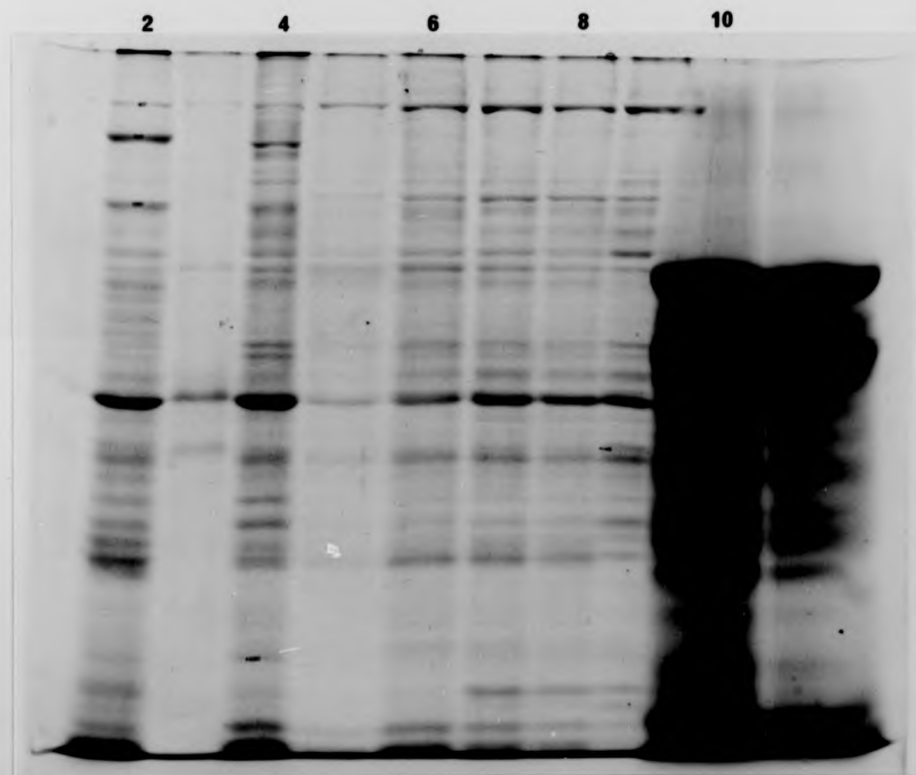
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membranes

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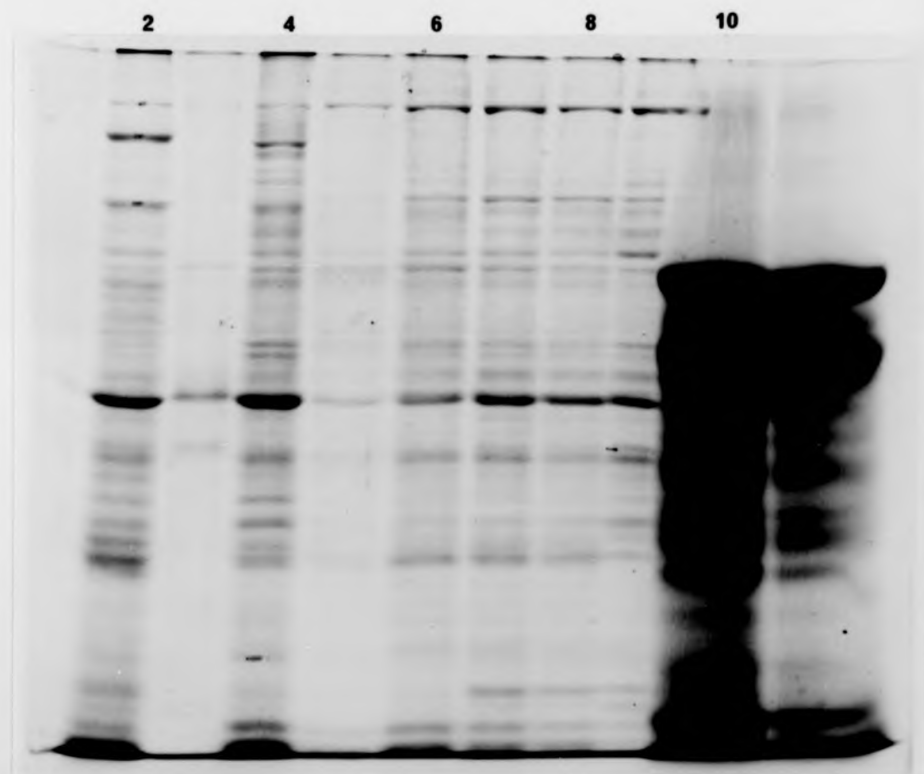
were stained more heavily relative to those of the cercariae. This may represent increased concentration of the protein or more than one protein migrating at a similar rate. Three proteins appeared to be unique for adult schistosomes and were calculated to have molecular weights of approximately 58,000-60,000, 23,000 and 11,000. A protein of approximately 29,000 molecular weight was unique for females.

Iodination of schistosome surfaces:lactoperoxidase method

The lactoperoxidase-catalyzed iodination procedure (LPO-iodination) used in these studies is essentially that described by Hubbard and Cohn (1972) as modified by Butters and Hughes (1975). The optimal conditions of time, temperature and reagents were determined and are outlined in the Materials and Methods section of this Chapter. The numbers of organisms and the quantity of ^{125}I was varied as noted in several experiments but the ratio of reagents to iodine was maintained. The viability of the schistosomes was determined (Materials and Methods) before and after iodination and only batches of organisms with greater than 94% viability were used in the experiments which follow.

Comparative studies of iodine incorporation by schistosomes surfaces. Electrophoresis on cylindrical (disc) gels

This experiment was designed to compare the relative amounts of radioactivity incorporated into the externally exposed protein components on the surfaces of skin penetrated schistosomula (SS) and adult worms. The schistosomes were labelled with either ^{125}I or ^{131}I, and then analyzed separately or co-electrophoresed.

174

Double labelling and co-electrophoresis facilitated comparison
of the relative amounts of iodine incorporated into the different
protein components. Electrophoresis was in 7.5% cylindrical gels
as described in Materials and Methods.

Figure 11 shows the incorporation of ^{125}I into surface
components of male and female pairs, while Figure 12 shows ^{125}I
and ^{131}I incorporation into surface components of the separate
sexes.   The low incorporation of iodine into the surface compo-
nents of the living worms makes interpretation of the results
difficult. However, the surface proteins of the males and females
appear to be very similar with 8-10 major peaks of radioactivity.

Figure (11   ) also compares the ^{125}I-labelled surface
components of living adult worms with the labelled components of
freeze/thaw (f/t) surfaces, isolated by the method of Kusel (1972)
as described in Materials and Methods. Note that the f/t
surfaces were isolated both from adult worms collected 6 weeks
after cercarial infection "pre-immune" and from adult worms collected
16 weeks after infection "immune". The differences between the
radioactively-labelled components of living worm surfaces and
those of f/t membranes are more easily interpreted. Firstly,
the total iodine incorporation of f/t surfaces is increased over
that of living worm surfaces. Secondly, although the gels can
not be compared exactly, there appears to be a major component
(or components) (fractions 34-42) in the "pre-immune" f/t mem-
branes which is not iodinated on the surface of living adults.

FIGURE 11: <u>Radioactively-labelled surface components of adult worm</u>

<u>pairs and isolated adult surfaces</u>

<u>Gel I</u>. Fifty living adult worm pairs were radioactively-labelled by
the lactoperoxidase method using 400 Ci ^{125}I, the surfaces were isolated
by the freeze/thaw technique of Kusel (1972) and solubilized in SDS
sample mix buffer containing 2-mercaptoethanol as described in Materials and
Methods. The sample was electrophoresed on a 7.5% cylindrical (disc) gel.

<u>Gel II</u>. Freeze/thaw surfaces iodinated after isolation from adult
worms collected from "immune" mice 16 weeks after cercarial infection.

<u>Gel III</u>. Freeze/thaw surfaces iodinated after isolation from
adult worms collected from "pre-immune" mice 6 weeks after cercarial
infection.

adult worm

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ion from

cercarial
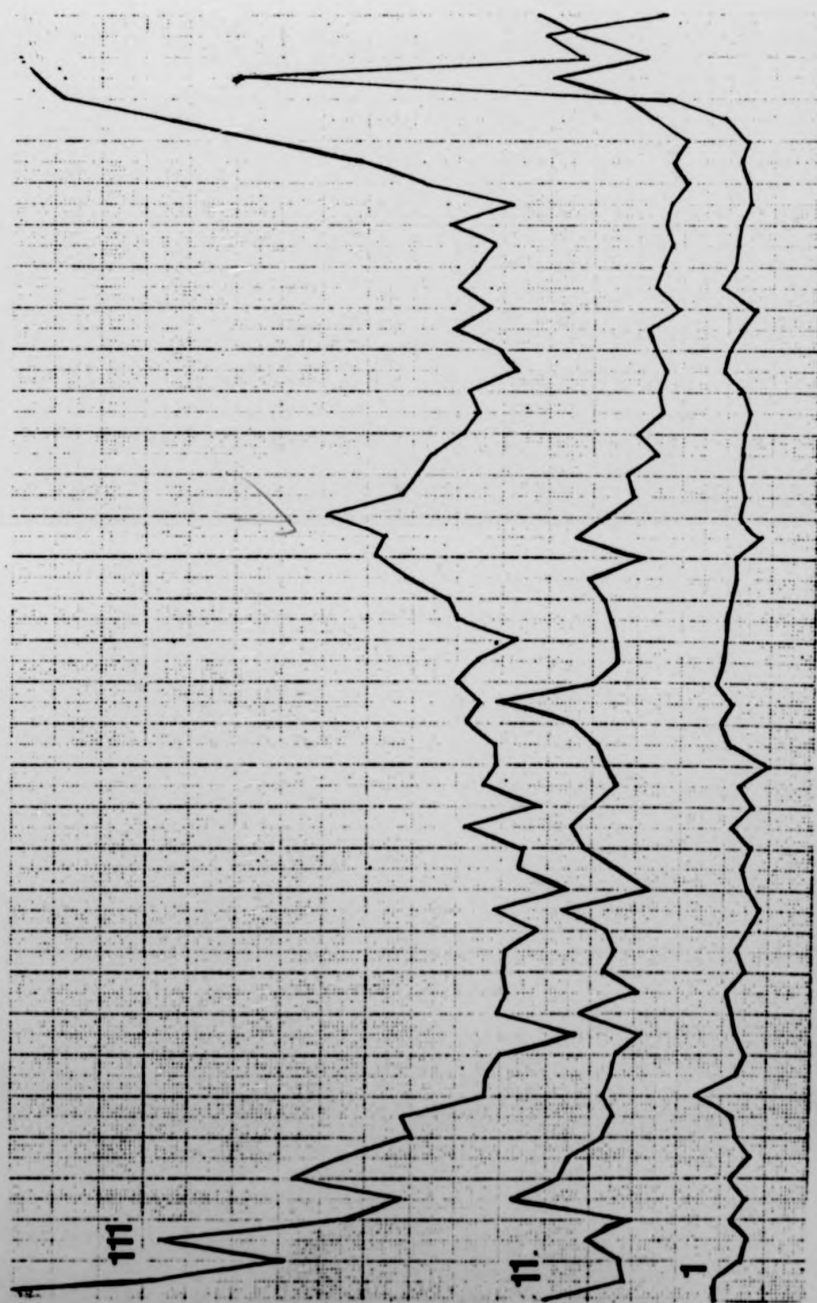
FIGURE 12: <u>Radioactively-labelled surface components of male and female</u>

<u>adult schistosomes</u>

Freeze/thaw isolated surfaces of adult males (^{131}I) and adult females (^{125}I) electrophoresed on 7.5% cylindrical (disc) gels.

male and female

and adult

) gels.

$♀ \, ^{125}I$

$♂ \, ^{131}I$
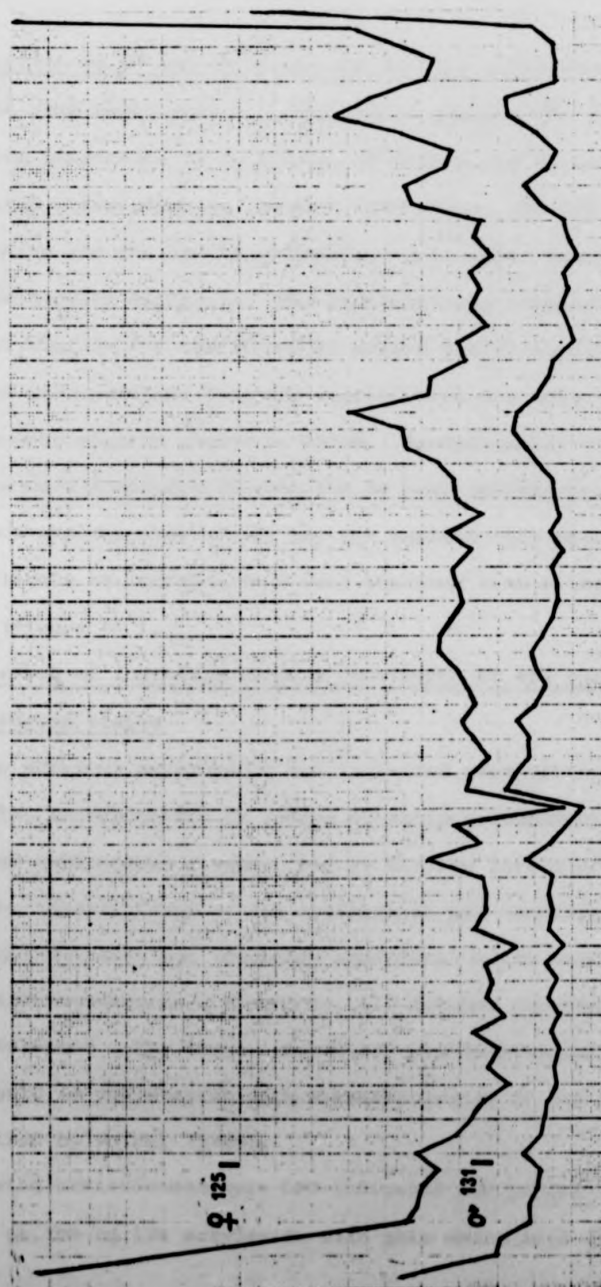
Comparison of 30 and 60 minute and 24 hour schistosomula surfaces

The next experiment was designed to compare the iodinated
surface components of 30 minute, 60 minute and 24 hour mechanically
prepared schistosomula. As described above, the low incorporation
of iodine and the use of cylindrical gels makes exact interpretation
of the results difficult. The radioactively labelled components
appear similar for the 30 and 60 minute schistosomula and the high
iodine incorporation into the region where low molecular weight
components migrate should be noted. Exceptionally low levels of
iodine were incorporated into the 24 hour schistosomula surfaces;
obvious experimental errors can not explain this phenomenon and the
same levels of incorporation were obtained with a repeat experi-
ment (Figure 13).

Comparison of iodinated surface components of the various schistosome stages

The previous experiments had indicated some difference in the
relative incorporation of iodine by surface components of the ·
various schistosome stages. Due to the low levels of incorporation
and the insensitivity of the cylindrical gel technique, the results
were inconclusive but suggested additional study using more
sensitive techniques. Therefore, all further experimentation
was performed using higher resolution one-dimensional discontinuous
slab gels in conjunction with autoradiography of the dried gels as
described by Maizel (1970).

Living schistosomes were LPO-iodinated and processed by SDS-
PAGE on 10% or 12% acrylamide slab gels which were then stained

178

FIGURE 13:Comparison of iodinated surface components of 30 minute,
60 minute and 24 hour schistosomula.

The three types of schistosomula were iodinated by the lacto-
peroxidase technique under identical conditions. The specimens were
analysed, following solublization and reduction, on 7.5% cylindrical
(disc) gels.

179

30 Min.

COUNTS I125 OR I131

SLICE NUMBER

60 Min.

SLICE NUMBER
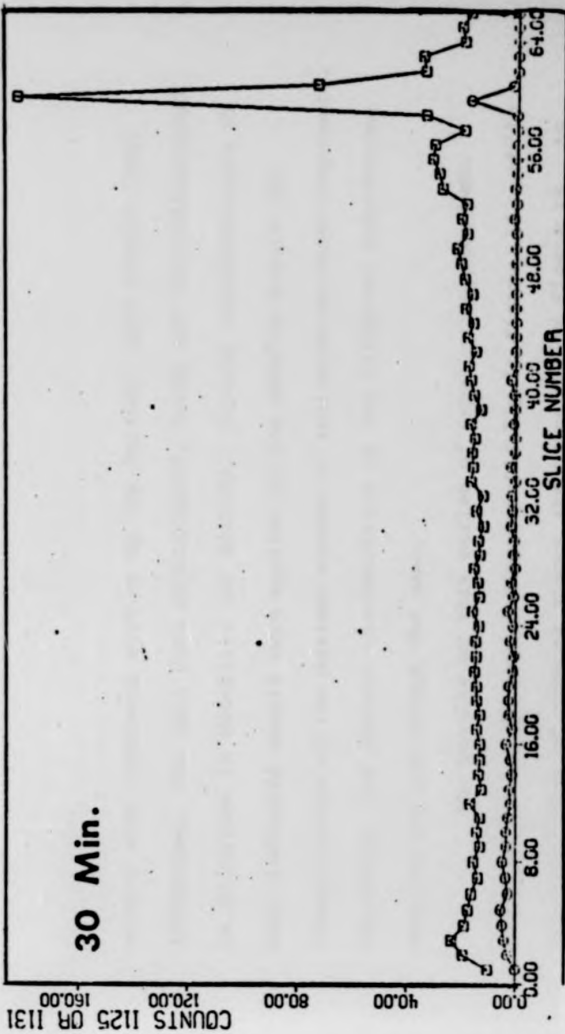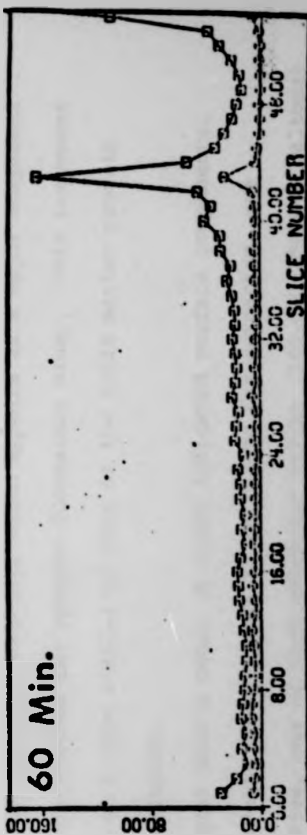
24 Hr.

SLICE NUMBER
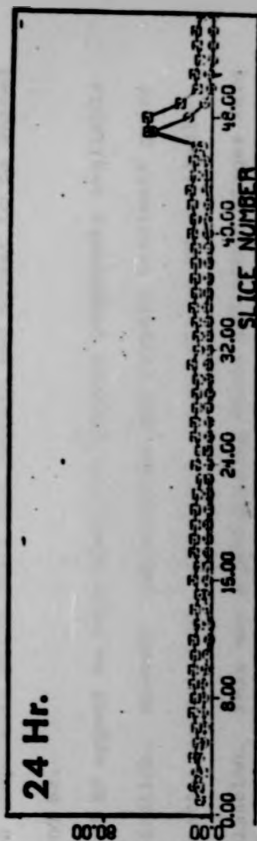
either with Coomassie Blue or by the Periodic Acid Schiff (PAS) technique. The gels were photographed, dried and autoradiographed as described in Materials and Methods. Several concentrations of each iodinated sample were applied to the gels to ensure the visualization of the maximum number of iodinated surface components. Therefore, the protein concentration of the different schistosome samples are not always the same.

The results of this series of experiments have been compiled and are presented diagrammatically in Figure 14 , while autoradiograms of several stages are presented in Figure 15 , 16 and 17 . The most interesting feature is that all stages have an iodinated surface component which appears as a major component on the gels stained for protein (Coomassie Blue). This component migrates at a rate similar to that of the actin marker protein (47,000 daltons).

Cercariae have a total of eight iodinated surface components. Two components which appear to be unique for cercariae, are PAS positive, one of high molecular weight (160,000-180,000 daltons) and another of low molecular weight (11,000-15,000 daltons); cercariae have two components which stain for protein and appear to be shared by MS and SS.

MS and SS appear to have identical surface components available for iodination. However neuraminidase and trypsin treatment prior to iodination produced two additional components on SS available for iodination. This was a preliminary study and other stages were

FIGURE 14: <u>Diagrammatic representation of the lactoperoxidase-iodinated</u>

<u>surface components of the various schistosome stages</u>

The figure presents diagrammatically the lactoperoxidase-iodinated surface components detected on autoradiograms of the samples following SDS-PAGE. Duplicate gels were stained with either Coomassie Blue for protein (P) or by the PAS method for carbohydrate (C).

ase-iodinated

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ase-iodinated

s following

le Blue
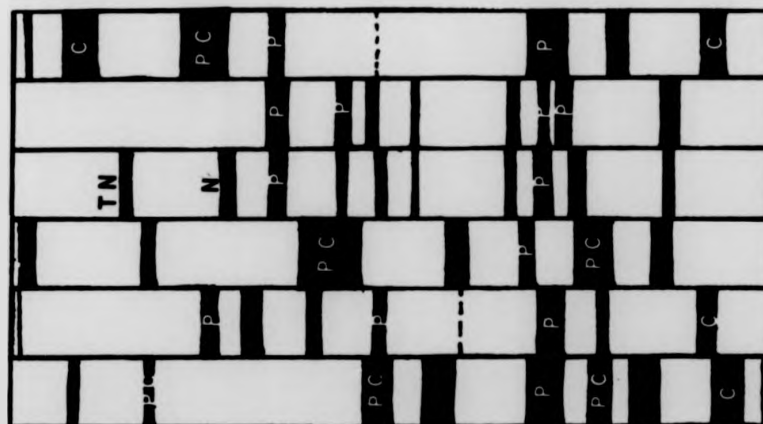
CERC
MS
SS
LS
CS
ADULTS

FIGURE 15: Lactoperoxidase-iodinated surface components of cercariae and cultured schistosomula.

Organisms were iodinated by the lactoperoxidase method, dissolved in SDS, reduced with 2-mercaptoethanol and processed by SDS-PAGE. The figure shows an autoradiogram of a 10% slab gel.

Tracks 2 and 3   -   Cercariae

Track 5   -   Cultured Schistosomula

Track 8   -   Lipid extract of MS

Sample mix buffer was applied to all other tracks.

cercariae and

od, dissolved

OS-PAGE. The
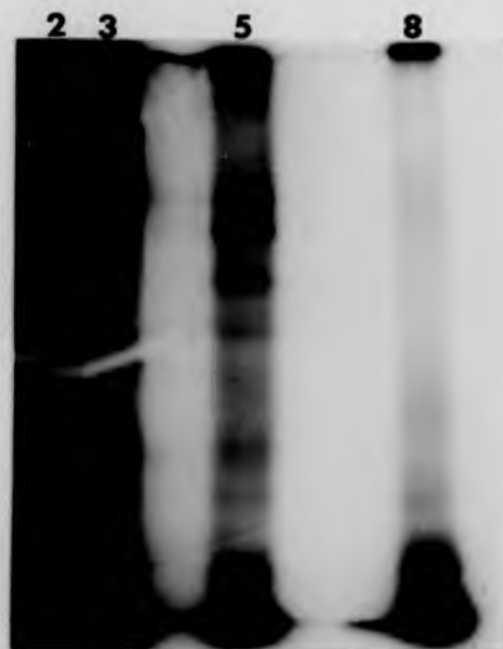
of cercariae and

thod, dissolved

SDS-PAGE. The
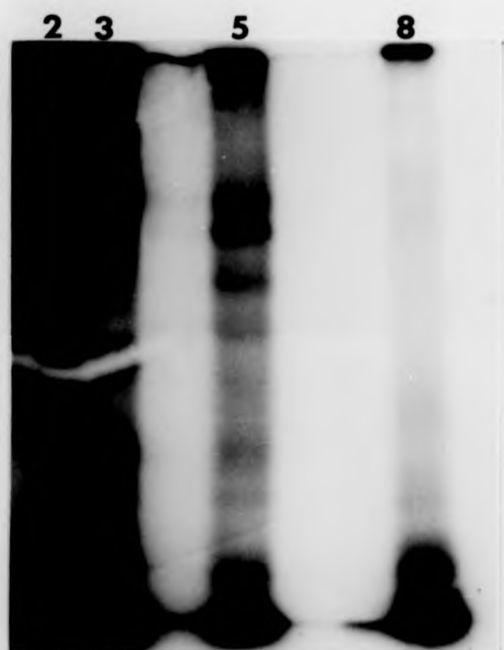
FIGURE 16: <u>Lactoperoxidase-iodinated surface components of mechanically</u>
<u>prepared schistosomula</u>

The figure shows an autoradiogram of a 10% slab gel containing samples
of various concentrations of iodinated mechanically prepared schistosomula.
The protein component which migrates similarly to actin is indicated (a).

nts of mechanically

gel containing samples

repared schistosomula.

in is indicated (a).

ents of mechanically

gel containing samples

prepared schistosomula.

in is indicated (a).
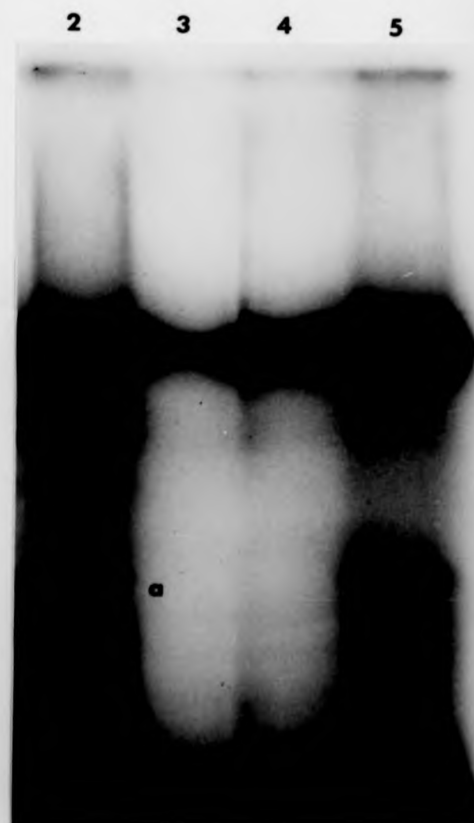
FIGURE 17: Lactoperoxidase-iodinated surface components of lung
            schistosomula and skin-penetrated schistosomula

        An autoradiogram of a 10% slab gel containing samples of iodinated
lung schistosomula (LS) and skin-penetrated schistosomula (SS). Note
high radioactivity at the dye front.

lung

f iodinated

S). Note

LS   SS

lung

of iodinated

S). Note

not enzyme treated prior to iodination.

LS and CS both share componant band with MS and SS. In addition, LS possesses two high molecular weight components, one of which may be similar to an adult schistosome component. CS have two unique protein positive components and a low molecular weight component which is PAS positive.

The adult schistosomes have eight surface components which are iodinated. Two high molecular weight components appear to be unique to the adult stage. The adults also possess a unique low molecular weight (11,000-17,000 daltons) PAS positive component. Protein positive components of molecular weights of 65,000, 55,000, 40,000 and 30,000 daltons probably represent components similar to those of other stages. The 65,000 and 40,000 dalton components were also PAS positive.

Comparison of surface components labelled by the LPO-and the Bolton and Hunter iodination procedures

A preliminary study involving a comparison of the surface proteins labelled by the LPO and Bolton and Hunter procedures gave some very promising results. Under identical experimental conditions batches of equal numbers of living MS were iodinated by the two procedures and analyzed together by SDS-PAGE on a 10% slab gel. The same number of protein bands which stained to approximately the same intensity with Coomassie Blue were resolved in the two samples (Figure 18). Autoradiography of the gel revealed that the Bolton and Hunter reagent labelled the same

FIGURE 18: <u>Comparison of lactoperoxidase-catalyzed and Bolton and</u>

<u>Hunter reagent iodination procedures</u>

Equal numbers of organisms were iodinated by either the lactoperoxidase-catalyzed iodination procedure or with the Bolton and Hunter reagent. The organisms were solubilized in SDS following reduction by 2-mercapto-ethanol and equal quantities were analyzed by SDS-PAGE on a 10% acrylamide gel. The gels were stained with Coomassie Blue, dried and processed for autoradiography.

The figure shows the Coomassie Blue positive components.

Track 2   —   cercariae

"   3   —   MS   LPO

"   6   —   MS

                          B and H
"   7   —   MS

Bolton and

the lactoperoxidase-

nter reagent.

n by 2-mercapto-

n a 10%

ue, dried

ents.

H
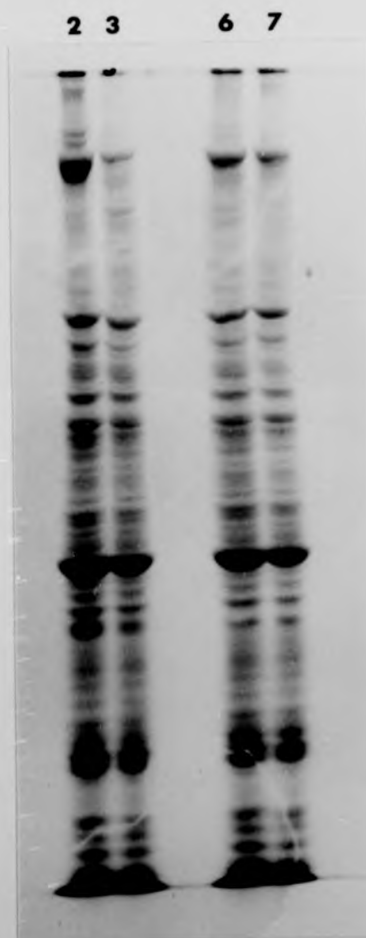
and Bolton and

ther the lactoperoxidase-

Hunter reagent.

tion by 2-mercapto-

E on a 10%

Blue, dried

ponents.

PO

and H

components as the LPO-iodination procedure but to a much greater intensity. In addition, the Bolton and Hunter reagent labelled several bands not observed with the LPO technique (Figure 19).

Galactose oxidase, ^{3}H-borohydride labelling of glycoproteins and glycolipids

Treatment of living schistosomes with galactose oxidase followed by reduction with tritiated sodium borohydride (NaB^3H_4) labels galactosyl and N-acetylgalactosaminyl residues which are exposed on surface components. Following tritium labelling, the schistosomes were solubilised and the components analysed by electrophoresis on 10% acrylamide slab gels. The gels were stained with Coomassie Blue or the PAS technique and then processed and dried for fluorography as described in the Materials and Methods.

The labelling patterns of the various schistosome stages were compared and are presented diagrammatically in Figure 20 . The two iodinated PAS positive components (molecular weight 160,000-180,000 and 11,000-15,000 daltons) of cercariae were also labelled with tritium, these bands appear to be unique for cercariae. A third tritium-labelled component (molecular weight 100,000) of cercariae was not iodinated by the lactoperoxidase procedure, was Coomassie Blue negative and PAS positive.

MS and SS have two components (60,000 and 51,000 daltons) which are labelled with tritium but the incorporation was very low. These components were also iodinated and were stained by Coomassie Blue.

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These components were also iodinated and were stained by Coomassie
Blue.

187

FIGURE 19:   <u>Comparison of lactoperoxidase catalyzed and Bolton and Hunter</u>

              <u>reagent iodination procedures</u>

Autoradiogram of gel described in Figure 18.

Track 2   -   cercariae
                                  LPO
   " 3   -   MS

   " 6   -   MS
                                 B and H
   " 7   -   MS

188

Bolton and Hunter

LPO

B and H

Bolton and Hunter

LPO

B and H
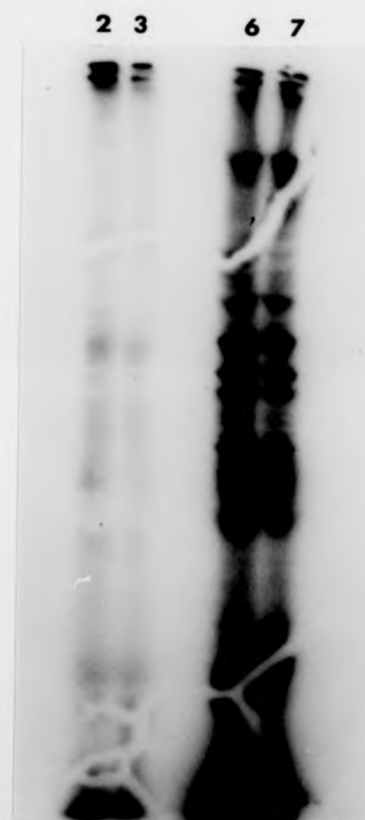
Figure 20   Diagrammatic representation of the galactose oxidase-
            tritium borohydride labelling of surface components of
            the various schistosome stages

        The figure presents diagrammatically the labelling of
schistosome surface components by the galactose oxidase-tritiated
sodium borohydride procedure.

CERC

MS

SS

LS

CS

ADULTS

LS and CS have a tritium labelled component which appears to be similar to the 60,000 daltons component of MS and SS. However, with LS and CS the component is more heavily labelled and appears as a very diffuse band in LS and a thin band in CS. In addition, LS and CS possess a weakly tritium labelled component band in the 30,000 dalton molecular weight range.

The adults have three tritium labelled components with molecular weights of 110,000, 55-60,000 and 30,000 daltons. The last component may correspond to a similar component in LS and CS, while the 55-60,000 dalton surface component appears to be common to all the schistosomula stages but was not observed with cercarial preparations.

DISCUSSION

The experiments described in this Chapter have shown that the technique of slab gel electrophoresis in the presence of SDS can be successfully applied to the analysis of both total proteins and radioactively labelled components of various schistosome stages. The use of this type of gel system permits more sensitive comparison of samples, and must be considered an advancement over previously reported separation systems.

In addition, it has been possible to apply several different labelling techniques to the study of surface components of living schistosomes, which had not previously been attempted. The relative merits of the different procedures will be discussed in the sections which follow.

Total protein composition of the schistosome stages

When the protein composition of the various schistosome stages was compared by one dimensional SDS-PAGE and Coomassie Blue staining, the total number of protein bands obtained ranged from 45 to 55 depending on the concentration of the gel, the size of the sample, etc. This variation may explain the differences in the number of protein bands observed in other studies using different one dimensional systems. Forty five to fifty five is a minimal estimate of the number of proteins present and two dimensional SDS-PAGE or isoelectric focussing would certainly resolve some of the present protein bands into two or more proteins.

The majority of the proteins were common to all stages and may be those present in tissues such as muscles and nerves.

Two of them co-migrated with tadpole-tail myosin and actin marker proteins, but further studies are necessary to show that the schistosome bands are identical to myosin and actin.

Recent work has shown that actin and myosin, the contractile proteins, are also major components of the cytoskeleton in non-muscle cells (Lazarides and Weber, 1974; Weber and Graeschel-Stewart, 1974). Four important cellular processes have been postulated to involve actin and myosin, 1) movement of cell organelles, 2) cell division, 3) maintenance of cell morphology and 4) cell locomotion. Actin from non-muscular sources has a molecular weight of approximately 45,000 daltons and is very similar to muscular actin. Myosin, molecular weight of 460,000 and 200,000 with 54,000 and 20,000 subunits, from the two sources differs considerably.

Several protein bands were noted which were unique to a particular developmental stage and it is assumed that these proteins are components of structures unique to that stage. Cercariae have a unique high molecular weight protein band ($\cong$ 160,000-180,000 daltons). This protein may be associated with the cercarial glycocalyx, which is not present in any other stage. Alternatively, it may represent some structure associated with the cercarial tail, which is lost during cercarial transformation. This protein band was observed in a sample of tails but not in a sample of cercarial bodies. Although the amount of total protein of the two samples was the same, this component could have been enriched in the tail preparation and diluted to undetectable amounts in the

body preparation. Therefore, it is not possible to determine whether this band is derived from the cercarial glycocalyx or from some component present only in cercarial tails.

The proteins unique to the adult schistosomes could be associated with several organ systems which are absent in the immature stages, including the intestine, the genital organs, etc. The protein which appears to be unique to females could represent some feature of the reproductive process, or even the ova.

The complex nature of these organisms, represented by the large number of proteins resolved by one-dimensional SDS-PAGE, would certainly suggest that some form of separation of target antigens is necessary for future research on immunization.

Iodinatable surface components of the schistosome stages

Before discussing the results obtained using lacto-peroxidase catalyzed iodination, and the Bolton and Hunter reagent, as probes to study the proteins of the schistosome surface, a brief description will be given of the present knowledge concerning the structure and chemical composition of the schistosome surface.

The outer membrane of the cercaria is trilaminate and is covered by loosely stranded material, the glycocalyx (Hockley and McLaren, 1973). The glycocalyx appears to consist of polysaccharides, and is PAS positive.

During penetration and the first 3 hours within the skin, the cercarial tail and glycocalyx are eliminated and the surface membrane becomes heptalaminate. The new membrane consists of two closely apposed trilaminate membranes (Hockley et al., 1975), and

is formed from membraneous vacuoles which originate in subtegu-
mental cells. The vacuoles pass into the tegument, where they
enlarge and fuse with the tegumental outer membrane. The interior
of the vacuole opens to the worm surface and the heptalaminate
membranes of the vacuoles become the outer membrane of the tegu-
ment. At the same time the original trilaminate membrane of the
cercaria is formed into microvilli which are presumably cast off
the schistosomulum surface (McLaren and Hockley, 1976). After
the rapid formation of the new heptalaminate outer membrane
within 60-90 minutes, the large vacuoles are not seen in the
tegument, they are replaced at about 3 hours by smaller membraneous
bodies which presumably contribute to the outer membrane during
growth of the schistosomulum. The membraneous bodies are very
numerous in the tegument of the adult worm; they appear to be
involved in the repair of damaged outer membrane and it is also
suggested that the outer membrane of the adult worm is being
continually replaced by new membrane derived from the membraneous
bodies (Hockley, 1970; Hockley and McLaren, 1973; Wilson and
Barnes, 1974). Perez and Terry (1973) have suggested that this
sloughing may be one mechanism for counteracting antibody attack.

Histochemical studies of the <u>S.mansoni</u> tegument have been
reviewed by Stirewalt (1976). In general, the surfaces of the
digenetic trematodes contain acid mucopolysaccharides (Lee, 1966)
and, on the basis of a positive reaction between the surface
and colloidal iron, Morris and Threadgold (1968) suggested that
this was also true for the tegument of adult <u>S.mansoni</u>. Smith

Reynolds and von Lichtenberg (1969) suggested that PAS positive
material and proteins are present in the tegument, while Pearse
(1968) has shown that colloidal iron has affinity for most proteins
and therefore, is not a reliable index of acidic mucopolysaccharide.
Wilson and Barnes (1974) and Wheater and Wilson (1976) have used
both light and electron microscopic histochemical reactions to
confirm that the tegument is composed mainly of neutral mucopoly-
saccharide or glycoprotein.

Meyer, Meyer and Bueding (1970) used isotopically labelled
precursors to demonstrate that S.mansoni synthesizes phospho-
lipids containing choline and ethanolamine but do not synthesize
glycolipid. Wheater and Wilson (1976) used the Plasmal Reaction,
which detects choline and ethanolamine, to show large quantities
of phospholipid on the male dorsal tegument. In addition, they
observed trace amounts of glycolipid on the male dorsal tegument
when sections of adults were submitted to the Bruckner reaction.

A glycolipid fraction unique to adult schistosomes was obtained
by Kusel (1972), when he compared lipid extracts of radiolabelled
cercariae, schistosomular and adult surfaces. Considered together,
the findings described above are compatible with the suggestion
of Clegg, Smithers and Terry (1972) that developing schistosomes
incorporate host glycolipid into their tegument and that this glyco-
lipid serves as an "immunological disguise".

In view of the probable role of the schistosome surface in evad-
ing the host immune response it is important to isolate and
characterise the major components of the tegument. Such a
molecular analysis was initiated by the studies of Kusel (1970, 1972)
the tegument of S.mansoni. He developed methods for isolating

195

"surface membranes" from cercariae, schistosomula and adult
schistosomes which he subsequently analyzed by SDS-PAGE using
cylindrical gels. Kusel (1972) later increased the sensitivity
of the analysis by iodinating the proteins with the chloramine T
procedure following delipidization of the isolated surface membranes,
which he suggested was necessary for efficient iodination. Using this
technique, Kusel observed seven major protein components. In
addition, he suggested that the low molecular weight PAS positive,
Coomassive Blue negative component migrating near the gel front,
which is heavily labelled with iodine is glycolipid. This material
was not observed with his cercariae or schistosomula preparations
and thus Kusel suggested that this material may be host derived.
Lenard (1970) detected erythrocyte membrane glycolipid in a
similar position on acrylamide gels.

The turnover of adult schistosome surface membrane components
has also been studied by Kusel and coworkers (Kusel and Mackenzie,
1975; Kusel et al., 1975). These studies consisted of labelling
worms with ^{3}H leucine and recovering the radioactively labelled
components from the culture medium. A percentage of the labelled
components in the culture medium could be precipitated with hyper-
immune anti-schistosome serum (Clegg and Smithers, 1972) and
furthermore, isolated schistosome membranes could compete in this
precipitation. Therefore, it was suggested that at least some
of the antigens in the culture medium represented components
released into the medium during membrane turnover. The following

molecular weights were given for the components in the culture medium: 110,000; 90,000; 55,000; 45,000; 40,000; 32,000 and 23,000 daltons. Both adults and schistosomula release these components into the culture medium. However, schistosomula of less than 19 days of age release little of this material (Kusel 1975).

In the present study living schistosomes of the various developmental stages were iodinated initially with the lactoperoxidase method of Hubbard and Cohn (1972) and later with the Bolton and Hunter reagent (Bolton and Hunter, 1973). The iodinated organisms were analyzed on acrylamide slab gels in the presence of SDS following reduction by 2-mercaptoethanol. Seven to nine labelled bands were detected in autoradiograms of the gels; several of the bands appeared to be unique for a certain developmental stage while others were shared by all or some of the schistosome stages.

Interpretation of the results is complicated by the fact that living organisms have a continual turnover of membrane material (Kusel et al., 1975) and this appears to be increased in medium lacking proteineous components such as serum or lactalbumin hydrolysate (Wilson and Barnes, 1974). In addition, incorporation of iodine in the lactoperoxidase reaction appears to be low relative to the amounts of iodine incorporated by membranes of erythrocytes (Hubbard and Cohn, 1972). Also, the number of exposed surface components available for iodination is greater than in other membrane systems for example, erythrocyte membranes, and thus extensive enrichment techniques to increase labelled components

results in loss of resolution with the system using SDS-PAGE followed by autoradiography of the gels. Therefore conclusions as to the exact number and molecular weights of surface components are not possible at present. Although technical problems were encountered when living schistosomes were surface labelled, the use of living organisms is important. It is felt that this technique is the one most likely to give relevant information concerning parasite surface and host antibody interaction.

The increased efficiency of iodination by the Bolton and Hunter reagent would permit better and more extensive comparisons of surface components, especially from the aspect of host antigens and acquisition of other host components. Further studies must be made to ensure that this technique represents surface labelling. However, when used in conjunction with LPO-iodination the major surface components could be identified and characterized.

Labelling of glycoprotein and glycolipid components of schistosome surfaces

The galactose oxidase, tritiated borohydride labelling of the surfaces of the schistosome stages has detected the presence of several glycoproteins unique to various stages. The presence of glycolipid material is suggested but further study would be necessary to characterize these components.

In conclusion, when living schistosomes were labelled either by the lactoperoxidase technique, which iodinates external proteins containing exposed tyrosine residues, or by the galactose oxidase tritium borohydride technique, which labels glycoproteins and

glycolipids with exposed galactosyl and N-acetylgalactosaminyl re-
sidues , the following components were detected by SDS-PAGE:

i) All stages possess a Coomassie Blue positive protein component
which is iodinated by the lactoperoxidase procedure and has a
molecular weight of 47,000 daltons.

ii) All stages have some radioactive label in the region of low
molecular weight components which could represent a lipid component
or possibly free iodine.

iii) Cercariae have eight surface components which are iodinated
by lactoperoxidase. Three of these components are also labelled
by tritium in the galactose oxidase-^{3}H borohydride procedure. Two
of the tritium labelled components are Coomassie Blue negative
indicating that these particular glycoproteins are present in the
surface in small amounts.

iv) MS and SS share eight lactoperoxidase labelled surface
components at least three of which are also present in cercariae.
The two surface components with molecular weights of 60,000 and
51,000 daltons, which are also tritium labelled in the galactose
oxidase-^{3}H borohydride procedure show very low incorporation of
radioactivity, suggesting either that these components are not
readily available to the external surface, have very short carbo-
hydrate portions or that the organisms are actively sloughing off
this material.

v) LS have seven components, which are iodinated by the lacto-
peroxidase procedure and three can also be tritium labelled.

One   component is   shared with MS, SS and CS and possibly with
the adult schistosomes.

vi) CS possess nine lactoperoxidase iodine labelled components,
two of which are tritiated in the galactose oxidase procedure and
shared with the other stages. A third tritium labelled component
has a molecular weight of approximately 17,000-19,000 daltons.

vii) Adult schistosomes have eight lactoperoxidase labelled
surface components,three of which are also labelled by the
galactose-oxidase-^{3}H-borohydride procedure and have molecular
weights of 110,000, 55-60,000 and 30,000 daltons. The high
molecular weight components appear to be unique to this stage, while
the other two may be similar to components observed in the other
stages.

viii) There is a major component in the adult f/t surface prepara-
tion which is not iodinated in the surfaces of living adults.
This may represent a component present only on the cytoplasmic
face of the surfaces or may have been exposed during the f/t .
preparation of the surfaces.

CHAPTER 6:   GENERAL DISCUSSION

Schistosomiasis, a disease affecting 200 million people, has become the object of intensive research in recent years. Several interesting phenomena are associated with this disease, these include a situation termed "concomitant immunity", the "acquisition of host antigens" and the modulation of the pathological response.

With schistosomiasis the theory of concomitant immunity assumes that the continued presence of the adult organisms stimulates an immune response which is effective against the invading juvenile stage, the schistosomula. It is also suggested, and evidence supports the idea, that the target of the host immune response is the surface of the schistosomula and that organisms successful in evading this response have modified their surface. Several mechanisms for this modification have been suggested and include the acquisition of host material as an antigenic disguise and restructuring of the surfaces during maturation of the organism.

The aims of this research were to examine biochemically and immunologically the developing schistosome surface and to investigate the interaction of the host humoral response with the surfaces of the various schistosome stages. The use of living organisms was considered most representative of the in vivo situation and therefore, it was essential that these organisms be easily obtained as uniformly developed specimens in sufficient quantities for adequate studies. Furthermore, in order to establish a reference point, the initial experiments would require organisms which were uncontaminated by host material.

The standard techniques for preparing schistosomula, that is by allowing cercariae to penetrate either intact or isolated rodent skins, did not meet the requirements outlined above. Thus it was decided to investigate the techniques and the resulting organisms from several recently reported procedures for artificially preparing schistosomula. Detailed studies suggested that with certain modifications the preparation of schistosomula by mechanical separation of cercarial tails and bodies resulted in organisms which met the generally accepted criteria for transformation from cercariae to schistosomula. These mechanically prepared schistosomula (MS) were used as the reference organism for all subsequent biochemical and immunological studies.

Initially, the humoral responses of mice, the animals most frequently used in experimental schistosomiasis, were investigated with respect to factors which might affect the humoral response, such as mouse strain differences, size of cercarial infection and duration of infection. The response was studied from two aspects: 1) the immunoglobulin class or subclass produced specifically against the schistosomula surface and 2) the effect on the surfaces of the various schistosome stages. The results suggested that 1) CBA mice produce a lower humoral response later in the course of the infection than do Parkes mice, 2) IgM responses appear relatively late but remain at high levels for the duration of the infection, 3) the levels of the IgG subclasses specific for schistosome surfaces increase only 3-5-fold while according to Sher et al., (1977) total IgG levels increase many-fold and 4) the levels of IgA and IgD appear to be increased above the normal levels. Other interesting observations concerned 1) non-

202

specific adsorption of immunoglobulins, 2) correlation of increased binding of host material with a decrease in binding of antibody to the parasite surface and 3) the sloughing or shedding of the parasite surfaces during interaction with antibodies.

The dynamic nature of the schistosome surface in relation to host-like antigenic material was studied further using radioactively labelled antibody components. The resulting increased sensitivity of these techniques allowed the following observations to be made. Firstly, certain types of host material, possibly glycolipids related to blood group substances, become an integral part of the surface. Secondly, other host material, e.g. immunoglobulins, is loosely adsorbed to the surface and, thirdly, material synthesized by the parasite and cross-reacting with mouse α_2 macroglobulin is also present on the surface.

The sloughing of the schistosome surface during the host immune attack was investigated in several ways. Failure to detect parasite antigens following sloughing of the surface suggested that the antigens involved in that specific reaction were either released into the medium or were modified in some way and were not recognised by the antibody. Viability studies in vivo and in vitro suggested that loss of surface material during the sloughing process was not detrimental to the worm; furthermore the presence of microvilli would suggest active repair of surfaces after elimination of the antibody material similar to the removal of the glycocalyx during transformation from cercariae to schistosomula.

The biochemical studies of the various schistosome surfaces suggested several important differences in the numbers and types of proteins, glycoproteins and glycolipids exposed. Some of these components appear unique to certain stages such as the glycocalyx of the cercariae while other components may represent acquired host material as in the case of the cultured schistosomula. In addition, the schistosome stages share many (more than 55) protein components.

The studies were limited by the quantities of the organisms available and the problems inherent in the use of a living multi-cellular organism. However, the aim of this research was to demonstrate and study exposed surface components and to emulate the in vivo host-parasite relationship. The possible interference by lipid containing components in the incorporation of surface labels such as iodine or tritium is suggested and may represent an even more complex evasive system against antibody attack.

When the results of the immunological and biochemical studies presented in this research are considered together, the following general conclusions can be made.

1) The dynamics of host and parasite interaction are very complex and involve factors inherent in both the host and the parasite. Generalizations about the immune response and extrapolations from one host parasite system to another are therefore no longer acceptable and the parameters must be carefully defined for each specific interaction.

2) The effective host immune response involves more than humoral antibody production, and these studies support previous suggestions

that cellular mechanisms are also necessary.

3) The parasite surface is a complex structure capable of adsorbing, eliminating and mimicing the antigens of the host. Although some progress has been made here towards separating and characterising the components of the surface by 1° SDS-PAGE on slab gels in conjunction with specific radioactive labelling techniques, further research will depend upon developing better techniques to isolate the surface antigens and to label them more efficiently. The preliminary studies using the Bolton and Hunter reagent reported here suggest that this reagent should be explored as a more efficient surface label for proteins. In addition, radioactively labelled lectins could be used as sensitive and specific probes for characterization of carbohydrate surface components such as glycoproteins and glycolipids.

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211

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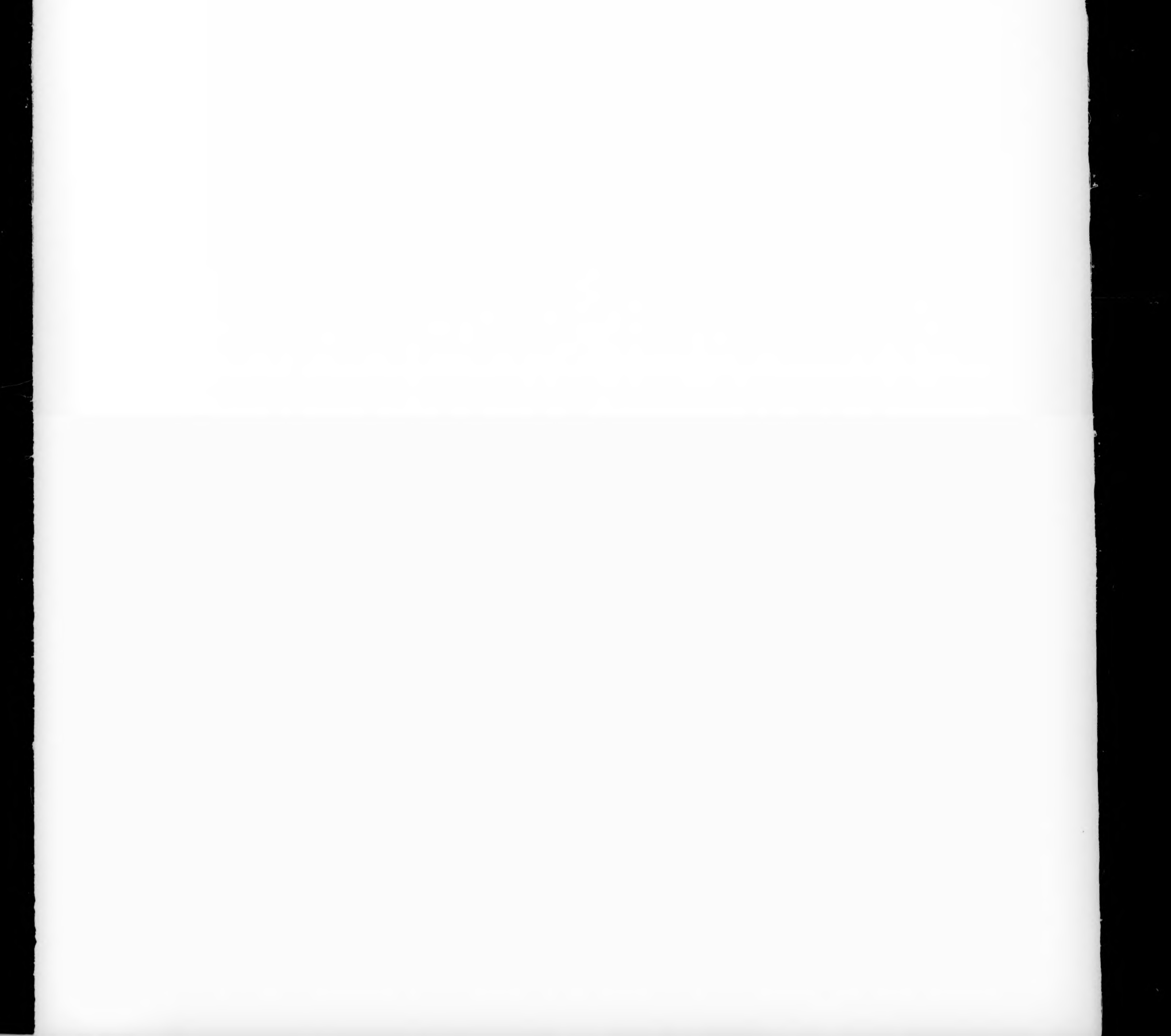   <u>71</u>, 4561.

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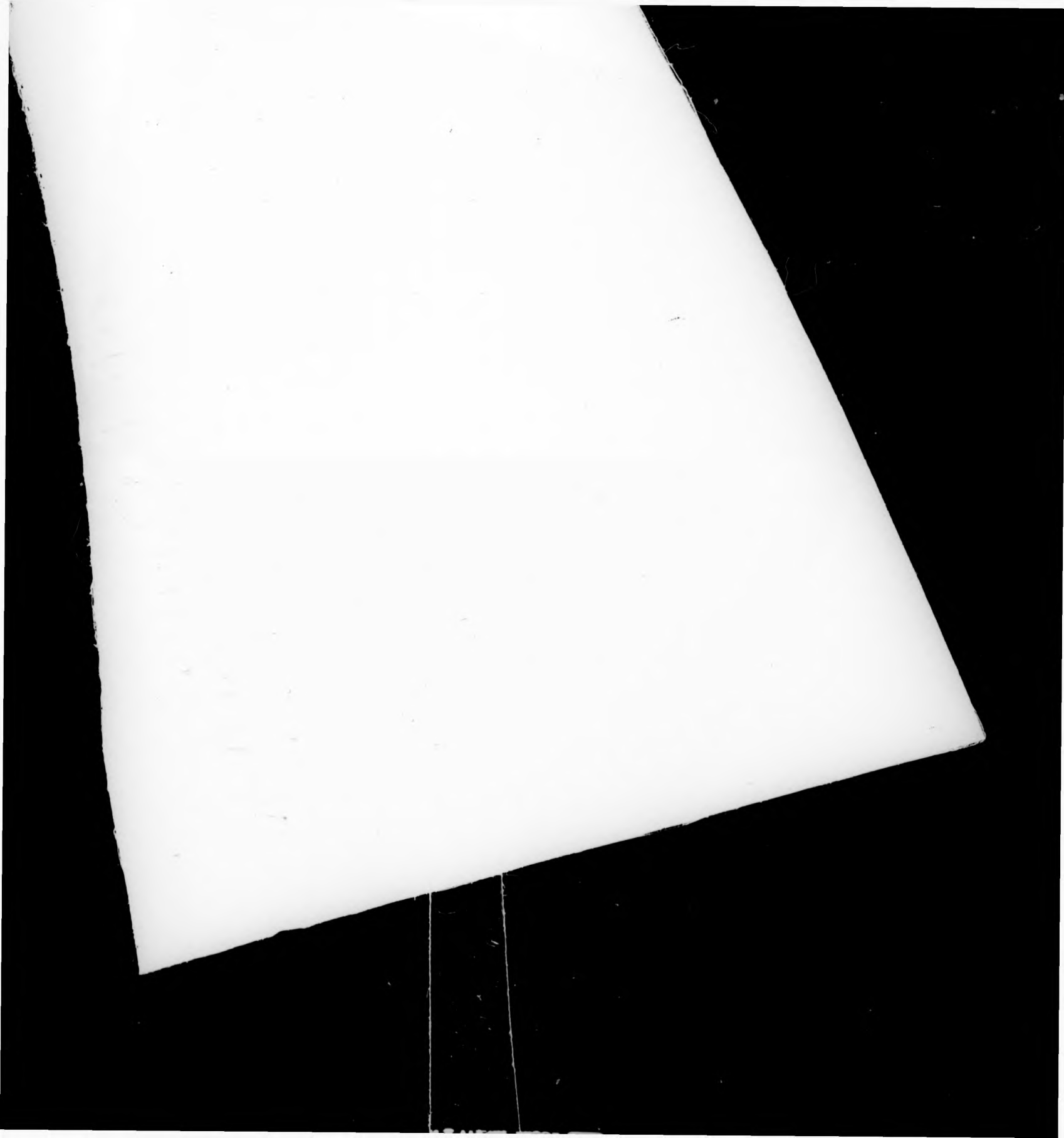
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