



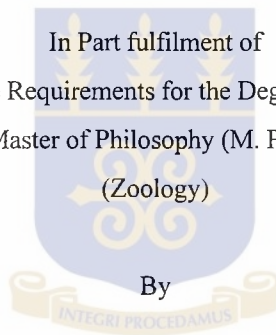
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**CHARACTERIZATION OF ANTI-*SCHISTOSOMA*  
*HAEMATOBIIUM* MONOCLONAL ANTIBODIES AND  
INVESTIGATIONS INTO THEIR REACTIVITY IN THE  
WESTERN IMMUNOBLOT ASSAY**

A Thesis Presented to  
The Board of Graduate Studies,  
University of Ghana, Legon  
Ghana.

In Part fulfilment of  
the Requirements for the Degree of  
Master of Philosophy (M. Phil.)  
(Zoology)



By

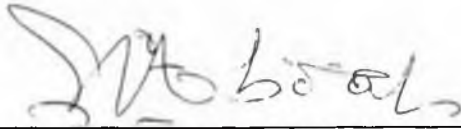
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## DECLARATION

I do hereby declare that except for references to other people's work which I have duly acknowledged, this exercise is a result of my own original research, and this thesis, either in whole, or in part, has not been presented for another degree elsewhere.



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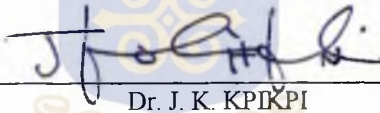
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**ABBREVIATIONS**

ABTS	-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
APS	-Ammonium per sulphate
DAB	-Diaminobenzidine tetrahydrochloride
DE-52	-Diethylaminoethyl cellulose
DMSO	-Dimethylsulfoxide
DNA	-Deoxyribonucleic acid
EDTA	-Ethylenediaminetetracetate
ELISA	-Enzyme-linked immunosorbent assay
FBS	-Foetal bovine serum
FITC	-Flourescein isothiocynate
gm	-Gramme(s)
HAT-medium-	Hypoxanthine, aminopterin and thymidine medium
HEPES	- N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid
hr	-Hour
HRPO	-Horseradish peroxidase
HT-medium -	Hypoxanthine and thymidine medium
IFAT	-Indirect immunoflourescent antibody test
Ig	-Immunoglobulin
IgA	-Immunoglobulin A
IgD	-Immunoglobulin D
IgE	-Immunoglobulin E
IgG	-Immunoglobulin G
IgM	-Immunoglobulin M
l	-Litre
2-ME	-2-mercaptoethanol
MoAb	-Monoclonal antibody
ml	-Millilitre(s)
min	-Minutes
MW	-Molecular weight
μ	-microns
μg	-micrograms
nm	-Nanometres
NC	-Nitrocellulose
NMIMR	-Noguchi Memmorial Institute for Medical Research
PBS	-Phosphate buffered saline
PEG	-Polyethylene glycol
pH	-Negative logarithm base of hydrogen ion concentration
SDS	-Sodium dodecyl sulphate
SDS-PAGE	-Sodium dodecyl sulphate polyacrilamide gel electrophoresis
TBS	-Tris buffered saline
TEMED	-N,N,N',-N'-tetramethylethylenediamine
WHO	-World Health Organization
Xg	-Times gravitational force

## SUMMARY

*Schistosoma haematobium* antigens are the least studied amongst the three major human schistosomes (*S. haematobium*, *S. mansoni* and *S. japonicum*), mainly because of difficulty in maintaining the life cycle of this parasite in the laboratory. As a result, work on identification of potentially diagnostic and protective *S. haematobium* antigens lags behind.

The work reported in this thesis was aimed at characterizing monoclonal antibodies recently produced against *S. haematobium* antigens so as to determine whether any of them are useful for specific diagnosis of urinary schistosomiasis or for protection against the disease.

Nineteen monoclonal antibodies (MoAbs) recently generated against *S. haematobium* soluble egg and infected human urinary antigens were used in this study. Earlier attempts to characterize six of the MoAbs had revealed that one of them (Sh3/15.28) was *S. haematobium* species-specific, but it could not detect the antigen in infected human urine, and was therefore not suitable for developing highly desirable non-invasive, field applicable assays. Moreover, four out of the six MoAbs failed to bind the antigens that they detect in the western immunoblot assay, thereby making it difficult to further characterize them by this technique.

From the studies reported in this thesis, fifteen of the MoAbs were found to be of the IgM class, three were of IgG1 and one was of IgG3 subclass. Cross-reactivity studies using crude antigens of *S. haematobium*, *S. mansoni*, *S. japonicum*

and *Necator americanus* (hookworm) eggs or worms showed that two MoAbs (Sh2/15.F and Sh3/15.28) were *S. haematobium* species-specific, and another one (Sh3/44.3) was specific to the Egyptian strain of *S. haematobium*. Sh3/44.3 detected antigens in soluble egg antigens of an Egyptian strain of *S. haematobium* (SEA<sub>Egy</sub>) obtained from the World Health Organization (WHO) but failed to detect antigens in both egg and adult worm antigen extracts of Ghanaian strain(s) that were tested. Six MoAbs namely, Sh1/71.7, Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10 were *Schistosoma* genus-specific, while the remaining ten bound cross-reactive antigens in *S. haematobium* and *S. japonicum*. Biochemical analysis of the antigens showed that seven out of eight MoAbs namely, Sh2/15.F, Sh3/15.13, Sh3/34.10, Sh4/14.3, Sh4/16.45, Sh5/32.30 and Sh5/34.10 bound protein epitopes which were also present in ammonium sulphate precipitated proteins (P<sub>2</sub>J) from the urine of individuals infected with Ghanaian strain(s) of *S. haematobium*. The eighth antibody (Sh3/44.3) bound a *S. haematobium* Egyptian strain-specific antigen and, therefore, could possibly not detect any antigens in P<sub>2</sub>J. All the remaining eleven MoAbs bound glycoprotein antigens. Characterization of the antigens detected, by the indirect immunofluorescent test (IFAT), showed that four MoAbs bound antigens located on the surface membranes of Ghanaian strain(s) of *S. haematobium* miracidia while, three others bound internally located (possibly cytoplasmic) antigens. The remaining twelve MoAbs bound both surface and internal antigens. Further characterization using the western immunoblot assay showed that reactivity of the MoAbs was influenced by the biochemical nature of the antigens detected, the

antibody concentration used as probes for antigen detection and the amount of current used in electrophoresis. Out of the nineteen MoAbs investigated, seventeen reacted under varying conditions of complete denaturing whilst the remaining two (Sh3/38.2 and Sh3/44.5) failed to bind antigens under both denaturing and non-denaturing conditions. All the MoAbs that detected glycoproteins epitopes (except those with generally low reactivity) reacted when diluted in Tris-buffered saline, pH 8.0 containing 2% skimmed milk as blocking agent. On the other hand, most of the MoAbs which detected relatively low molecular weight polypeptides (<40kDa) could only do so at high antibody concentrations, particularly when undiluted. Interestingly, none of the MoAbs could detect antigens in P<sub>2</sub>J by the western immunoblot assay.

The two *S. haematobium* species-specific MoAbs, Sh2/15.F and Sh3/15.28 detected both the West African (Ghanaian) and North African (Egyptian) strains of the parasite, thereby suggesting that they would be suitable for diagnosis in different geographical areas. However, only Sh2/15.F could detect antigens in *S. haematobium* infected human urine, indicating that this antibody may be more suitable for diagnostic purposes. Furthermore, the ability of Sh2/15.F to detect antigens by the dot-ELISA, adds to its probable usefulness, since the MoAb may then be suitable for developing field applicable membrane-based dipstick assays. Western immunoblot analysis showed that Sh2/15.F bound a polypeptide antigen of approximate molecular weight of 29 kDa, whilst Sh3/15.28 bound a glycoprotein epitope located on 53, 56.7 and 66 kDa antigens

Even though the *S. haematobium* Egyptian strain-specific MoAb Sh3/44.3 is not likely to be useful in diagnosis, it may be potentially useful in studies intended to characterize *S. haematobium* strains. On the other hand, *Schistosoma* genus-specific MoAbs that could detect urinary antigens may be suitable for the development of genus-specific assays for use where species-specific identification is not critical. Furthermore, genus-specific MoAbs that detected protein antigens may also be useful in studies towards the search for schistosomiasis protective antigens. This is because it is generally accepted that the most suitable vaccine candidate antigens for the disease are the cross-reactive proteins.

Two of the MoAbs (Sh3/15.13 and Sh4/16.45) that detected cross-reactive antigens in *S. haematobium* and *S. japonicum* could also bind the antigen in *S. haematobium* infected human urine and may therefore be useful for diagnosis of urinary schistosomiasis since its distribution does not coincide with that of the disease caused by *S. japonicum*.

In conclusion, therefore, this study has shown that some of the MoAbs generated against *S. haematobium* are suitable for the development of non invasive field-applicable diagnostic assays for urinary schistosomiasis and/or for intestinal schistosomiasis. Furthermore, the identification of *S. haematobium* antigens that are likely to be of protective value would facilitate studies aimed at controlling schistosomiasis.

# **CHAPTER 1**

## **INTRODUCTION**

## 1.1 General Introduction

Schistosomiasis is an important socio-economic disease of man and livestock caused by trematode parasites of the genus *Schistosoma*. The disease is mostly endemic among rural communities in tropical and subtropical areas (Doumenge, Mott, Cheng, Villenave, Chapuis, Perrin and Reaud-Thomas, 1987). It is estimated that at present about 250 million people world-wide are infected and a further 600 million people are at risk (Vogel, 1973; Nash, Cheever, Ottesen and Cooks, 1982; Wurapa, Barakamfitye and Mott, 1989; Markovics, Ram, Grossman, Ziv, Lantner and Schechter, 1994). According to Bergquist (1987), the highest infection rates are found in Brazil, Egypt and Ghana. In addition, schistosomiasis has been found to be directly or indirectly involved in 800,000 deaths per year (Grzych, Roussel-Velge and Capron, 1989). According to WHO (1985), the disease is still spreading because of increasing snail habitats created mainly by water resource developments. For example, in Ghana the construction of the Akosombo dam led to the creation of the Volta lake with accompanying changes in the ecology of the area leading to extensive proliferation of aquatic plants suitable for breeding the planorbid intermediate snail host of schistosomiasis and, therefore, increased disease transmission (Odei, 1965; Derban, 1984; Okoh, 1994).

Today, schistosomiasis is one of the most widespread parasitic diseases of man. It is endemic in seventy-five (75) countries distributed in Africa, Madagascar, South America, India, South East Asia, the Far East, parts of the Middle East and



the countries bordering the Mediterranean including a small focus in Portugal. In Africa, urinary schistosomiasis is endemic in 38 countries whilst intestinal schistosomiasis is found in 36 countries. The distribution of the two diseases overlap in 34 African countries (Wurapa *et al.*, 1989) and in parts of Southwest Asia (Sturrock, 1987).

In man, schistosomiasis may be caused by *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. mekongi* and *S. intercalatum*. The acute disease is characterized by Katayama fever, anaemia, or fluid/electrolyte problems in severely toxæmic victims. A real threat of ectopic Central Nervous System involvement leading to myelitis or cranial palsies exists.

Chronic schistosomiasis caused by *S. haematobium*, is characterized by haematuria (bloody urine), which is caused by haemorrhages resulting from penetration of the bladder wall by schistosome eggs, frequent micturition and occasional dysuria (painful micturition). The illness is usually mild but the symptoms are intractable. Late complications of *S. haematobium* infection may include calcification of the bladder, hydronephrosis, hydro-ureter and carcinoma of the bladder. On the other hand, chronic schistosomiasis caused by *S. mansoni* and *S. japonicum* are characterized by enlargement of the liver and the spleen, diarrhoea with blood and mucus in the stools resulting from ulcerative and polypoidal lesions in the colon, scattered granulomatous foci of the gut and liver that could lead to pipestem fibrosis and cirrhosis of the liver and ascites.

In domestic animals, schistosomiasis is caused by *S. mattheei*, *S. curassoni*, *S. spindale*, *S. nasali*, *S. leiperi*, *S. indicum*, *S. bovis*, *S. incognitum* and *S. japonicum*.

Owing to its importance, various strategies have been tried in an attempt to control schistosomiasis. These include; Chemotherapy (Cook, Jordan and Bartholomew, 1977), provision of sanitary facilities for safe and acceptable human waste disposal (Jordan, 1977; Jordan and Webbe, 1982), control of human and/or animal access to contaminated water, environmental modification and chemical or biological destruction of intermediate snail hosts (Jobin, 1979), and a programme of continuing health education. However, these efforts aimed at controlling schistosomiasis has achieved limited success due in part to logistical constraints and cost of their proper application (Manson, 1989). Consequently, there has been a shift of emphasis from transmission control or eradication of the disease to morbidity control (Bergquist, 1995). In addition, it has been widely communicated in recent times (Peters and Kazura, 1987; De Clercq, Sacko, Vercruyse, Diarra, Landoure, vanden-Bassche, Gryseels and Deelder, 1995) that effective management of schistosomiasis requires the development of accurate diagnostic tools and eventually the identification of protective antigens.

Diagnosis of schistosomiasis is however beset with several problems. For example, the standard method for identification of schistosome infections in man is by microscopic demonstration of schistosome eggs in excreta or biopsy specimen. In the case of human urinary schistosomiasis, routine diagnosis is normally

achieved by examination of terminal urine for the presence of blood or eggs of the parasite, whilst the presence of blood or schistosome eggs in human stool specimen may indicate intestinal schistosomiasis. This method of identifying parasite eggs is very specific, but it is not sensitive enough because of periodic fluctuation of egg output, the lodging of eggs in tissues and the small numbers of eggs excreted. Furthermore the use of bloody excreta for the diagnosis of infections could be misleading since bloody urine or stool could be precipitated by other disease conditions such as genito-urinary tract infections and dysentery.

Other methods used for the diagnosis of schistosomiasis involve detection of host antibodies produced against parasite antigens or detection of circulatory parasite-specific antigens. Several tests including complement fixation (CF), indirect haemagglutination assay (IHA), thin layer immunoassay, gel precipitation, indirect immunofluorescent assay (IFA) and enzyme-linked immunosorbent assay (ELISA) have been developed and used in detecting host antibodies (Hillyer and Gomez de Rios, 1979; Maddison, 1986). These tests are generally very sensitive, but they also have several limitations. For example, the presence of specific antibodies does not always indicate an active infection, since antibody titres remain high for a long time after spontaneous recovery or treatment. Furthermore, antibody levels seldom show a good correlation with intensity of infection and are of inadequate specificity.

In view of the limitations with antibody detection, the identification of parasite antigens in infected hosts has been found to have two main advantages.

Firstly, detection of parasite species-specific antigens permit the development of highly specific tests suitable for identifying active infections and whose sensitivity could also be enhanced through the use of monoclonal antibodies (MoAbs). Secondly, the appearance of circulatory parasite antigens in the urine of infected persons open up avenues for the development of simple, field applicable MoAb-based tests. The prospects of MoAbs in the development of such assays is one of the reasons that led the WHO Scientific Working Group (SWG) on schistosomiasis to recommend the use of MoAbs in those reagents in the overall strategy for schistosomiasis control (Bergquist, 1984). Another reason is that MoAbs have been found to play an important role in studies towards the identification of protective vaccine candidate antigens for schistosomiasis. Unfortunately, work towards identification of novel *S. haematobium* antigens was reported to lag behind the progress made with *S. mansoni* or *S. japonicum* (Bergquist, 1995).

The need to identify more novel diagnostic or protective schistosome antigens especially in the case of *S. haematobium* therefore continues to call for more work. In a recent study, several monoclonal antibodies were produced against *S. haematobium* (Amanor, Bosompem, Arishima, Assouku and Kojima, 1996; Bosompem, Arishima, Yamashita, Ayi, Anyan and Kojima, 1996a) and some were shown to be *S. haematobium* species- or *schistosoma* genus-specific. More MoAbs remain to be characterized whilst the molecular weight characteristics of some of the potentially useful specific antigens have not been successfully determined by western blotting experiments.

The aim of the work to be conducted here, therefore, was to characterize 19 monoclonal antibodies generated against *S. haematobium* antigens.

**1.2****The objectives of the study**

- 1.2.1.** To determine the specificities of anti-schistosome MoAbs through cross-reactivity studies with *S. haematobium*, *S. mansoni*, and *S. japonicum*.
- 1.2.2.** To investigate the ability of the MoAbs to detect urinary *S. haematobium* antigens.
- 1.2.3.** To characterize the antigens detected by the MoAbs using western immunoblot analysis and biochemical studies, and to localize them using immunocytochemical methods.
- 1.2.4.** To investigate the conditions under which those antigens which do not react in the western immunoblot assay will be made to do so as a prelude for utilizing the assay in diagnosis or for isolating semi-purified antigens for protective studies.

**1.3****Justification**

The characterization of Anti-*S. haematobium* MoAbs would provide needed information on the usefulness of the detected antigens in diagnosis and/or protection against schistosomiasis. Furthermore, investigations into the reactivity of these MoAbs in the western immunoblot assay would facilitate identification of the useful epitopes and their further exploitation through protein and gene sequencing, and gene cloning and recombinant DNA technology for purposes of improved diagnosis or vaccination against the disease.

## **CHAPTER 2**

### **LITERATURE REVIEW**



## 2.1 Schistosomes and Schistosomiasis

Human schistosomiasis is caused by flukes of the genus *Schistosoma* which dwell in the blood vessels of the infected host. The location of the parasites in the host depends on the schistosome species involved. For example, *S. haematobium* localizes in the vesical plexus around the urinogenital system whilst, *S. mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi* are normally found in the hepatic portal system around the gastro-intestinal tract (Sturrock, 1987).

The first schistosome to be described was *Schistosoma haematobium*. Adult worms of this parasite were discovered in the veins of a man at autopsy in Cairo by the German surgeon Theodor Bilharz in 1851 and described in 1852. The disease caused by the schistosomes parasites, Bilharziasis, was later named after him. Elucidation of the schistosome life-cycle, however, was not made until 1913 when Miyairi and Suzuki showed that *S. japonicum* developed in the hydrobid snail *Oncomelania hupensis nosophora* (Rollinson and Southgate, 1987).

## 2.2 Classification and morphology of schistosomes

Schistosomes are members of the family Schistosomatidae (Webbe, 1982), which are dioecious Digenea parasites of vertebrates, transmitted by fresh water snails (Sturrock, 1987). These parasites derive their name from a general feature of the family the ventral groove also called gynaecophoric canal, or schist, which is formed by ventrally flexed lateral outgrowth of the male body within which the

adult male clasps the cylindrical female to give the pair an elongated nematoid form suited to living in the blood vessels (Sturrock, 1987; Rollinson and Southgate, 1987). The family can be divided into three subfamilies; the Schistosominae, Bilharziellinae and Gigantobilharziinae (Table 1). Of the twelve genera within the family, seven are confined to birds and five to mammals, but only the genus *Schistosoma* is associated with man.

Eighteen species belonging to the genus *Schistosoma* are recognized and placed in four different groups also called species complexes by Kuntz (1955) based on the common relationships of the parasite species with particular snail host genera and on zoo-geographical distribution patterns as well as the morphology of the parasite's egg. The groups are the *S. haematobium*, *S. mansoni*, *S. japonicum* and *S. indicum* species complexes.

Epidemiological studies have shown that the genus *Schistosoma* has achieved the greatest geographic distribution and diversification in terms of numbers of recognized species and different hosts parasitized (Rollinson and Southgate, 1987). As comprehensively reviewed by Sturrock (1987), the overall distributional range of the different schistosome species is primarily influenced by the presence or absence of suitable mammalian and intermediate snail hosts. Unsuitable ambient temperatures have been particularly noted to affect this distribution as it affects intramolluscan schistosome development. This temperature effect is likely to contribute to the absence of *S. intercalatum* in places where both the mammalian and snail hosts exist. In the case of *S. mansoni* and *S. haematobium*,

Table 1: Classification of Schistosomes (After Manson, 1989; Rollinson and Southgate, 1987)

Kingdom	Animalia										
Subkingdom	Metazoa										
Phylum	Arthropoda	Nematoda	Mesozoa	Platyhelminthes	Acanthocephala	Pentastomida	Annelida				
Class	Aspidogastrea	Monogenea	Digenea	Cestoda	Turbellaria						
Superorder	Epitheliorcystida			Anepitheliorcystida							
Order	Strigentida			Echinostomati							
Family	Diplostomatidae	Bucephalidae	Schistosomatidae	Spirochidae	Cyclocoelidae	Strigeidae					
Subfamily	Gigantobilharzinae		Schistosominae			Bilharziellinae					
Genus	Ornithobilharzia	Microbilharzia	Orientalobilharzia	Austro-bilharzia	Schistosoma	Schistosomatium	Bivettobilharzia	Heterobilharzia			
Species	hippopotami	rodhaini	leiperi	matheei	japonicum	mansoni	haematobium	intercalatum	bovis		
	indicum	curassoni	margrebowiei	spindalei	incognitum	mekongi	sinensium	nasale			

however, the presence of suitable intermediate snail hosts alone is a very strong indicator for the presence of the parasites or their potential introduction. The most important human schistosomes are *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. mekongi* and *S. intercalatum*. In domestic animals schistosomiasis is caused by *S. mattheei*, *S. curassoni*, *S. spindale*, *S. nasali*, *S. leiperi*, *S. indicum*, *S. bovis*, *S. incognitum* and *S. japonicum*. *S. japonicum* is the only important schistosome pathogen of man which also infect domestic animals mainly cattle, sheep and goats. On rare occasions man may also be infected by the animal schistosomes; *S. bovis*, *S. curassoni*, *S. margrebowiei*, *S. mattheei* and *S. rodhaini* (Sturrock, 1987).

### 2.2.1 The *S. haematobium* group

The *Schistosoma haematobium* group of parasites are distributed in Africa and adjacent islands of Zanzibar and Madagascar, parts of the Middle East including Iran, Iraq, Lebanon, Saudi Arabia, Syria, Turkey and North and South Yemen and a small focus in India and Mauritius (as reviewed by Iarotoski and Davis, 1981; WHO, 1985; Wilkins and Gilles, 1987; Rollinson and Southgate, 1987). The group comprises seven species, namely, *S. haematobium*, *S. intercalatum*, *S. mattheei*, *S. bovis*, *S. curassoni*, *S. margrebowiei* and *S. leiperi*. Members of this group show marked differences in the shape of their eggs and kinds of definitive hosts. In Africa some members of this group occur in all countries except Rwanda and Burundi. In affected areas schistosomiasis is often endemic in only a part of a country where it coincides with the distribution of the snail hosts (Wilkins and Gilles 1987).

There is evidence for geographical variation in many characters associated with the *S. haematobium* group of species. For example, laboratory studies with hamsters have revealed differences between strains of the parasites that differ in many biological features including, intermediate host specificity, infectivity of cercariae, adult worm growth rates and maturation times, egg productivity and the distribution of egg in infected host organs (Wright and Knowles, 1972).

As has been comprehensively reviewed by Brown (1980), *S. haematobium* in the tropical regions of Africa is generally transmitted by snails of the *Bulinus africanus* group. In the Mediterranean area and South West Asia, transmission is mainly by the tetraploid members of the *B. truncatus/tropicus* complex and in Arabia and Mauritius by members of the *forskalii* group of snails. In West Africa, all three of these snail groups are known to act as intermediate hosts and in Arabia the *B. truncatus* group is also implicated. Of particular significance is the observation that *S. haematobium* from North Africa and the Middle East develops in *B. truncatus* and the parasite from tropical Africa develops in snails of the *B. africanus* group, but with few exceptions, neither of these forms can develop in the intermediate host of the other. Frandsen (1979), therefore, emphasized that each schistosome species in Africa is composed of a complex of genetically different populations each being highly adapted to the transmission conditions in their respective distributional areas.

Despite the marked differences between members of the *S. haematobium* group of parasites natural hybridization has been reported to occur in man. For example, pairing between male *S. haematobium* and female *S. intercalatum* in

Cameroun and between male *S. haematobium* and female *S. mattheei* in parts of Southern Africa were reported by Sturrock (1987). Also Chu, Kpo and Klumpp (1978) reported possible hybridization between the biologically different strains of *S. haematobium* in parts of Ghana. These observations may in all suggest antigenic polymorphism among this group.

### **2.2.2 The *S. mansoni* group**

*Schistosoma mansoni* is the most important parasite that causes intestinal schistosomiasis in man in both the New and Old Worlds (WHO, 1985). The parasite occurs in Libya, Oman, Saudi Arabia, North and South Yemen and Madagascar. It is also distributed discontinuously over the greater part of Africa south of the Sahara. In the Caribbean, it is endemic in Puerto Rico, St. Lucia, Guadeloupe, Martinique, Dominican Republic, Antigua and Monstreat. In South America, intestinal schistosomiasis exists in Brazil, Surinam and Venezuela (Rollinson and Southgate, 1987).

Of the seventeen well-defined species of *Biomphalaria* in the Americas, only *B. glabrata*, *B. amazonica* and *B. tenagophila* have been found naturally infected with *S. mansoni* (Rollinson and Southgate, 1987). However, *B. peregrina* (Paraense and Correa, 1973) was found to be susceptible to infection in the laboratory. Snails from different geographical areas tend to show variation in levels of susceptibility to different strains of *S. mansoni* (Basch, 1976; Michelson and Dubois, 1978) and populations of snails from a given area may vary in their

susceptibility to parasites isolated from anyone of the intermediate snail hosts. In Africa, twelve species of *Biomphalaria* are recognized (Brown, 1980) and all those that have been tested appear to show some compatibility with at least certain strains of *S. mansoni*. *B. pfeifferi* shows a broad compatibility and is, therefore, regarded as an important intermediate host, where as species such as *B. alexandrina* appears to be susceptible only to the local *S. mansoni* from Egypt (Frandsen, 1979).

### **2.2.3 The *S. japonicum* group.**

*S. japonicum* is responsible for a grave, debilitating and chronic form of intestinal schistosomiasis which affects both man and domestic animals. The disease is widespread in the Far East where it is endemic in parts of China, Taiwan, Indonesia and the Philippine Islands of Leyete, Samar, Mindanao, Bohol, Mindoro and Luzon (Olveda and Domingo, 1987 and Rollinson and Southgate, 1987). In Japan, the parasite is limited to three main areas, namely Kufu, Katayama and the basin of the Chikugo River.

*S. japonicum* is a true zoonosis and occurs as a natural parasite of a large number of mammalian species which play an important role in the epidemiology of the disease. On mainland China, 31 species of wild mammals including carnivores, rodents, primates, insectivores and artiodactyls have been found with natural infections (Mao and Shao, 1982). However, domestic animals, especially cattle, pigs and dogs, have been found to play the most important role in the epidemiology of the disease.

Throughout its range, *S. japonicum* is transmitted by populations of polytypic *Oncomelania hupensis* which is an amphibious snail. There are six subspecies (Davis, 1980) namely *O. hupensis chiui* and *O. hupensis formosana* in Taiwan, *O. hupensis hupensis* in mainland China, *O. hupensis lindoensis* in Sulawesi, *O. hupensis quadrasi* in Philippines and *O. h. nosophora* in Japan. Other members of the group are *S. mekongi* (Voge, Bruckner and Bruce, 1978) which is endemic on Khong Island in the Mekong River in southern Laos and in many parts of northern and central Cambodia. *S. mekongi* is transmitted by the aquatic snail *Tricula aperta*; and *S. sinensium* which is endemic in the Szechuan Province of China (Pao, 1959) where it infects only field rats and transmitted by species of *Tricula*.

### **2.3 The life cycle of schistosomes**

As has been comprehensively reviewed by Sturrock (1987) and Rollinson and Southgate (1987) the schistosome life cycle consists of a succession of stages namely, the egg, miracidium, first and second stage sporocysts, cercaria, schistosomulum and adult worms. The first and second stage sporocysts reproduce asexually within the snail, whilst the adult worms reproduce sexually in the mammalian hosts. The eggs pass out of the body of the definitive host in the excreta (urine or faeces), and on encountering fresh water, they hatch into free-living ciliated embryos known as miracidia. The free-swimming miracidia seek and find compatible snail species which they penetrate within a few hours after hatching.



Within the tissues of the snail, each miracidium transforms into a mother sporocyst, which divide asexually to give rise to large number of second stage sporocysts each of which in turn produces a number of fork-tailed free-swimming larvae called cercariae. The time of development from miracidia penetration to cercarial release is influenced mainly by the prevailing ambient temperature, varying from many months at below 18°C to less than three weeks as temperatures approach 30°C. The cercariae which alone are capable of infecting the mammalian host, are released in daily bursts over a fairly long period that may last several months if the snail survives the infection. Mammalian infection begins with cercarial penetration of intact skin or subcutaneous membranes. Following entry the cercaria transforms into a tailless worm-like schistosomulum which migrates along the blood vascular system and develops into the mature adult worm. Sex is determined at the time of egg fertilization and is, therefore, the same for all the offspring of any one egg. In humans, the prepatent period is six to twelve weeks depending on the schistosome species.

#### **2.4. Morphology and ultrastructure of the schistosome egg**

Schistosome eggs are generally oval in shape and non-operculate. They are characterized by a pointed expansion of the shell to form either a lateral or a terminal spine. This characteristic is one of the most important features considered in the identification of the different schistosome species using the morphology of the eggs. Kusel (1970) provided a detailed morphological description of *S. mansoni*

eggs and showed that the miracidium within the egg is suspended by four vacuoles which together with the miracidium are surrounded by a vitellin membrane. Earlier on, Inatomi (1962) had shown that schistosome eggs possess sub-microscopic egg-shell pores through which various egg antigens including enzymes could be released. Indeed, it has been reported that numerous interacting factors, namely, the spine of the egg, proteolytic enzymes secreted by the miracidia inside the egg blood pressure, and peristalsis may be involved in the passage of eggs through the walls of vessels and parenchyma of the intestines or bladder (Smith, 1974; Bloch, 1980). The number of eggs excreted daily by schistosomes is estimated as the number of eggs per worm pair. This estimate differs greatly between different species of the parasite. For example, Loker (1983) estimated 560-2200 for *S. japonicum*, 66-495 for *S. mansoni* and 22-203 for *S. haematobium*.

Once excreted, the eggs hatch to release the miracidia under suitable environmental conditions which normally increase the chances of contacting suitable snail hosts. Studies by Erasmus (1972), Morgan (1972), Bair and Etges (1973) have shown that the major factors involved are temperature, light and osmotic pressure.

## **2.5 Morphology and ultrastructure of the miracidium**

Electron microscopy studies by several workers (Pan, 1965; Schutte, 1974a and Eklun-Natey, Wuest, Swiderski, Striebel and Huggel, 1985), have thrown more light on the morphology and ultrastructure of the miracidium, which represents the

first free larval stage in the life-cycle of schistosomes. A newly released miracidium is generally pyriform, and varies between 150 and 180  $\mu\text{m}$  in length and 70 and 80  $\mu\text{m}$  in width. The body of this larval stage is covered with 21 or 22 ciliated, enucleated cells responsible for its swift movement in water. The anterior extremity has a hemispherical structure called tetraborium, which is formed of anastomosed membrane folds with at least 12 ciliated sensory organelles. The secretory ducts of the apical and lateral glands emerge at this point.

The miracidium has an excretory system which comprises an anterior and posterior pair of flame cells and a common duct which opens laterally in the posterior third of the body. It is likely that a large amount of soluble egg antigens found in the urine of infected persons are secreted by the miracidium via this route. It is generally accepted that penetration of miracidia into snail tissues occurs by a mechanical action, (Jourdan and Theron, 1987), however, the role of histolytic secretions of the glandular cells of the miracidia could be implicated.

## **2.6 Cercarial penetration and development of schistosomes**

Schistosome cercariae can penetrate the unbroken skin of man and other mammals within minutes (Cousin, Stirewalt and Dorsey, 1981). During the penetration process, the cercaria loses its tail and external layer and changes from a fresh water organism into one which can survive only in salt water and is then called schistosomulum. Structural changes during transformation from cercaria to schistosomulum in the host tissues have been reviewed extensively by Stirewalt

(1974) and McLaren (1980). The syncytial surface of the cercaria is bounded by a normal trilaminar plasma membrane, external to which is a 1-2µm thick, fibrillar glycocalyx. This is shed in the form of numerous microvilli up to 4µm long, and simultaneously replaced by a multilaminar configuration (McLaren, 1980). The schistosomulum moves through the tissues into the lymph and blood vessels and ultimately reaches the lungs where they remain for several days. They then migrate to the liver via the bloodstream or directly through the tissues (Wilson, 1987). In the liver, they grow into adult male and female schistosomes, depending on the sex of the cercariae. Male and female worms pair up and pass down the mesenteric or vesical venules depending on the schistosome species. Each worm pair produces 300-3000 eggs per day and may do so for many years (Wilson, 1987). Approximately half of these eggs are excreted from the body, and the remainder remain trapped in host tissues (Warren, 1973). Thus during the course of an infection, the host is exposed to antigens from the schistosomulum, the adult worms as well as the parasite's eggs.

## **2.7 Schistosome antigens**

During a normal infection, the schistosome presents a complex array of antigens to its host. These include soluble antigens in secretions and excretions, and structural antigens on the surface, of the different developmental stages of the parasites that occur in the host. Numerous investigations within the last few years have contributed to a better knowledge of schistosome biochemical structures and

significant progress has been made in identification, isolation and in some instances biosynthesis of potentially protective and diagnostically useful antigens (Rumjanek, 1987; Kelly; 1987; Dissous, Balloul, Pierce and Capron, 1987; Bergquist, 1995)

Different stages of the schistosome life cycle have been implicated by various investigators to present the major antigenic stimuli to the host. Kelly (1987) listed the adult worm, egg and schistosomulum as most important parasite stages in this respect, but further argued that adult worm and egg antigens by far constitute the major stimuli. Even though these antigens play a major role in host immune responses to schistosomes, however, Dissous *et al.* (1987) reported that it is the surface membranes of cercariae and the newly transformed schistosomula that present the major targets of protective host immune responses.

The young schistosomulum has been found to be susceptible to a wide variety of cytotoxicity mechanisms involving specific antibodies and effector cells (Butterworth, Taylor, Veith, Vada, Dessaint, Capron, Joseph and Topier, 1982; Capron, Dessaint, Capron, Joseph and Topier, 1982), and as a result the antigens exposed at the surface of the schistosomulum have been extensively studied. The development of hybridoma technology has for example, allowed the selection of protective monoclonal antibodies and the identification of their corresponding target antigens. Also studies conducted by several workers including Kusel, Sher, Perez, Clegg and Smithers (1975), Haguya, Murrel, Taylor and Vannier (1979), Dissous, Dissous and Capron (1981), Aronstein and Strand (1983) on antigens recognized by sera from patients infected with *S. mansoni*, *S. haematobium* and *S. japonicum* have

revealed about 20-30 antigenic polypeptides of adult worms and about 20 glycoprotein egg antigens of each of the three schistosome species. There is now accumulating evidence that both polypeptides and carbohydrate epitopes are involved in the expression of immune response to schistosomes and are responsible for both cellular and humoral responses to schistosome infection. Progress in molecular cloning of parasite antigens and studies using hybridoma-derived antibodies has facilitated the identification and production of large amounts of schistosome antigens for use in immunodiagnosis, and immunization and vaccination trials (Dissous *et al.*, 1987).

#### 2.7.1 Schistosomula polypeptide surface antigens

Dissous *et al.* (1981) identified a set of polypeptide surface antigens ranging between Mr 32-40,000 by radioiodination of intact live schistosomulum, followed by immunoprecipitation with immune rat sera. The antigens were later shown to consist of four components by polyacrylamide gel electrophoresis but only one subset of these polypeptides were recognized as antigenic by infected animals and human patients (Smithers, Simpson, Yi, Omer-Ali, Kelly and McLaren, 1987; Dissous, Grzych and Capron, 1982). Other schistosomula surface antigens were identified by iodogen-catalysed radioiodination (reviewed by Simpson and Smithers, 1985). Sera from chronically infected mice were found to recognize a slightly different profile of antigens compared with sera from mice immunized by exposure to radiation-attenuated cercariae. Collectively, however, both sera were

found to precipitate antigens of  $M_r > 200,000$ , 92-94000, 32-38000, 20000, 17000 and 15000. Due to its reproducibility and ease of application, iodogen-catalysed labelling has been used extensively. However, the use of other labelling techniques have led to the identification of new schistosomula surface antigens. For example, Taylor, Haguya and Vanier (1981) used the diazonium salt of  $^{125}\text{I}$  iodosulphanilic acid, to identify three major schistosomula surface antigens of  $M_r$  15, 28, and 69. Tremendous progress in the identification of new schistosomula surface antigens has, however, been made through the use of monoclonal antibodies. Harns, Mitsuyama, Haguanel and David (1985) precipitated an antigen of  $M_r$  22,000 from solubilized schistosomula surface membrane-enriched fractions labelled with the Bolton-Hunter reagent using a monoclonal antibody that bound the surface of live schistosomulum. In a similar study, Dissous *et al.* (1982) precipitated an antigen of approximate  $M_r$  38000 which was shown to be recognized during chronic infections of both rodents and man and Simpson and Smithers (1985) identified a  $M_r$  17000 and a complex of  $M_r$  24-30000 major *S. haematobium* schistosomulum surface antigens which appeared to be species-specific since they were not recognized by human anti-*S. mansoni* serum.

Three *S. mansoni* proteins have been identified in the 27-28 kDa range. One of these antigens (P28) present in adult worm antigen extract was particularly studied for its protective effect against mouse, hamster, rat and monkey schistosomiasis (Dissous *et al.*, 1987). Western blot analysis revealed that this antigen is present in four schistosome species: *S. mansoni*, *S. japonicum*, *S.*

*haematobium* and *S. bovis* (Balloul, Grzych, Pierce and Capron, 1987b). The functional properties of the anti-P28 antibodies were first demonstrated *in vitro* by their ability to mediate killing of schistosomula in the presence of eosinophils. Using Recombinant technology, Balloul, Sondermeyer, Dreyer, Capron, Grzych Pierce, Carvalho, Lecocq and Capron. (1987a) cloned and expressed complementary DNA (cDNA) encoding this 28 kDa antigen in *Escherichia coli*, thereby facilitating its exploitation as a protective antigen.

Balloul *et al.* (1987a) isolated the 28 kDa antigen by electro-elution of adult *S. mansoni* proteins from polyacrylamide gels. Antisera produced in rats against the 28 kDa protein recognized *in vitro* translation products from adult worm mRNA showing the same electrophoretic mobilities in SDS-PAGE and isoelectricfocusing ( $P_i$  6.3-6.5). This result suggested that the P28 protein was not processed after the translation step (Balloul, Pierce, Grzych and Capron, 1985). Immunofluorescence studies demonstrated that P28 was mainly located in the parenchyma of schistosomula and adult worms, including the dorsal spines of the parasite. However, immunoprecipitation of a 28 kDa labelled molecule from surface-radioiodinated schistosomula using anti-P28 antibodies, indicated that the antigen could be briefly exposed at the surface of schistosomula and may represent an excreted or secreted metabolite of parasites. In similar studies using monoclonal antibodies Harns *et al.* (1985) identified a major surface antigens of *S. mansoni* of similar molecular weight of 28kDa which was shown to be actively synthesized by



developing schistosomulum but, however, found to be present in extracts of all four stages of the life-cycle.

### 2.7.2 Schistosomula Carbohydrate surface antigens

In studies with schistosome antigens the emphasis was laid on protein or glycoprotein antigens. This was because of the possibility of synthesizing them in large quantities in microorganisms using recombinant DNA technology to provide the basis of an anti-schistosome vaccine. Nevertheless, the importance of existing glycolipid and polysaccharide antigens has not been ruled out (Simpson, James and Sher, 1983). A number of studies have shown that carbohydrate components are significant contributors to the antigenicity of the schistosomula surface. Omer-Ali, Magee, Kelly and Simpson (1986) showed that under conditions of antibody excess, sera from chronically infected mice bound *S. mansoni* schistosomulum surface antigens at a 2-3 fold higher level than sera from vaccinated mice. Treatment of the schistosomula with reagents such as trifluoromethanesulfonic (TFMS) and sodium periodate which selectively remove or modify carbohydrates indicated that most of the antibodies in sera from chronic infections reacted with specific carbohydrate epitopes not recognized by sera from vaccinated animals. These epitopes were shown to be in antigens of M<sub>r</sub> 17000, 32000, 38000, and >200000.

In studies with non-species-specific monoclonal antibodies that bound carbohydrate antigenic epitopes in glycoprotein antigens Yi, Omer-Ali, Kelly, Simpson and Smithers (1986) suggested that carbohydrate epitopes may be responsible for inducing the non-species-specific concomitant immunity whereas

polypeptide epitopes are more likely to be involved in species-specific immunity. The immunogenic stimuli for concomitant immunity could, therefore, also come from either eggs or adult worms, since both share carbohydrate epitopes with the schistosomulum (Hamburger, Lustigman, Arap Siongok, Ouma and Mahmoud, 1982; Omer-Ali *et al.*, 1986; Yi *et al.*, 1986). Nevertheless, several workers have demonstrated the presence of IgM blocking antibodies in the sera of chronically infected rats and humans that are directed against carbohydrate epitopes (Grzych, Capron, Lambert, Dissous, Torres and Dissous, 1985; Yi *et al.*, 1986; Capron, Pearce, Balloul, Grzych, Dissous, Sondermyer and Lecocq, 1987). The role of these blocking antibodies may explain in part the susceptibility to reinfection by *S. mansoni* in humans whilst the loss of the antibodies may ultimately lead to the acquisition of immunity (Capron *et al.*, 1987).

The importance of a group of 30-40 kDa antigens has also been demonstrated during infection of various animal species including rats, mice, monkey and humans (Dissous *et al.*, 1981; Dissous and Capron, 1982; Simpson *et al.*, 1983). One of these antigens (38 kDa) was selectively precipitated by a rat monoclonal IgG2a (IPLSm1) that induced *in vitro* eosinophil-mediated cytotoxicity and protected naive rats by passive transfer (Dissous *et al.*, 1982; Grzych, Capron, Bazin and Capron, 1982). The protective epitope was characterized on a 115 kDa molecule in adult worm metabolic products, thus providing a molecular support for concomitant immunity (Dissous and Capron, 1983). It was also identified on high molecular weight components of cercariae (Dissous *et al.*, 1982) and miracidia

(Dissous, Grzych and Capron, 1986) surfaces. These observations suggested that the epitope could involve carbohydrate chains linked to different proteic or proteolipidic structures. Moreover, the sensitivity of the epitope to periodate treatment and the inability of IPLSml to precipitate *in vitro* translation products from parasite messenger RNA (mRNA) confirmed the hypothesis of a carbohydrate nature of the epitope. The major immunogenicity of the 38 kDa carbohydrate moieties was confirmed in mouse and human schistosomiasis (Omer-Ali *et al.*, 1986; Khalife, Capron, Capron, Grzych, Butterworth, Dunne, and Ouma, 1986).

### 2.7.3 Schistosome egg antigens

The main pathologic feature of hepatosplenic schistosomiasis involves the formation of granulomatous inflammation around eggs entrapped in the tissues and portal vasculature. The immune response also leads to fibrosis which affects the organ's architecture and circulation in the affected organs (Warren, 1972 and Kelly, 1987). In *S. mansoni* infections, the granulomatous reaction around the eggs is essentially a cell-mediated immune response to antigens normally secreted by mature viable eggs (Boros and Warren, 1970; Hang, Warren, Boros, 1974). Warren (1972) showed that the soluble egg antigens (SEA) in the supernatant of ultracentrifugated egg homogenate can elicit granulomatous hypersensitivity and other immunologic reactions characteristic of intact eggs. The crude SEA preparation was shown by Carter and Colley (1978, 1979) and Pelley, Pelley, Hamburger, Peters and Warren (1976) to contain multiple glycoproteins and non-

glycoconjugated proteins. Also, studies with SEA glycoproteins (purified from SEA by affinity Chromatography on immobilized Concanavalin A) suggested their possible importance in the induction of granulomatous hypersensitivity as well as the elicitation of other delayed type responses (Pelley *et al.*, 1976; and Carter and Colley, 1979). Furthermore Carter and Colley (1979) showed that only the glycoprotein fraction of SEA was capable of eliciting T-cell responses.

Investigations by Pelley *et al.* (1976), showed that sera from mice with light chronic *S. mansoni* infections identified three major serological antigens which formed the bulk of the glycoprotein fraction obtained with ion exchange chromatography on Concanavalin A. These antigens were designated MSA<sub>1</sub>, MSA<sub>2</sub> and MSA<sub>3</sub> and their molecular weights estimated by gel filtration to be 50, 450 and 80 respectively.

Hamburger, Pelley and Warren (1976) investigated the specificity of these antigens using antigen-binding radioimmunoassay and showed that antibodies that reacted with MSA<sub>2</sub> and MSA<sub>3</sub> showed-cross-reaction with cercarial, but not adult worm antigens of *S. mansoni*. However they both cross-reacted with SEA from *S. japonicum* and *S. haematobium*. In contrast, MSA<sub>1</sub> appeared to be both egg stage- and species-specific. Further investigations with MSA<sub>1</sub> suggested that it may be a major immunopathological egg antigen (Kelly, 1987).

Three glycoprotein antigens of *S. japonicum* were also identified by Boros and Warren (1970) who demonstrated the sensitizing activity of Concanavalin A binding fraction of *S. japonicum* SEA. The molecular weights of the antigens were

determined by gel filtration to be 590, 245 and 46 kDa. The 46 kDa antigen was less sensitizing compared with the others. Boros and Warren (1970) and Warren, *et al.* (1975) studied *S. japonicum* and *S. mansoni* SEA and reported that SEA from *S. japonicum* elicited an immediate (antibody-mediated) inflammatory response when injected into the footpads of *S. japonicum* infected mice, whereas, *S. mansoni* SEA elicited a delayed type (cell-mediated) response. An enhanced granulomatous reaction was only observed when mice were pre-sensitized with eggs or SEA of *S. japonicum* injected subcutaneously as against intraperitoneal injection in the case of *S. mansoni*. Owashi and Ishi (1982) also purified a glycoprotein antigen of approximate  $M_r$  90 from SEA of *S. japonicum*. The antigen showed eosinophilic chemotactic activity which appeared to be dependent on the integrity of the carbohydrate moiety.

#### **2.7.4 Hepatotoxic egg antigens**

It has been observed that T-cell deprived mice infected with *S. mansoni* do not develop granulomatous reactions around eggs deposited in tissues within seven days of infection, however, they suffer an acute hepatotoxicity reaction (Byram, Doenhoeff, Musallam, Brink and von Lichtenberg, 1979). Injection of such mice with serum from chronically infected mice prevents the liver damage (Doenhoeff, Musallam, Bain and McGregor, 1979). Using chronic sera Dunne, Lucas, Bickle, Peresan, Madgwick, Bain and Doenhoeff (1981) identified 12 of the *S. mansoni* egg antigens by immunoelectrophoresis. Also, work with a series of sera with partially

overlapping specificities, showed that recognition of one particular antigen was required for sera to protect against liver damage. Subsequently, Dunne and Doenhoff (1983) purified and characterized this antigen coded  $w_1$  as a non-glycosylated polypeptide of approximate  $M_r$  22-26. This antigen appeared to be stage-specific and has proved a valuable immunodiagnostic reagent (Kelly, 1987).

### 2.7.5 Circulating schistosome antigens

Several studies have been conducted to detect circulating antigens in schistosome infected mammals. These studies are found necessary because existing assays utilizing crude or partially purified schistosome antigens to detect anti-schistosome antibodies do not give information suitable for the estimation of worm burden (Mott, 1982), or for identification of active infection. However, similar to the egg detection method, detection of circulatory antigens in body fluids of the host would provide a basis for the identification of active infection and give a better correlation to infection intensity (Deelder, Kornelis, Marck, Van Eveliegh and Egmond, 1980).

Berggren and Weller (1967) described a circulating schistosome antigen in the serum of mice and hamsters heavily infected with *S. mansoni*. The antigen was later characterized by Gold, Rosen and Weller (1969) who also demonstrated its presence in the urine of infected hamsters. They found that the antigen is anodic (on the basis of its mobility in immunoelectrophoresis) and is heat-stable and dialysable. Nash (1974) reported that, the circulating antigen was a large molecular weight

substance, most likely a polysaccharide. It was demonstrated in *S. mansoni*, *S. haematobium* and *S. japonicum* homogenates and in the serum of mice and hamsters heavily infected with *S. mansoni* and *S. japonicum* (Bawden and Weller, 1974). Nash (1974) and von Lichtenberg, Bawden and Shealy (1974) also showed that the same antigen was present in the epithelial cells of the schistosome gut. Deelder, Klappe, Van den Aardweg and Van Meerbeke (1976) confirmed the presence of circulating anodic antigens (CAA) in *S. mansoni* infected hamsters and also demonstrated the occurrence of a lower molecular weight circulatory cathodic antigen (CCA). Both the CAA and CCA were demonstrated in adult worm extracts as well as in the excretory and secretory products of the worms by Deelder *et al.* (1976).

## **2.8 Electrophoretic separation of proteins**

One- and two-dimensional gel electrophoresis of proteins are normally performed so as to provide answers to questions like: Is the protein pure? Does the protein have subunits? How many protein subunits are there? What are the molecular weights of the protein? Both electrophoretic methods have high-resolution which yield protein whose sequence can be determined after either electroelution or electroblotting onto polybrene-coated or derivatized glass fiber sheets both of which are compatible with a gas-phase protein sequencer. Alternatively, polyvinylidene difluoride membrane filter may be used for blotting the protein for sequencing. Separated proteins can be electroeluted from

polyacrylamide gels and utilized in diverse experiments including those aimed at improved diagnosis or in the search for protective antigens.

For a protein without subunits or one with identical subunits, the detection of single protein band after polyacrylamide gel electrophoresis (one-dimensional gel electrophoresis) or a single spot after two dimensional gel electrophoresis indicates that the protein is pure. However if the protein consist of multiple subunits of different molecular sizes purity may be confirmed by detection of a single stainable band after gel electrophoresis under nondenaturing conditions. Once a protein is demonstrated to be pure, an estimate of the molecular size of the units can be determined by subsequent electrophoresis under denaturing conditions in the presence of the anionic detergent “sodium dodecyl sulphate” (SDS) and by comparison to the molecular sizes of standard proteins.

### **2.8.1 One-dimensional Polyacrylamide gel electrophoresis**

#### **2.8.1.1 *SDS-denaturing discontinuous method***

Polyacrylamide gels are formed following polymerization of monomeric acrylamide into polymeric acrylamide chains and the cross-linking of these chains by N, N'-methylene-bisacrylamide. The polymerization reaction is initiated by the addition of ammonium persulfate and it is accelerated by N,N,N'-.N'-tetramethylethylenediamine (TEMED) which catalyzes the formation of free radicals from ammonium persulfate. It is advisable to degas the gel solution



before the polyacrylamide catalysts are added since dissolved oxygen inhibits the polymerization process.

The most widely used polyacrylamide gel electrophoresis system is the denaturing SDS discontinuous method (SDS-PAGE) described by Laemli (1970).

In this technique buffers of different pH and composition are used to generate a discontinuous pH and voltage gradient in the gel. Since the discontinuous system concentrates the protein in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

#### **2.8.1.2** *The electrophoresis principle*

The sample first passes through a stacking gel into which it is loaded. The stacking gel has large pores and is made with buffer containing chloride ions (called the leading ions) whose electrophoretic mobility are greater than the mobility of the proteins in the sample. On the other hand the electrophoretic buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility are less than the mobility of the proteins in the sample. The net result is that the faster migrating ions leaves a zone of lower conductivity between itself and the migrating protein. The higher voltage gradient in this zone allows the proteins to move faster and to stack. After leaving the stacking gel the protein enters the separating gel which has smaller pores and is made with a buffer with higher salt concentration and higher pH. In this separating gel, therefore, the

glycine ions migrate past the proteins and the proteins are separated according to molecular size in denaturing gel containing 0.1% SDS. In a non denaturing gel, the proteins are separated based on molecular shape, size, and charge.

### **2.8.1.3** *Preparation and characteristics of sample and gel*

Proteins are denatured by heating in the presence of a low-molecular weight thiol (2-mercaptoethanol) and SDS. Under these conditions the subunits of protein are dissociated and their biological activities are lost. It is therefore advisable to use gel electrophoresis as a last step in the purification of a protein whose identity is based on a functional assay. Most proteins bind SDS in a constant weight ratio, leading to identical charge densities for the denatured proteins. Hence, the proteins migrate in the polyacrylamide gel according to size, not charge. Most proteins are resolved on polyacrylamide gel containing 5 to 20% acrylamide and 0.2 to 0.5% bisacrylamide. It is recommended that for discontinuous polyacrylamide gel electrophoresis under denaturing conditions, 5% gel should be used for SDS-denatured proteins in the molecular size range of 60,000 to 200,000, and 10%, 15% gel for proteins in the range of 16,000 to 70,000 and 12,000 to 45,000, respectively. Over these ranges the relationship between the relative protein mobility and  $\log_{10}$  molecular weight of the protein is linear. The true estimate of the molecular size of an unknown protein (its subunits) may therefore be made by comparing the relative mobility of the

unknown protein to protein in a calibration mixture. It is important to note, however, that if two or more proteins have identical molecular size they will not be resolved regardless of charge differences between them. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will be resolved into different bands.

### **2.8.2 Nondenaturing polyacrylamide gel electrophoresis**

In nondenaturing polyacrylamide gel electrophoresis, the protein subunits are not dissociated and the biological activity of the protein is maintained. This type of electrophoretic separation is used for assessing the purity of a protein complex or a multimeric protein which will be identified as a single protein band.

In this case, however, a true estimate of the molecular size ( $M_r$ ) of the native protein cannot be made, because the separation is based on molecular size, shape and charge. Such gels are only used to confirm the purity of the nondenatured form of a protein.

### **2.8.3 Common problems encountered in polyacrylamide gel electrophoresis**

If an electrophoretic separated protein will be electroeluted or electroblotted, then the highest purity reagents available should be used. After a separating gel is poured, it may be stored with an overlay of the same buffer used in preparing the gel. The stacking gel should be poured immediately prior to use;

otherwise, there will be a gradual diffusion-drive mixing of buffers between the two gels which will cause a loss of resolution. It is recommended that the unknown protein of interest should be present in 0.2 to 10 $\mu$ g in a complex mixture of protein if the gel is to be stained by Coomassie Brilliant Blue, however, up to 100 $\mu$ g of a complex protein mixture can be loaded onto a slab gel. Below is a list of some of the most important problems likely to be encountered in the electrophoretic analysis of proteins by SDS-PAGE.

In situations when the protein bands curve upwards at both sides of the gel (called "smiling"), it is recommended to increase heat transfer from the gel by adding more buffer to the lower buffer chamber up to the level of the sample wells or by cooling the lower buffer below ambient temperature, and by stirring it. Another problem involves the spread of protein bands laterally from gel lanes. This spreading which is due to diffusion of sample out of the wells may be corrected by reducing the time interval between sample application and running of the gel. Alternatively, the acrylamide percentage in the stacking gel should be increased from 4% to 4.5% or 5% or the operating current in the stacking gel should be increased by 25%. On the other hand, the protein bands may be uneven because the stacking gel is not adequately polymerized or the bands may be distorted because of the presence of salt in the protein sample. This problem may be corrected by degassing the stacking gel solution thoroughly or by increasing the ammonium sulphate and TEMED concentrations by 25%. Also, excess salt in the sample may be removed by dialysis, gel filtration or precipitation. Another

cause of skewed protein bands is an uneven interface between the stacking and separating gels which can be prevented by not disturbing the separating gel surface when overlaying it with buffer.

Vertical streaking of protein bands may be corrected by decreasing the amount of sample loaded onto the gel, or by further purifying the protein so as to reduce the amount of contaminating protein applied or by reducing the operating current by 25%. Where streaking is caused by precipitation in the sample, it may be corrected by centrifugation of the sample or by reduction of the percentage of acrylamide. When the protein bands are diffuse, it is advisable to increase the operating current by 25 to 50% or use a higher percentage of acrylamide.

Double protein bands are observed when the protein is partially oxidized. Such oxidation can be reduced by increasing the concentration of 2-mercaptoethanol in the sample buffer or by preparing fresh protein samples.

In situations where the tracking dye band is diffuse, it is advisable to prepare new buffers and acrylamide monomer stocks. If the run takes too long the buffers may be too concentrated or the operating current too low, while if the run is too short, the buffers may be too dilute or the operating current too high.

## **2.9 Interactions between schistosome antigens and host immune system**

### **2.9.1 Consequences of schistosome infection- General overview**

After maturing in a permissive host, schistosome worm pairs take up residence in characteristic venous habitats depending on the parasite species, and

engage in permanent copula and egg-laying for many years. Their life span in humans has been estimated to average 3.5-12 years with some worms surviving for 30 years or longer (Smyth and Halton, 1983). Consequently, schistosomiasis is a disease of long chronicity which normally begins with cercariae penetration upon contact with contaminated water during childhood and progresses into adult life. Nevertheless, it has been observed that schistosomiasis prevalence peaks around age 15-17 and then decreases with increasing age (Rollinson and Southgate, 1987).

The schistosome parasite (adult worms) have an ability to evade the host's immune system, which probably, explain the chronic nature of the disease (Colley and Colley, 1989). The mechanisms involved in this immune evasion include rapid turnover of membrane components, enzymatic cleavage of attached antibodies, tegumental structural developments, the coating (or masquerade) of the parasite surface with host antigenic components [such as ABO blood group antigens, serum components and major histocompatibility (MHC) antigens], antigenic mimicry and direct immunoregulatory mechanisms, including anti-idiotypic networks.

Even though a newly infected host is exposed to a wide variety of parasite antigens, only a few infected persons ever develop the acute febrile illness which begins one or two months after first cercarial exposure. This acute disease is referred to as "toxaemic schistosomiasis" in Brazil or as "katayama fever" in Japan (Lichtenberg, 1987). Most infected children have only minor early symptoms or none at all, and, they may continue in apparent good health during the subsequent chronic phase of the disease even though progressive pathological changes occur internally. Eventually, in five or more years, individuals with heavy parasite burdens begin to suffer advanced fibrovascular lesions to target organs such as the

urinary bladder and genito-urinary tract in the case of *S. haematobium* infections, or the liver and gastrointestinal tract in the case of *S. mansoni* or *S. japonicum* infections. The prevalence of severe symptomatic schistosomiasis varies from one endemic setting to another. In heavily infected populations, it may reach 5% or more, with the bulk of infected persons continuing indefinitely in the subclinical state (von Lichtenberg, 1987). It has been well established by many investigators including von Lichtenberg (1962), Warren (1987), Warren, Domingo and Cowan (1967), and Erasmus (1987) that the key parasite factor in the pathogenesis of schistosomiasis is the eggs, many of which fail to reach the lumen of the gut or urinary tract but lodge in the host tissue and for a short period of time induce an intense localised inflammatory response. This response has been found to be an immunological reaction of the delayed hypersensitivity type (Boros and Warren, 1970, Davis, Mahmoud and Warren, 1974; Phillips, Di Conza, Gold and Reid, 1977; Cheever, Duvall and Hallack, 1984; Olds and Stavitsky, 1986). Related to the inflammation is the induction of collagen synthesis and the appearance of fibrosis and scarring (Dunn and Kamel, 1981; Wyler, Stadecker, Dinarello and O'Dea, 1984 and Olds, Griffin and Kresina, 1985). Consequently, fibrosis is important in both hepatosplenic and obstructive urinary tract diseases.

### **2.9.2 Immunity to schistosomes**

Human schistosomiasis is usually a well-tolerated infection (Colley, 1987). Results of numerous epidemiological studies indicate that individuals living in

endemic areas are repeatedly exposed to low levels of infection over long periods of time (Sturrock, Kimani, Cottrell, Butterworth, Sietz, Siongok and Houba, 1983; Wilkins, Goll, Marshall and Moore, 1984a,b; Butterworth, Dalton, Dunne, Mugambi, Ouma, Richardson, Arap-Siongok and Sturrock, 1984; and Butterworth, Capron, Cordingley, Dalton, Dunne, Karinki, Kimani, Koech, Mugambi, Ouma, Prentice, Richardson, Arap-Siongok, Sturrock and Taylor., 1985). Colley (1981) pointed out that the exposure of such individuals (who may eventually become infected) to schistosome antigens may begin as early as *in utero* and may involve maternal immune reactants against parasite antigens. Also, Vermund, Bradley and Ruiz-Tiben (1983) observed that schistosome infected individuals are parasitized for long periods by the relatively large and prolific parasites. In such chronic infections, the adult worms may survive for as long as 3.5-12 years during which they produce large numbers of eggs and, excretory and secretory antigens (Smyth and Halton, 1983) and the passage of the eggs across tissues is known to be dependent upon highly immunogenic enzymes released from the miracidia (von Lichtenberg, 1987). This pattern of chronic exposure to schistosomes and their antigens leads to a wide variety of complex immunoregulatory interactions between the parasite and host (Colley, 1981 and 1987). Studies with patients and experimentally infected laboratory animals (reviewed by Anderson, 1987) have, however, revealed that mammals are able to mount specific immunological responses against schistosomes even though the expressed immune capabilities of



each individual are affected by epidemiological, parasitological, perinatal and immunogenetic influences (Anderson, 1987).

Early schistosome infection begins with cercaria penetration and von Lichtenberg (1987), reported that this produces very little immune reaction, but in repeated infections local hypersensitivity responses may occur. However, development of acute or early infections is reported to be associated with a sudden exposure of the host to an upsurge in parasite antigen production mainly as a result of excretory and secretory materials released from the tegument and intestines of the adult worms and enzymes from the eggs (von Lichtenberg, 1987). The release of these potent immunogens produces a strong cell-mediated responses and often have circulatory immune complexes (Ottesen, Hiatt, Cheever, Sotomayor and Neva, 1978; Hiatt, Sotomayor, Sanchez, Zombrana and Knight, 1979; Lunde, Ottesen and Cheever, 1979; Nash, Garcia-Coyco, Ruis-Tiben, Nazaro-Lopez, Vazquez and Torres-Borges, 1983; Gazzinelli, Lambertucci, Katz, Rocha, Lima and Coley, 1985). However, despite the abundant evidence for immunological responsiveness to infection there is, in man, very little clear-cut evidence for acquisition of protective immunity and infections persist for long periods.

Colley, Garcia, Lambertucci, Parra, Katz, Rocha and Gazzinelli (1986) demonstrated that despite the strong responses to schistosome antigens seen during acute or early infections, the establishment of chronic infections led to the development of various regulatory mechanisms which negatively modulate the degree of immune responsiveness expressed to schistosome antigens. For example,

the levels of some anti-schistosome antibodies decline and stabilize as infections progress (Gazzinelli *et al.*, 1985) or may appear to plateau during chronic infections (Nash *et al.*, 1983). However, as was found by Wyler (1983) some patients never effectively express this modulation, leaving them to respond vigorously throughout infection.

Several investigators working on the pattern of reinfection in human communities with patients of varying ages following treatment, have provided valuable information on immunity to schistosomes. For example, Sturrock, Kimani, Cottrell, Butterworth, Sietz, Siogok and Houba (1983) and Butterworth *et al.* (1985) identified groups of Kenyan children who resisted reinfection with *S. mansoni* despite considerable contact with infected waterbodies. Also Wilkins, Blumenthal, Hagan, Hayes and Tulloch (1987), showed that reinfection among Gambian children infected with *S. haematobium* tended to be significantly greater in those under 10 years of age than in 10-14 year-olds, despite similar levels of water contact. Crombie and Anderson, (1985) using animal models, provided supportive evidence for this phenomenon when they demonstrated that parasite establishment declined as the duration of exposure to infection increased, in a manner related to the accumulated sum of past experience of infection. This trend for reinfection to decrease with increasing age, after allowance for variation in exposure, was interpreted as evidence for the slow acquisition of a degree of immunity with age, in a manner dependent on past experience of infection (Wilkins *et al.*, 1987).

In studies using animal models and attenuated cercariae Smithers and Terry (1967), and Dean (1983), showed that resistance to reinfection correlated with the presence of eggs in the tissues or with factors related to egg-associated pathology (Dean, Bukwoski and Cheever, 1981). Peresan and Cioli (1980), however, transferred adult worms intrahepatically into naive rhesus monkeys, baboons and mice and showed that immunity was mostly induced by the adult worm stage without a necessary prior exposure to cercariae or schistosomula as collaborated by Smithers and Terry (1969) and Webbe, James, Nelson, Smithers and Terry (1976). This immunity has been referred to as "concomitant immunity" because in this situation, schistosomula of challenge infection were destroyed whilst the egg-laying adult worms of a primary infection remained unharmed (Smithers *et al*, 1987). Sprent (1959), Damian (1964) and Smithers and Terry (1967), attributed this phenomenon to the acquisition by the adult worms of a coating of host-derived macromolecules that masked the adult worm's antigens.

In contrast to "concomitant immunity", Bickle, Taylor, James, Nelson, Hussein Andrews, Dobinson and de C. Marshall (1979) demonstrated a high level of immunity following exposure of mice, rats and several primate species to irradiated cercariae or schistosomula, which they called "vaccine immunity". This immunity was found to be species-specific and in several situations could be consistently shown to be passively transferred with serum (Mangold and Dean, 1986). It is clear that this type of immunity is not associated with the adult stage of the parasite nor with egg induced pathology, instead, similar to concomitant

immunity, it is the migrating larvae or immature juveniles which constitute the target (McLaren, Pearce and Smithers, 1985).

McLaren *et al.* (1985) reported that the crucial difference between concomitant and vaccine immunity appears to be their relative specificities. Concomitant immunity crosses the species barrier (Smithers and Doenoff, 1982), whereas vaccine immunity is species-specific (Moloney and Webbe, 1987).

## **2.10 Methods for Diagnosis of schistosomiasis**

Several authors (WHO, 1983a,b,c) have communicated the need for more simple, rapid, sensitive and reproducible diagnostic tests for schistosomiasis not only for epidemiological studies and evaluation of drug efficacy, but also for the management of infected individuals (Hoffman, Lehman, Stott, Warren and Webbe, 1979; Peters and Kazura, 1987; Kremsner, Enyong, Krijger, De Jonge, Zotter, Thalhammer, Muhlschlegel, Bienzle, Feldmeier and Deelder, 1994). The currently available schistosomiasis diagnostic tests may be divided into four categories. Firstly, there are the clinical signs such as frequent and painful micturition, haematuria and proteinuria in urinary schistosomiasis, and diarrhoea with blood and mucus, and hepatosplenomegally in the case of intestinal schistosomiasis. Secondly, there are the parasitological methods which are based on microscopic demonstration of parasite eggs in host excreta or tissues. Thirdly, there are serological assays based on the detection of circulatory antigens or anti-parasite

antibodies, and lastly, there are imaging techniques for demonstration of pathological changes resulting from the disease (Peters and Kazura, 1987).

### **2.10.1 The Parasitological Diagnostic methods**

#### **2.10.1.1 *Demonstration of *S. haematobium* eggs in urine***

Urine may be examined directly for *S. haematobium* eggs by microscopy. The method is relatively simple and rapid but it is not sensitive enough. One way to increase the sensitivity of this method is by examination of the sediment from large volumes of urine (Peters and Kazura, 1987). Such urine sediment may be obtained by centrifugation or by sedimentation. In field situations where fresh urine samples cannot be processed immediately within few hours, the excreted eggs may be preserved by adding an equal volume of 0.002% carbolfuchsin which also contained 10% (v/v) absolute ethanol, and 5% (v/v) phenol. When ready for examination, the treated urine may be subjected to centrifugation to obtain a sediment for direct inspection or it may be passed through Whatman filter paper (No.1 grade) attached to a vacuum apparatus. Air-dried filters are placed egg side up under a microscope and examined.

Peters (1976) introduced an improved urine filtration method. This new technique involved passing urine through a suitable membrane filter that traps schistosome eggs. The eggs were then observed microscopically for identification of schistosome species and counted for estimation of the intensity of infection.

Polycarbonate and polyamide membrane filters have been shown to be very suitable for this purpose (Peters, 1976 and Mott, 1982). The procedure involves collection of urine between 1100 and 1400 hours, when *S. haematobium* egg excretion is known to be highest. A fixed volume of urine usually 10ml is drawn into a syringe and passed through a membrane filter secured in a swinnex support chamber. The filter is removed from the chamber with forceps and the side with trapped eggs laid face down on a microscope slide. When ready for microscopy, a drop of saline is placed on the filter to improve the refractive index (Peters, Warren, and Mahmoud, 1976). The sensitivity of this method is reported to be fairly good, especially when 10ml of urine is used (Peters *et al.*, 1976), however, the time consuming nature of the procedure limits its field applicability.

#### **2.10.1.2 Faecal examination for schistosome eggs**

Most of the eggs laid by adult worms of *S. mansoni*, *S. japonicum* and *S. intercalatum* are either excreted in the faeces or trapped in the tissues of the gut wall. Diagnosis of these infections are, therefore, mostly based on direct examination of faeces or biopsy specimen of the rectal mucosa for parasite eggs (Peters and Kazura, 1987).

The simplest of the faecal examination methods is the direct faecal smear. By this technique, approximately 2mg of stool is emulsified following the addition of one or two drops of 0.9% NaCl solution and transferred onto a glass slide and spread with a coverslip before the smear is scanned for parasite eggs by microscopic

examination. This technique is not sensitive enough mostly because of the small amount of specimen examined. Also, the presence of large amounts of fibre may interfere with visualization of the parasites eggs. For these reasons, Peters and Kazura (1987) concluded that although the method is simple, it is mainly useful for identification of heavily infected persons.

Garcia and Shimuzi (1981) reported that an improved method of stool examination (the formal-ether method) which leads to the preservation of specimen is also simple to perform. By this technique, approximately half a teaspoon of fresh stool is placed in 10ml of 10% formalin and allowed to "fix" for at least 30 minutes.

The preserved specimen is then passed through two layers of gauze into a centrifuge tube. The filtered stool is sedimented and washed twice by centrifugation at 290 *Xg* in 0.9% NaCl and the final pellet resuspended in 7ml of 10% formalin. The mixture is then added with 3ml of ether and shaken for 30 seconds in a capped tube before centrifugation again at 290 *Xg* for 2-3 min. This separates the mixture into four layers consisting of a small sediment containing schistosome eggs at the bottom with a layer of formalin above it followed by faecal debris and ether on top. The sediment containing parasite eggs is recovered and examined by microscopy. Even though the method is very sensitive, it is semi-quantitative and, therefore, not suitable for accurate determination of intensity of infection (Peters and Kazura, 1987).

Bell (1963) introduced the "Bell method" for detection of schistosome eggs in faeces which was the first to be extensively used in field studies (Teesdale and

Amin, 1976; Jordan, Bartholomew and Petras, 1981). In this method, a sufficient volume of stool is added to 90ml of a formalin-glycerol solution to bring the final volume to 100ml. The preparation is then mixed at medium speed in a blender for 15-20 seconds to give a creamy suspension. An aliquot of 1ml (representing 0.1ml of stool) is placed on a 7 cm diameter piece of filter paper that is fixed on a suction apparatus and subjected to negative pressure to spread the specimen. The paper is then sprayed with ninhydrin, which stains the egg blue. Before counting the eggs by microscopic examination, the paper is wetted with distilled water to improve the refractive index.

Another technique (the Kato method) is generally preferred for detecting the eggs of intestinal parasites in stool specimen. In the standard Kato method (Kato and Miura, 1954; Kato, 1960; Komiya and Kobayashi, 1966; Martin and Beaver, 1968; Katz, Chaves and Pellgrino, 1972 and Warren, Mahmoud, Cummings, Murphy and Houser, 1974), a sample of stool is taken with a wooden spatula and forced through a stainless steel mesh to remove particulate and fibrous material. The specimen is then applied to fill a hole in a template that is set on a glass microscope slide. The template is carefully lifted off the slide to leave an intact plug of faeces. The stool specimen is then covered by a 25 x 35mm cellophane coverslip previously impregnated with 50% (v/v) glycerol in water containing 3% malachite green. The slide is subsequently turned face down on a flat surface, and pressed gently but firmly to spread the stool specimen evenly. The prepared slide is left for about 30min in the light to clear before it is examined microscopically for parasite



eggs. This technique is rapid and suitable for large epidemiological surveys in which sensitivity needed to detect light infections is not crucial.

### **2.10.1.3**      *Demonstration of schistosome eggs in host tissue*

The absence of schistosome eggs in faecal or urine specimen do not necessarily exclude the possibility of infection (Peters and Kazura, 1987). This is because eggs may not be found in the faeces or urine but may be present in various host tissues such as the rectal mucosa, liver, lungs, urethra and occasionally even in the central nervous system. Hence, the diagnosis of schistosomiasis by examination of biopsy specimen has proven very useful. By this approach, urethra biopsy may be taken where urinary schistosomiasis is suspected, while rectal biopsy is suitable for diagnosing intestinal schistosomiasis. The procedure involves the excision of 1-2mm snips of tissues which is subsequently sandwiched between two glass slides and examined for parasite eggs by microscopy. This technique is, however, more time consuming, expensive and requires the involvement of skilled medical personnel.

### **2.10.2** Immunodiagnosis of schistosomiasis

Parasitological diagnosis of schistosomiasis by microscopic examination of urine or stool for parasite eggs has been the most widely used method of identifying infected individuals (WHO, 1992). However, the validity of the parasitological results obtained in any epidemiological study, depends on many factors, including

the ability of a diagnostic laboratory to perform accurately the daily tasks of collecting samples, preparing and reading slides and recording the results, whilst avoiding the possibility of mislabelling specimens or contaminating apparatus (Jordan and Goddard, 1982). Moreover, the technique is labour-intensive, and relatively insensitive as it is influenced by daily fluctuations in the rate of egg excretion which tend to render especially negative results unreliable, particularly in areas characterized by low intensities of infection. Furthermore, schistosomes are less fecund than most helminths, and majority of their eggs are retained in tissues (Kloetzel, 1963). As a result, accurate determination of schistosomiasis requires more than one stool or urine examination (Ruiz-Tiben, Hillyer, Knight, Gomez de Rois and Woodall, 1979). It is for these reasons that the call for simpler, more rapid, specific and sensitive field applicable techniques for the diagnosis of schistosomiasis remains.

Virtually all well-established immunological assays such as complement fixation (CF), indirect haemagglutination assay (IHA), thin layer immunoassay, gel precipitation, indirect immunofluorescent assay (IFA) and enzyme-linked immunosorbent assay (ELISA) have been applied to identification of schistosome species and diagnosis of schistosomiasis (Kagan, 1968). However, very few of these tests have been advocated for large-scale use in diagnosis mostly because of limited field applicability and in some cases inadequate sensitivity. For example, complement fixation tests employing extracts of lyophilized worms were comparatively as specific as immunofluorescence utilising adult worm sections, yet

they could detect antibody in only 70% of infected cases investigated (Reviewed by Smithers and Doenhoff, 1982). One other important test developed for diagnosis of schistosomiasis is the Circumoval Precipitin Test (COPT) introduced by Oliver-Gonzales (1954). This test which initially utilized fresh *S. mansoni* eggs was, later simplified through the use of lyophilised eggs by Tanaka, Matsuda, Blas and Nonsenas (1975). The technique was made even more field applicable following the introduction of the use of air-dried schistosome eggs (Kamiya, 1983).

Smithers and Doenhoff (1982) reported that both the ELISA and radioimmunoassay have improved the sensitivity of serodiagnostic procedures for schistosomiasis. However, ELISA has several advantages over radioimmunoassay. These advantages include the stability of antigen antibody complexes and the use of enzymes in place of radioactive labels. In different studies with ELISA, McLaren, Draper, Roberts, Minter-Goedbloed, Lighthart, Teesdale, Amin, Omer-Ali, Bartlet and Voller (1987) found that schistosome egg antigens were more reactive than worm antigens and was better for detecting antibodies in acute or early infections. On the other hand, using the MSA<sub>1</sub> antigen obtained from *S. mansoni* eggs Hamburger, Pelley and Warren, (1976), Hillyer and Pelley (1980), showed that the radioimmunoassay was stage-specific and more sensitive in detecting parasite-specific antibodies in chronic infections. Nevertheless, McLaren, Long, Goodgame and Lilleywhite (1979) using antibody detection ELISA, reported that 82-100% of intestinal schistosomiasis patients in St. Lucia could be diagnosed even under circumstances where up to three different stool examinations were required to reveal

some infections. It has, however, been pointed out that although antibody detection is the most sensitive serodiagnostic approach (Kelley, 1987), its field applicability is limited by the inability to differentiate active infections from previously cured ones (Nantulya, Musoke, Rurangirwa and Molloo, 1984). The technique is also limited by extensive cross-reactivity between *S. mansoni*, *S. japonicum* and *S. haematobium* especially when crude parasite antigens were used (McLaren *et al.*, 1987). To overcome this lack of specificity, efforts have been made to use more purified parasite antigens (McLaren, Lilleywhite, Dunne and Doenhoff, 1981), but no *Schistosoma* species-specific assay based on antibody detection have so far been developed. It is widely accepted that problems associated with antibody detection assays could be overcome by detecting parasite antigens instead (Gold, Rosen and Weller, 1969; Bawden and Weller, 1974).

Nash (1974) and Deelder *et al.* (1980) studied and reported on circulatory schistosome antigens which could be exploited in diagnosis of schistosomiasis. Two important circulatory antigens, both of them proteoglycans associated with the gut of adult schistosomes have been identified, and named, circulatory anodic antigen (CAA) and circulatory cathodic antigen (CCA) on the basis of their electrophoretic mobilities (Deelder *et al.*, 1976). Although both antigens are not strong immunogens, Feldmeier, Nogueira-Queiros, Doehring, Dessaint, de Alencar, Daffalla and Capron, (1986) using polyclonal antisera in ELISA were able to detect CCA at very low concentrations in a two-site radioimmunoassay. More recently, several investigators including De Jonge, Fillie, Hilberath, Krijger, Lengeler, De

savigny, van Vliet and Deelder (1989) and Kremsner *et al.* (1994) used MoAbs produced against the CAA and CCA and showed that they could be used to detect the antigens in both urine and sera of patients. The advantages of antigen detection in the management of schistosomiasis have since been clearly communicated by Kremsner *et al.* (1994) who reported that quantitative measurement of schistosomal antigens in human specimen can be used to : (1) diagnose infection; (2) measure the severity of pathological manifestations; (3) monitor impact of chemotherapy and (4) investigate ongoing transmission in endemic areas.

Even though neither the CAA nor CCA are specific to any of the schistosome species, it is generally accepted that *Schistosoma* genus-specific assays are desirable because of the common drug (praziquantel) treatment. However, it may be argued that schistosome species-specific assays are necessary, for example, to differentiate between urinary and intestinal schistosomiasis in hyperendemic areas of Africa (WHO, 1985). Recent advances in the production of *Schistosoma* species-specific MoAbs with diagnostic potential (Amanor *et al.*, 1996) further demonstrates the applicability of MoAbs in the diagnosis of schistosomiasis.

## **2.11 Monoclonal antibodies (MoAbs)**

Monoclonal antibodies are homogeneous immunoglobulins with specific reactivity to a single antigenic epitope against which it was derived (Billings, 1985).

The underlying principle of their production involves the immortalization of a single B-lymphocyte by fusing it with a non-secreting tumour lymphocyte *in vitro*

(Kohler and Milstein, 1975, 1976). The extraordinary specificity of MoAbs makes them useful in various immunological applications such as in diagnosis. As a result, MoAbs are now being used in immunoparasitology; (1) as probes for the detection and localization of antigen, and analysis of its organization and availability; (2) for studies of antigenic heterogeneity (variability) in parasite populations; (3) for detection of cloned DNA in various vectors; and (4) for parasite typing and parasite detection (Mitchell, 1984; Sikora and Smedley, 1984; and Goding, 1986). This considerable potential of MoAbs has already been widely exploited in the study of the most common parasitic diseases such as malaria (Yoshida, Nussenweig, Potocnjak, Nussenzweig and Aikawa, 1980; Rener, Carter, Rosenberg and Miller, 1980), leishmaniasis (McMahon-Pratt, Bennet and David, 1982) and trypanosomiasis (Nantulya, Musoke, Rurangirwa, Saigar and Minja, 1987; Bosompem, Assoku and Nantulya, 1996*b*). In Schistosomiasis, MoAbs have become useful tools in the develop of standardizable immunodiagnostic reagent of high specificity and for identification and characterization of immunoregulatory, and protective antigens (Taylor and Butterworth, 1982; Smith *et al.*, 1982; and Amanor *et al.* 1996).

## **2.12 Purification of monoclonal antibodies**

Large quantities of MoAbs can either be obtained as cell free supernatant from culture of MoAb secreting hybridoma cells *in vitro*, or in ascitic fluid as a result of the passage of the cell *in vivo* (Clark and Waldmann, 1986). The most

commonly used media for growth of MoAb-producing hybridoma cells *in vitro* are, however, usually supplemented with Fetal Bovine serum (FBS) which may form about 10% of the complete medium. This serum supplement contains large amounts of albumins, bovine immunoglobulins and soluble cellular debris which may include proteases and nucleases. These usually serve as contaminants in the supernatant containing the antibodies and may interfere in some of the assays that utilize MoAbs. In ascitic fluid, likely contaminants include irrelevant host immunoglobulins and larger amounts of proteases and nucleases (Campbell, 1984).

It is, therefore, necessary to purify MoAbs from culture supernatant and ascites, especially when the MoAbs are required as standard reagents in immunoassays or for characterization of the MoAbs themselves (Goding, 1986). Campbell (1984) observed that one instance in which purification of an antibody may not be necessary is where all that is required is specificity (Campbell, 1984). This is because each batch of antibody can be tested for titre and used directly.

One of the oldest and most useful methods of purification of immunoglobulins is based on the observation that they are precipitated by lower concentrations of ammonium sulphate than most other serum proteins (Goding, 1986). Thus, for example, at 40% saturation most immunoglobulins are freely precipitated and separated from the main contaminating protein which is albumin (Campbell, 1984). Even though precipitation of immunoglobulins by ammonium sulphate does not result in a homogeneous antibody preparation the procedure is known to be gentle, effective and simple, and it provides a substantial enrichment,

thereby reducing the protein load on subsequent purification steps (Campbell, 1984).

Knowledge of antibody class and subclass has been reported to be very important in determining the methods for further purification of MoAbs (Goding, 1986). Perhaps, the simplest means of determining antibody class is by the double diffusion method described by Ouchterlony (1967). Alternatively, the antibody class might be determined by radioimmunoassay, enzyme-linked immunosorbent assay or indirect immunofluorescence utilizing class-specific antibodies. Once determined IgG MoAbs are best purified further by ion exchange chromatography. This technique is one of the most useful since by simple gradient elution the correct compromise between yield and purity can be made without guesswork (Goding, 1986). Apart from its simplicity the high capacity of ion-exchange gels permit fractionation of large quantities of MoAbs at a go. For example, a 10 ml column of DEAE-Sephacel will easily handle 100-200 mg protein, and the recovery could be as good as 100%.

On the other hand, IgM MoAbs are best purified further by Gel filtration (Goding, 1986). By this method the proteins are separated according to their molecular weights (Fischer, 1969). Even though this procedure is also simple to perform and allows good recoveries, it usually results in greater dilution. Gel filtration may also be used during purification of IgGs, as an adjunct to other methods in situations where a higher degree of purification is needed.



### **2.13 Usefulness of MoAbs in immunological studies**

Conventional animal antisera contain the products of many different antibody-secreting clones with specificity for several antigenic determinants. Consequently extensive cross-reaction is known to occur between such polyclonal antibodies and antigens from different sources (Campbell, 1984). Polyclonal sera are, therefore, found to be unsuitable in applications in which specificity is an important requirement (Lopes and Alves, 1984). Fortunately, the introduction of MoAbs with extraordinary specificity of epitope detection has circumvented this problem and greatly improved the performance of immunological assays (Sikora and Smedley, 1984). Furthermore, MoAbs have been found to be useful in characterization and isolation of parasite antigens, leading to identification of diagnostic, immunoregulatory and protective antigens (Sikora and Smedley, 1984; Kremsner *et al.*, 1994; Bergquist, 1995; Amanor *et al.*, 1996).

### **2.14 Vaccination against schistosomiasis**

There is still no vaccine available for schistosomiasis because development in this area has proved very difficult (reviewed by Bergquist, 1995). The search for protective antigens against the disease has been lengthy partly because *S. mansoni* is the only schistosome species that can adapt with ease to the laboratory (Bergquist, 1995). For this reason, most schistosome antigens were initially identified in *S. mansoni*, even though some specific and polymorphic antigens in other species have been described. For example, variants of glutathione S-transferase (Sm28GST)

from all three major schistosome species affecting humans, as well as, *Schistosoma bovis*, have been identified and cloned. Recent advances in immunology and molecular biology have further increased the chances of identifying putative antigens which may provide effective immune prophylaxis against schistosomiasis (Simpson, Chandler, Kelly, Walder, Knight and Smithers, 1987; Bergquist, 1995). These new developments have already resulted with a long list of cloned *S. japonicum* antigens considerably improving the prospects for developing a vaccine against this parasite in parallel with one against *S. mansoni* (Brindley, Ramirez, Tiu, Wu, Wu and Yi, 1995). More recently, Bosompem *et al.* (1996a) have generated monoclonal antibodies to *S. haematobium* antigens and characterized them (Amanor *et al.*, 1996). Even though some of the monoclonal antibodies appeared to bind *S. haematobium* species-specific antigens work on identification of protective *S. haematobium* antigens continues to lag behind.

The search for protective schistosome antigens has been focused on the less protected larval (schistosomula) stage, partly because of the elaborate defence mechanisms employed by adult schistosome worms (Dissous *et al.*, 1987; Kelly, 1987). Several investigators (Bickle *et al.*, 1979; Bergquist, 1995) have shown that injection of live schistosomula attenuated by sub-lethal doses of radiation into experimental animals results in high levels of resistance in a subsequent challenge. This observation was attributable to the presence of particular antigens on the surface membranes of schistosomula. As a result, both carbohydrate and polypeptide epitopes expressed on the surfaces of newly transformed schistosomula

have been tried and found to induce protective immunity (Kelly, 1987). However, the carbohydrate epitopes are reported to be unsuitable as candidate antigens for vaccine production because of their similarity with schistosome egg glycoprotein antigens in terms of their ability to induce pathological responses (Warren, 1987). In spite of this, Dissous *et al.* (1982) identified a carbohydrate epitope on a major 38-kDa schistosomulum surface antigen, as defined by a protective rat monoclonal antibody was found to be capable of inducing a high level of protection against *S. mansoni* infections.

Polypeptide epitopes on the other hand appear to be more suitable for use in vaccine production. This is because they do not cross-react with the pathology inducing egg antigens (Hackett, Simpson, Knight, Ali, Payeres and Smithers, 1986). For this reason, a range of schistosomulum surface peptides have now been cloned and expressed in *E. coli* (reviewed by Simpson and Cioli, 1987). These include several major polypeptide antigens of  $M_r$  97, 32, 28, 25 and 20 kDa which have also been reported to be expressed by adult worms and are presented to host immune system during natural infections (Simpson, Hackett, Kelly, Knight, Payeres, Ali, Lilleywhite, Fleck and Smithers, 1986). According to (Simpson *et al.*, 1987) more schistosomula surface polypeptide antigens are becoming available from several laboratories.

Lanar, Pearce, James and Sher (1986) identified and cloned a 97 kDa antigen from *S. mansoni* adult worm parasites. This internally localized antigen known as paramyosin was found to resemble the  $\alpha$ -helical protein which forms the

core for myosin filaments in invertebrate muscle. This molecule is important not only because of its immunological potential as a vaccine immunogen, but also because of its probable physiological function for the parasite. As a core structure for myosin filaments, paramyosin is suspected to be an important molecular component in a "catch" mechanism aiding adult schistosomes in continuously maintaining themselves against the venule wall and thus avoiding dislodgment by the blood flow (Lanar *et al.*, 1986). Several other antigens have been discovered and are undergoing confirmatory testing and subsequent vaccine trials. These include SRP and gp68 from *S. mansoni* schistosomula and adult worms, respectively, and Sj26 from *S. japonicum*. JM8-36, an anti-idiotypic antibody and Fh(SmIII) from *Fasciola hepatica* adult worms have also been found to be useful in protection against schistosomiasis (Colley and Colley, 1989).

At present, the strongest candidate antigens for the production of vaccines for schistosomiasis are Glutathione-S-transferase a 28 kDa molecule (Sm28GST) that is found in parenchymal cells but expressed transiently on schistosomulum and adult worm surfaces, Paramyosin (Sm97) and an irradiation-associated vaccine antigen (IrV-5) (Bergquist, 1995). Sm28GST, Sm97 and IrV-5 have been found to induce 30-60%, 30% and 50-70% protection in mice respectively (Pearce, 1988; Bergquist, 1995).

## **CHAPTER 3**

### **GENERAL MATERIALS AND METHODS**

### 3.1 Resuscitation and cloning of hybridoma cells

A freezing vial containing  $2 \times 10^6$  hybridoma cells in 1ml of stabilizing medium was retrieved from liquid nitrogen storage at  $-196^\circ\text{C}$  and thawed rapidly in a water bath at  $37^\circ\text{C}$ . The thawed cell suspension was quickly transferred into sterile centrifuge tubes containing 10ml of growth medium. This medium was composed of Dulbecco's Modified Eagle's Medium (DMEM), modified by addition of 4mM L-glutamine, 1ml of 5 mM HEPES (N-2-Hydroxyethylpiperazine-N-2-Ethanesulfonic acid) in 200ml of medium, and supplemented with 10% (v/v) heat-inactivated ( $56^\circ\text{C}/30\text{min}$ ) Foetal Bovine Serum (FBS). The contents of the tubes were gently mixed to dilute out the DMSO contained in the stabilizing medium [growth medium containing 7.5% (v/v) dimethyl sulphoxide (DMSO) (E. Merk, Darmstadt Germany)]. The tubes with their contents were then centrifuged at  $400\times g$  for 5 min. The supernatant was discarded and the cell pellet resuspended in fresh medium in a  $25\text{cm}^2$  tissue culture flasks (Costar, Cambridge, MA, USA, or Falcon, Oxnard, California) and incubated at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator (MCO 175, Sanyo, Japan.) at 5% carbon dioxide concentration.

Hybridoma cells maintained in culture were cloned to ensure monoclonality and continuous secretion of MoAb. Cells in  $25\text{cm}^2$  tissue culture flasks were first counted in a Neubauer Counting Chamber using Trypan blue as vital stain. Cell suspensions were diluted with either growth-medium or phosphate buffered saline, pH 7.4 containing Trypan blue which was used at a final concentration of 0.1 to 0.5%. Live cell counts were made using a compound microscope under  $100\times$

magnification. The cells were cloned by limiting dilution in growth medium to give 1 cell per 50 $\mu$ l of medium in each of the wells of 96 well tissue culture plates. Prepared plates were incubated at 37°C in a CO<sub>2</sub> incubator and left undisturbed for about 10 days. The plates were then examined for viable cell colonies by viewing the bottom of the wells against light or by observing under an inverted microscope (Olympus, Tokyo, Japan). Wells with single viable cell colonies were marked and the cells transferring into 0.5ml of growth medium in 24 well tissue culture plates. The cells were allowed to grow and increase in number and 1ml of fresh growth medium added to each well. The culture fluids were then allowed to become acidic (yellow) before they were tested for antibody activity by micro-plate ELISA. Antibody positive culture fluids were also concentrated by ammonium sulphate precipitation and used for the determination of immunoglobulin class by the double immunodiffusion technique as described by Ouchterlony (1967).

### **3.2 Cryopreservation of hybridoma cells**

Cloned MoAb secreting hybridoma cells in logarithmic phase of growth were centrifuged at 400xg for 5min. The pelleted cells were resuspended in stabilizing medium to give 2 x 10<sup>6</sup> cells/ml and 1ml aliquots pipetted into 2ml cryo-preservation vials before freezing in liquid nitrogen using a Cryo-controller version 2.01 (Department of Biomedical Engineering, University Hospital, Copenhagen, Denmark). The Cryo-controller was programmed to freeze cells over a period of

1hr during which time specimen were subjected to a temperature gradient from room temperature to  $-140^{\circ}\text{C}$ . The frozen vials were removed and immediately transferred onto labelled stabilisation racks and immersed in liquid nitrogen at  $196^{\circ}\text{C}$ .

### **3.3 Propagation of hybridoma cells**

Cloned hybridoma cells secreting MoAbs were grown in 24 well plates with medium changes every three days until about  $1 \times 10^7$  cells could be transferred into  $25\text{cm}^2$  tissue culture flasks. The cells were multiplied and transferred to  $75\text{cm}^2$  tissue culture flasks in 20 - 25ml of growth medium containing between  $1 \times 10^5$  to  $1 \times 10^6$  cells per ml and maintained by subculturing into other flasks when the medium turned acidic. In order to produce more MoAbs, hybridoma cells were propagated in  $125\text{cm}^2$  tissue culture flasks in 40 - 50 ml of growth medium. All the cultures were incubated at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator as described earlier (Section 3.2). MoAb containing culture fluids were harvested by centrifugation at  $400\times g$  for 5 min and stored frozen at  $-20^{\circ}\text{C}$ .

### **3.4 Purification of monoclonal antibodies**

MoAb containing culture supernatant stored at  $-20^{\circ}\text{C}$  were retrieved and thawed at  $37^{\circ}\text{C}$  in a water bath. The thawed culture supernatants were concentrated by filtration using the Amicon concentration chamber (Amicon Corporation, Ireland) fitted with a 30,000 MW cut-off ultra-filtration disk membrane (Sigma,



USA), and the immunoglobulins were precipitated by slow addition of an equal volume of saturated ammonium sulphate  $[(\text{NH}_4)_2\text{SO}_4]$  while mixing. The precipitates formed were then pelleted by centrifugation at 900xg for 30 min and dissolved in minimum amount of distilled water. The antibody solutions were transferred into 1000 MW cut-off dialysis membrane (Spectro Medical Industries Incorporated, USA) and dialysed overnight against phosphate buffered saline (PBS) (9mM  $\text{NaH}_2\text{PO}_4$ , 0.9mM  $\text{Na}_2\text{HPO}_4$ , 15mM NaCl, pH 7.4) with one buffer change. MoAbs concentrated by this process were subjected to further purification as described below.

### **3.5 Gel filtration**

Precipitated and dialysed IgM MoAbs were further purified by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) which was prepared according to the manufacturer's instructions. Ten millilitres of dialysed antibody was applied onto a 2.6 x 100cm column and eluted with PBS pH 7.4, at a flow rate of 40ml/hr. The eluates were collected 10ml/tube and stabilised by addition of NaCl to a final concentration of 1M and assayed for antibody activity by microplate-based enzyme-linked immunosorbent assay (ELISA). Fractions containing antibodies were pooled and concentrated by filtration (Section 3.5).

### **3.6 Ion exchange chromatography**

Culture supernatants containing IgG MoAbs were also concentrated by the ammonium sulphate precipitation method but dialysed against 50 volumes of Tris-buffer (0.05M Tris, 0.01M NaCl, 0.02% NaN<sub>3</sub> pH 8.6) at 4°C for 24 hr with one buffer change. The IgG MoAbs were further purified by ion-exchange on diethyl aminoethyl cellulose (DE-52, Whatman, Kent, England). Samples were applied onto the column and washed with at least one column volume of dialysis buffer (Tris, pH 8.6). Bound MoAbs were then eluted with a linear gradient of 15-300 mM NaCl and the various fractions assayed for antibody. MoAb positive fractions were concentrated and stored as described for IgM antibodies.

## **CHAPTER 4**

### **CHARACTERIZATION OF ANTI-*S. HAEMATOBIIUM* MONOCLONAL ANTIBODIES**

## 4.1 Summary

Nineteen mouse monoclonal antibodies (MoAbs) produced against *S. haematobium* antigens were extensively characterized. IFAT studies showed that four MoAbs (Sh2/15.F, Sh3/12.1, Sh3/44.31 and Sh3/12.31) stained the surface membranes of *S. haematobium* miracidia while three others (Sh4/14.3, Sh3/15.13 and Sh4/16.45) stained only internal (possibly cytoplasmic) antigens. The remaining twelve MoAbs stained both the surface membrane and internal antigens. Proteinase-K digestion and periodate oxidation analysis showed that eleven MoAbs bound glycoprotein antigenic determinants while the remaining eight antibodies detected protein epitopes.

Cross-reactivity studies using crude antigen extracts from *S. haematobium*, *S. mansoni*, *S. japonicum* and *N. americanus* in micro-plate ELISA revealed that Sh2/15.F and Sh3/15.28 were *S. haematobium* species-specific. Both MoAbs detected antigens in soluble egg antigen extracts of Ghanaian (SEA<sub>Gh</sub>) and Egyptian (SEA<sub>Egy</sub>) strains of the parasite. Another MoAb, Sh3/44.3 could only bind antigens in SEA<sub>Egy</sub>. Six other MoAbs were found to cross-react with antigens of all the three major human schistosomes listed above and the remaining ten were found to cross-react with *S. haematobium* and *S. japonicum* antigens only. Micro-plate and dot-ELISA studies showed that seven MoAbs (Sh2/15.F, Sh3/15.13, Sh3/34.10, Sh4/14.3, Sh4/16.45, Sh5/32.30 and Sh5/34.10) detected soluble egg antigens of Ghanaian strains of *S. haematobium* (SEA<sub>Gh</sub>) which were also present in proteins

precipitated out of *S. haematobium* infected human urine (P<sub>2</sub>J). The other twelve MoAbs detected antigens in SEA<sub>Gh</sub> and SEA<sub>Egy</sub> but not P<sub>2</sub>J. Sh2/15.F could only detect antigens in P<sub>2</sub>J but not in SEA<sub>Egy</sub>. Generally only the MoAbs which bound protein epitopes could detect antigens in (P<sub>2</sub>J).

## 4.2 Introduction

Over the last decade there has been an explosion of interest in the development of new and improved immunodiagnostic tests for infectious diseases (De Clercq *et al.*, 1995). This is so because most diseases still require more simple and accurate field applicable diagnostic techniques which are now most likely to be developed because of the applicability of highly specific and sensitive MoAbs. The use of MoAbs also has the advantage of enhancing prospects for identification of protective antigens that may be useful in vaccines against diseases including schistosomiasis. In view of these, the World Health Organisation's Scientific Working Group on schistosomiasis, recommended support and incorporation of research with MoAbs into the overall strategy for schistosomiasis control (Bergquist, 1984).

Anti-schistosome monoclonal antibodies (MoAbs) have been produced and characterized by many workers including, Mitchell, Cruise, Garcia, Vadas and Munoz (1983), Sidner, Carter and Colley (1987), Yamashita, Watanabe, Hosaka, Minai, Saito and Sendo (1989), Bosompem *et al.*, (1996a) and Amanor *et al.*, (1996). The most promising of these MoAbs were the ones that bound circulating or urinary schistosomal antigens (De Jonge *et al.*, 1989; Kremsner *et al.*, 1994; Bosompem *et al.*, 1996a; Amanor *et al.*, 1996). Since they offered an opportunity to develop non-invasive immunodiagnostic assays using urine as test specimen. Deelder, De Jonge, Boerman, Fillie, Hiberath, Rotmans, Gertse and Schut (1989)

developed a highly specific and sensitive enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of a schistosome worm gut proteoglycan called circulating anodic antigen (CAA) in sera and urine of *S. mansoni* and *S. intercalatum* infected persons. Also, De Jonge *et al.*, (1989) used ELISA to detect CAA in the urine of *S. mansoni* and *S. haematobium* infected individuals. The cross-reactive nature of CAA, however, makes it unsuitable for specific diagnosis of any of the schistosomiasis, even though differential diagnosis may be necessary in hyperendemic areas of Africa (WHO, 1985) where *S. mansoni* and *S. haematobium* co-exist.

This study was, therefore, aimed at characterizing extant MoAbs produced against *S. haematobium* egg and urinary antigens (Bosompem *et al.*, 1996a) in order to find out if any of them is *S. haematobium* species-specific, and whether any could detect parasite antigens in the urine of infected individuals. Such MoAbs, could be useful in the development of more sensitive immunodiagnostic assays for urinary schistosomiasis.

### 4.3 Materials and Methods

#### 4.3.1 Parasites

Two strains of *S. haematobium*, one from Ghana (*Globosus* strain, Chu, Kpo and Klumpp, 1978) was obtained locally, and another from Egypt (*Bulinus truncatus truncatus* strain) was obtained from the WHO. *S. mansoni* and *S. japonicum* from the Philippines were obtained from *Biomphalaria glabrata* and *Oncomelania hupensis nosophora* snails, respectively. *Necator americanus* (Hookworm) eggs were obtained from school children from Ayikai-Doblo in Southern Ghana (Chu, Kpo and Klumpp, 1978).

#### 4.3.2 Preparation of Parasite Antigens

Crude antigen extracts were prepared from eggs and worms of *S. haematobium* and hookworm eggs. Frozen pellets of worms or eggs were thawed in extraction buffer consisting of 1mM phenylmethyl-sulfonyl fluoride PMSF, 0.2mM N-a-P-Tosyl-L-lysine chloromethyl Ketone-TLCK, and 0.05mM N-Tosyl-L-phenylalanine chloromethyl Ketone -TPCK (all from Sigma, Chemical Company, USA), dissolved in absolute ethanol, and diluted (1:100) with PBS, pH 7.4. Protease inhibitors (Leupeptin and E-64) were each added to a final concentration of 10µg/ml just before use. The thawed pellet was mixed by vortexing and disrupted on ice in a glass homogenizer (Kudoguki, Keiki, Tokyo, Japan) at 1200rpm for 5 min. Homogenized eggs or worms were centrifuged at 290 Xg for 5 min and the



supernatant collected as primary extracts. The pellet was resuspended in extraction buffer and the extraction process repeated to obtain secondary and then tertiary extracts for each antigen. The extracts were pooled and stored at -20°C until use.

#### **4.3.3 Urine specimen and precipitation of urine proteins**

Urine from 50 persons infected with the *Globosus* strain of *S. haematobium* from Ayikai-Doblo in Ghana (Chu, Kpo and Klumpp, 1978) were pooled for analysis. Diagnosis of schistosomiasis was made using the urine filtration and microscopy method (Peters *et al.*, 1976). The absence of *S. mansoni* was confirmed by microscopic examination of stool specimen.

Proteins were precipitated out of *S. haematobium* infected human urine using saturated ammonium sulphate 50%(v/v) as explained by Bosompem *et al.* (1996a) to obtain various fractions which were tested for the presence of schistosome antigens using dot-ELISA (see below), and for proteins (Lowry, Rosebrough, Farr and Randall, 1951).

#### **4.3.4 Coating of microtitre plates with antigens**

*Schistosoma haematobium* soluble egg antigens (SEA) were diluted in "coating buffer" consisting of 34.5mM NaHCO<sub>3</sub>, and 15.1mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.6, and dispensed into the wells of flat-bottomed 96 well microtitre plates 1µg/50µl/well. Crude *S. mansoni*, *S. japonicum* and *Necator americanus* egg or worm antigen extracts were coated similarly but with different concentrations of antigens ranging between 0.5 and 3µg of protein per well. Precipitated proteins from *S. haematobium* infected human urine P<sub>2</sub>J were also coated at 0.5µg/50µl/well. To

enhance binding the plates were incubated at 37°C until they became completely dry. Monoclonal antibodies were coated at 1 to 5µg/well in coating buffer at 4°C overnight. The optimum coating antigen and antibody concentrations were determined by checkerboard titration as described by Voller, Bidwell and Bartlett (1980).

#### **4.3.5. Antibody-detection micro-plate and dot-ELISA**

These procedures were used for screening hybridoma culture supernatants for antibody activity.

##### **4.3.5.1. *Micro-plate ELISA***

In this assay, polystyrene micro-plates (Sero-wel, Bibby Sterilin, UK) previously coated with *S. haematobium* SEA or P<sub>2</sub>J were emptied and rinsed once before incubation with test samples for 15min at room temperature (24-26°C). One hundred microlitres of hybridoma culture supernatants were tested per well in duplicates without dilution. Normal culture medium and pre-fusion serum diluted at 1:500 were added as negative and positive controls, respectively. The plates were rinsed once to remove excess unbound antibody, and then incubated with 50µl/well of goat anti-mouse HRPO conjugate diluted 1:500 in washing buffer [0.136M NaCl, 9.7mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4mM KHPO<sub>4</sub>, 2.7mM KCl, 0.6mM C<sub>7</sub>H<sub>5</sub>O<sub>2</sub>Na and 0.5%(v/v) Tween 80, pH 7.4] for 15min at room temperature. Each plate was washed 3 times with washing buffer to remove excess unbound conjugate, and the presence of

bound conjugate was revealed by the addition of substrate solution consisting of 40mM 2,2'-azino-bis (3-ethylbenzethiazoline-6-sulphonic acid) (ABTS) and 0.01%(v/v) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in 50mM citric buffer, pH 4.0. The substrate solution changed to green in wells with bound enzyme conjugates. The optical densities were read at 410nm wavelength using a Dynatech MR 600 micro-ELISA plate reader (Dynatech Laboratories Incorporated, USA).

#### **4.3.5.2 The Dot-ELISA Procedure**

In this assay, *S. haematobium* SEA and P<sub>2</sub>J were suspended in buffer and applied onto NC membrane filters in dots and air dried for 15 minutes. All incubations and washings in this assay were performed on a gentle rocker at room temperature. The antigen "dotted" membranes were cut into strips and placed in the wells of a slot tray and incubated for 1 hr with "blocking solution" containing 5%(w/v) skimmed milk in Tris-buffered saline (TBS) (50mM Tris and 150mM NaCl, pH 8.0). The blocking solution was discarded and the strips were incubated overnight with appropriate concentrations of antibodies diluted in blocking solution. The strips were washed in TBS, pH 8.0 to remove excess unbound antibodies. This was followed by a 1 hr incubation with goat anti-mouse immunoglobulin-HRPO conjugate diluted 1:500 in blocking solution. After three washes each lasting 10 min, the strips were incubated for 3 min in substrate solution containing 0.15% (v/v) H<sub>2</sub>O<sub>2</sub> and 0.05%(w/v) chromogen (3,3'-diaminobenzidine) in phosphate-Na<sub>2</sub>EDTA buffer (10mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Na<sub>2</sub>HPO<sub>4</sub> and 10mM Na<sub>2</sub>EDTA). The strips were

rinsed thrice with deionized water, after which the substrate reaction was stopped by immersing them for 5 min in deionized water acidified by the addition of a few drops of concentrated hydrochloric acid. The results were read visually, as positive reactions appeared as brown dots, and negative results remained colourless (data not shown).

#### **4.3.6. Determination of Immunoglobulin Class and Subclass**

The class and subclass specificity of the immunoglobulins (Igs) were determined using the double diffusion method described by Ouchterlony (1967). Commercially prepared antisera (Goat anti-mouse antibodies from Sigma, USA) against murine immunoglobulin isotypes; IgG1, IgG2a, IgG2b, IgG3, and IgM were used as recommended by the manufacturer. A 1% (w/v) agarose (Bethesda Research Laboratories, USA) gel was prepared by melting solid agarose in phosphate buffered saline (15mM NaCl, 1.1mM NaHPO<sub>4</sub> and 0.1mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 ). Five millilitres of the molten agarose was poured unto a microscopic slide and allowed to solidify. Wells were cut into the solid gel in a circular arrangement surrounding a central well and each filled with approximately 15µl of reagent. Antiserum was placed in the central well and culture supernatants concentrated twenty fold by ammonium sulphate precipitation were placed in the surrounding wells. A precipitin line formed in-between a sample well and the homologous antiserum in the central well. The precipitin reaction was allowed to develop in up

to 48 hours at room temperature in a moist chamber and observed by viewing the gels against light.

#### **4.3.7 Determination of the biochemical nature of antigenic epitopes**

##### **4.3.7.1 *Detection of Monoclonal antibodies specific for carbohydrate epitopes***

The micro-plate ELISA based periodate oxidation at acid pH described by Woodward, Young and Bloodgood (1985) was used in determining whether the antigenic epitopes detected by the specific MoAbs were carbohydrate in nature. Polystyrene micro-plates previously coated with SEA and P<sub>2</sub>J (Section 4.3.3) were rinsed once with washing buffer followed by a second rinse using 50 mM sodium acetate buffer, pH 5.4. Sets of wells were then incubated with varying concentrations of periodate (0, 0.5, 1, 5, 10, 15, and 20 mM) in sodium acetate buffer (100µl/well) for 1 hr at room temperature in the dark. The plates were rinsed once with sodium acetate buffer, and incubated (100µl/well) with 50 mM sodium borohydride in PBS, pH 7.4 for 30 min at room temperature, to reduce the aldehyde groups generated by periodate oxidation to alcohols and prevent non-specific cross-linking of antibodies to antigens. The wells were rinsed five times with washing buffer and, then incubated with MoAbs diluted appropriately, in the same buffer, at 100µl/well, for 1 hr at room temperature, followed by a five times wash with washing buffer to get rid of excess unbound antibody. Each well was then added with 50µl of HRPO-conjugated goat anti-mouse antibodies diluted at 1:500 in washing buffer and incubated for 15 min at room temperature. Again the plates

were washed five times and then incubation with substrate solution [40mM 2,2'-azino bis-(3-ethylbenz-thiazoline sulfonic acid) diammonium salt (ABTS) and 0.01% (v/v) hydrogen peroxide in 50mM citric acid buffer, pH 4.0]. The reaction was allowed to proceed for 30 min and the optical densities read at a wavelength of 410nm using a Dynatech MR600 micro-ELISA plate reader (Dynatech Laboratories Inc. Virginia, USA).

#### **4.3.7.2** *Detection of MoAbs specific for protein epitopes*

Monoclonal antibodies (MoAbs) with specificity for protein epitopes were detected using enzymatic digestion with proteinase-K according to the method described by Larose, Hamel, Lagac and Brodelur (1988) with modification.

Briefly, proteinase-K (Sigma, St.Louis MO, USA) was diluted in Dulbecco's PBS (pH 7.4) to prepare a stock solution of 2mg/ml concentration. 40 $\mu$ l of 2mg/ml preparations of each antigen extract to be tested were pipetted into six Eppendorf tubes labelled A-F. Tubes A, B, C, D, and E containing aliquots of an antigen preparation were added with different concentrations of proteinase-K each diluted in 40 $\mu$ l of PBS, pH 7.4 to give 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0.0625mg/ml of enzyme in the five tubes, respectively. Each tube labelled F was added with 40 $\mu$ l of Dulbecco's PBS, pH 7.4 without proteinase-K to serve as a control. All the tubes were then incubated at 37°C in a waterbath for 1hr, after which 3 $\mu$ l of each sample was pipetted onto nitrocellulose membrane strips in

dots. The strips were assayed in the dot-ELISA using MoAbs that are known to bind the various antigens as probes.

#### **4.3.8 Fixation of miracidia with paraformaldehyde**

Miracidia were hatched from *S. haematobium* eggs obtained from the urine of infected individuals. Large volumes of urine were left standing undisturbed overnight at 4°C and the supernatant decanted away. The sediment was collected into 50ml centrifuge tubes and spun at 500 Xg for 5 min. The pellets were resuspended in 1% saline and washed twice by centrifugation at 500 Xg for 5 min each. The final sediment was resuspended in distilled water and transferred into a flat bottom flask covered with aluminium foil to leave only the upper quarter of the neck. The flask was filled with distilled water up to a level above the darkened portion and placed in front of a light source. The miracidia hatched from the eggs were attracted to the light and concentrated in the small volume of exposed fluid. They were harvested with a pasteur pipette into 50ml tubes and paraformaldehyde diluted in PBS, pH 7.4 was added to give a final concentration of 1%. This preparation was kept at 4°C overnight to fix and stabilise membrane antigens after which the miracidia were washed thrice by centrifugation in siliconized tubes at 500 Xg for 15 min each. The final miracidia pellet was resuspended with a small volume of Dulbecco's PBS, pH 7.4 and the miracidia counted. Fixed miracidia were kept at 4°C until use.

#### 4.3.9. IFAT Procedure

Indirect immunofluorescence was performed with fixed miracidia (section 4.3.7) using a modification of the method described by van Meirvenne, Janssens and Magnus (1975). Briefly, seven microlitres of a  $2 \times 10^6$ /ml miracidia suspension were delivered using 50 $\mu$ l pipette into each of the wells on Teflon-coated multitest microscope slides (Cooper Wellcome, Fremhodgegen, Belgium) and fixed by gentle heating over a burner flame. The slides were then immersed in a bath of Dulbecco's PBS, pH 7.4 for 5 min to wash off loosely bound miracidia and the wells incubated for 15 min at room temperature with 30 $\mu$ l of different MoAbs diluted appropriately.

Some wells were incubated with pre-fusion serum diluted 1:100 or with fresh growth medium to serve as positive and negative controls, respectively. Incubated slides were washed twice by immersion in fresh buffer (2x10 min each) with one buffer change. Flourescein isothiocynate (FITC)-labelled goat anti-mouse immunoglobulin prepared as described by Clark and Shepard (1963) and as modified by Katende, Musoke, Nantulya and Goddeeries (1987), was diluted 1:50 in PBS and added to the wells and incubated for 15 min at room temperature. The slides were washed as before and mounted in 50% glycerol in Tris-buffered saline, (TBS)[50mM Tris and 150mM NaCl, pH 8.6], for microscopy. The miracidia were examined for fluorescence using the Olympus fluorescence microscope (Olympus, Japan), fitted with x20/1.30 oil phase contrast objective and x10 periplan eyepieces under epiploem illumination with a 200W ultra-high pressure mercury vapour lamp.



## 4.4 Results

### 4.4.1 Reactivity of MoAbs with *S. haematobium* Antigens

A list of the extant MoAbs used in this study, their isotypes and reactivity with *S. haematobium* soluble egg antigens (SEA) and ammonium sulphate precipitated proteins from infected human urine (P<sub>2</sub>J) as determined by micro-plate and dot-ELISA is provided in Table 2. Seven MoAbs namely, Sh2/15.F, Sh3/15.13, Sh3/34.10, Sh4/14.3, Sh4/16.45, Sh5/32.30 and Sh5/34.10 could detect antigens in (P<sub>2</sub>J). All of these antibodies except Sh2/15.F could detect soluble egg antigens of the Egyptian strain of *S. haematobium* as well (Table 2). The twelve other MoAbs (Sh1/71.7, Sh3/12.1, Sh3/12.31, Sh3/14.13, Sh3/15.28, Sh3/32.41, Sh3/38.2, Sh3/44.3, Sh3/44.5, Sh3/44.31, Sh3/45.17 and Sh3/45.55), could bind soluble egg antigens only (Table 2).

All the MoAbs except Sh3/44.3 could bind soluble egg antigens of the Ghanaian strain(s) of *S. haematobium*.

### 4.4.2 Range of Reactivity of the MoAbs

The aim of the experiments described here was to determine the suitability of the extant MoAbs as diagnostic reagents based on their ability to either specifically detect the *Schistosoma* genus or any of the constituent species, and react with parasite isolates from different geographical areas.

Table 2:

**Reactivity of MoAbs with *S. haematobium* Egyptian and Ghanaian strain soluble egg antigens and urinary antigens as determined by micro-plate and dot-ELISA**

Monoclonal antibody	Isotype	SEA <sub>Egy</sub>	SEA <sub>Gh</sub>	P <sub>2</sub> J
Sh1/71.7	IgM	+	+	
Sh2/15.F	IgG1		+	+
Sh3/12.1	IgM	+	+	
Sh3/12.31	IgM	+	+	
Sh3/14.13	IgM	+	+	
Sh3/15.13	IgM	+	+	+
Sh3/15.28	IgG1	+	+	
Sh3/32.41	IgM	+	+	
Sh3/34.10	IgM	+	+	+
Sh3/38.2	IgM	+	+	
Sh3/44.3	IgM	+		
Sh3/44.5	IgM	+	+	
Sh3/44.31	IgM	+	+	
Sh3/45.17	IgM	+	+	
Sh3/45.55	IgM	+	+	
Sh4/14.3	IgG1	+	+	+
Sh4/16.45	IgG3	+	+	+
Sh5/32.30	IgM	+	+	+
Sh5/34.10	IgM	+	+	+

SEA<sub>Egy</sub> = Soluble egg antigens from an Egyptian strain of *S. haematobium*.

SEA<sub>Gh</sub> = Soluble egg antigens from Ghanaian strain(s) of *S. haematobium*.

P<sub>2</sub>J = Ammonium sulphate precipitated proteins from urine of *S. haematobium* infected persons.

+ = MoAb reacts with antigen in at least one assay.

= MoAb does not react with antigen.

Table 3, summaries the reactivity of the MoAbs with antigens of different schistosome species as determined by micro-plate and dot-ELISA. With the exception of Sh2/15.F and Sh3/44.3 all the MoAbs reacted with soluble egg antigens of the Egyptian strain (SEA<sub>Egy</sub>) and Ghanaian strain(s) (SEA<sub>Gh</sub>) of *S. haematobium*. Sh2/15.F failed to react with SEA<sub>Egy</sub> whilst Sh3/44.3 could not detect SEA<sub>Gh</sub>. Sh2/15.F and Sh3/15.28 were found to be *S. haematobium* species-specific while Sh1/71.7, Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10 cross-reacted with antigens from *S. haematobium*, *S. mansoni* and *S. japonicum* worms or eggs (Table 3). None of the MoAbs reacted with the hookworm egg antigen extract (Table 3). Both *S. haematobium* species-specific MoAbs reacted with only egg-stage antigens but only one of them (Sh2/15.F) could detect parasite antigens in the urine of infected individuals. As shown in Table 3, ten MoAbs (Sh3/12.1, Sh3/12.31, Sh3/14.13, Sh3/15.13, Sh3/32.41, Sh3/44.5, Sh3/44.31, Sh3/45.17, Sh3/44.55 and Sh4/16.45) detected antigens in *S. haematobium* and *S. japonicum* eggs or worms but not in *S. mansoni* worms.

#### 4.4.3. Biochemical Nature of the Schistosome Antigenic Epitopes Bound by the MoAbs

Periodate oxidation of carbohydrate residues and proteinase-K digestion of polypeptide antigens were used to study the biochemical nature of the antigenic epitopes bound by the MoAbs. As shown in Table 4, periodate oxidation had no effect on the binding of eight MoAbs namely, Sh2/15.F, Sh3/15.13, Sh3/34.10, Sh

Table 3 Reactivity of MoAbs with several crude antigen extracts as determined by micro-plate ELISA and dot-ELISA

Monoclonal antibody	Reactivity with									
	SEA <sub>Eg</sub>	SEA <sub>Gh</sub>	ShW	ShW	SE	SjW	SmW	Pj	HW	HW
Sh1/71.7	+	+	+	+	+	+	+	+	+	+
Sh2/15.F	+	+	+	+	+	+	+	+	+	+
Sh3/12.1	+	+	+	+	+	+	+	+	+	+
Sh3/12.31	+	+	+	+	+	+	+	+	+	+
Sh3/14.13	+	+	+	+	+	+	+	+	+	+
Sh3/15.13	+	+	+	+	+	+	+	+	+	+
Sh3/15.28	+	+	+	+	+	+	+	+	+	+
Sh3/32.41	+	+	+	+	+	+	+	+	+	+
Sh3/34.10	+	+	+	+	+	+	+	+	+	+
Sh3/38.2	+	+	+	+	+	+	+	+	+	+
Sh3/44.3	+	+	+	+	+	+	+	+	+	+
Sh3/44.5	+	+	+	+	+	+	+	+	+	+
Sh3/44.31	+	+	+	+	+	+	+	+	+	+
Sh3/45.17	+	+	+	+	+	+	+	+	+	+
Sh3/45.35	+	+	+	+	+	+	+	+	+	+
Sh4/14.3	+	+	+	+	+	+	+	+	+	+
Sh4/16.45	+	+	+	+	+	+	+	+	+	+
Sh5/32.30	+	+	+	+	+	+	+	+	+	+
Sh5/34.10	+	+	+	+	+	+	+	+	+	+

SEA<sub>Eg</sub> = Soluble egg antigens from an Egyptian strain of *S. haematobium*.SEA<sub>Gh</sub> = Soluble egg antigens of Ghanaian strain(s) of *S. haematobium*.ShW = Crude worm extracts of Ghanaian strain(s) of *S. haematobium*.SmW = Crude *S. mansoni* worm extract.SjE = Soluble egg antigens of *S. japonicum*.SiW = Crude *S. japonicum* worm extract.Pj = Ammonium sulphate precipitated proteins from urine of *S. haematobium* infected persons.

+ = MoAb reacts with antigen in at least one assay.

- = MoAb does not react with antigen.

**Table 4**

**The nature of antigenic epitopes detected by the MoAbs as determined by periodate oxidation and proteinase-K digestion**

Monoclonal antibodies	Isotype	Sensitivity to		Nature of antigenic epitopes
		Periodate	Proteinase-K	
Sh1/71.7	IgM	+	+	*C and P
Sh2/15.F	IgG1		+	P
Sh3/12.1	IgM	+	+	*C and P
Sh3/12.31	IgM	+	+	*C and P
Sh3/14.13	IgM	+	+	*C and P
Sh3/15.13	IgM		+	P
Sh3/15.28	IgG1	+	+	*C and P
Sh3/32.41	IgM	+	+	*C and P
Sh3/34.10	IgM		+	P
Sh3/38.2	IgM	+	+	*C and P
Sh3/44.3	IgM		+	P
Sh3/44.5	IgM	+	+	*C and P
Sh3/44.31	IgM	+	+	*C and P
Sh3/45.17	IgM	+	+	*C and P
Sh3/45.55	IgM	+	+	*C and P
Sh4/14.3	IgG1		+	P
Sh4/16.45	IgG3		+	P
Sh5/32.30	IgM		+	P
Sh5/34.10	IgM		+	P

- + = sensitivity to periodate or proteinase-K.  
 = insensitivity to periodate or proteinase-K.  
 P = protein antigenic determinant.  
 C = carbohydrate antigenic determinant.  
 \* = possibly a glycoprotein.

3/44.3, Sh4/14.3, Sh4/16.45, Sh5/32.30, and Sh5/34.10. However, the binding of these same MoAbs was completely abrogated by proteinase-K treatment. Reactivity of the remaining eleven antibodies (Table 4) was affected by both periodate oxidation and proteinase-K digestion. Except for Sh3/44.3, all the MoAbs that bound protein epitopes reacted with P<sub>2</sub>J (Table 4).

#### 4.4.4. Immunolocalization of epitopes bound by the MoAbs in *S. haematobium* miracidia

Table 5, summarises the results of immunolocalization of the antigens detected by the anti-schistosome MoAbs in *S. haematobium* miracidia as determined by IFAT. Four MoAbs (Sh2/15.F, Sh3/12.1, Sh3/12.31 and Sh3/44.31) bound only antigens located on the surface of miracidia while three others (Sh3/15.13, Sh4/14.3 and Sh4/16.45) bound only internally located, possibly cytoplasmic antigens. The remaining twelve MoAbs bound both surface and internal antigens.

Table 5

**Immunolocalization of the *S. haematobium* antigens detected by the MoAbs using indirect Fluorescent antibody test (IFAT)**

Monoclonal antibody	Isotype	Specificity	Reactivity with miracidia*	
			Surface staining	Internal staining
Sh2/15.F	IgG1	Sh**	+	
Sh3/12.1	IgM	Sh&Sj	+	
Sh3/12.31	IgM	Sh&Sj	+	
Sh3/44.31	IgM	Sh&Sj	+	
Sh3/15.13	IgM	Sh&Sj	-	+
Sh4/14.3	IgG1	PanSh	-	+
Sh4/16.45	IgG3	Sh&Sj		+
Sh1/71.7	IgM	PanSh	+	+
Sh3/14.13	IgM	Sh&Sj	+	+
Sh3/15.28	IgG1	Sh	+	+
Sh3/32.41	IgM	Sh&Sj	+	+
Sh3/34.10	IgM	PanSh	+	+
Sh3/38.2	IgM	PanSh	+	+
Sh3/44.3	IgM	Sh	+	+
Sh3/44.5	IgM	Sh&Sj	+	+
Sh3/45.17	IgM	Sh&Sj	+	+
Sh3/45.55	IgM	Sh&SJ	+	+
Sh5/32.30	IgM	PanSh	+	+
Sh5/34.10	IgM	PanSh	+	+

+ = stained.

= not stained.

\* = miracidia of Ghanaian strain(s) of *S. haematobium*.

PanSh = Pan Schistosome.

Sh&Sj = *S. haematobium* and *S. japonicum*.

Sh = *S. haematobium* species-specific as determined by micro-plate, dot-ELISA and Western blotting.

## 4.5 Discussion

The aim of the study described in this chapter was to characterize nineteen murine MoAbs produced against *S. haematobium* in order to determine their usefulness as diagnostic reagents based on their specificity and the ability to detect schistosome antigens in infected human urine.

Two MoAbs, Sh2/15.F and Sh3/15.28 were found to be *S. haematobium* species-specific. They both reacted with antigens in Ghanaian and Egyptian strains of *S. haematobium*, thereby indicating that they would be suitable for detection of infection due to the parasite in different geographical areas. However, only Sh2/15.F could detect antigens in *S. haematobium* infected human urine, thus suggesting that this antibody may be more suitable for diagnostic purposes. Indeed diagnosis of schistosomiasis by detection of schistosomal antigens has been reported to have several advantages, including suitability for measuring the severity of pathological manifestations, monitoring impact of chemotherapy and investigating ongoing transmission in endemic areas (Kremsner *et al.*, 1994). Furthermore, the ability of Sh2/15.F to detect antigens by the dot-ELISA, adds to its probable usefulness. This is because the dot-ELISA has been found to be simple, specific, sensitive, field portable and reagent conservative (Pappas, 1988). It is likely that the inability of the other *S. haematobium* species-specific MoAb (Sh3/15.28) to detect the antigen in the precipitated urine protein fraction (P<sub>2</sub>J) is due to inefficient precipitation of the glycoprotein antigen by ammonium sulphate as compared to the



protein antigen detected by Sh2/15.F. This may as well explain why none of the eleven MoAbs that detected glycoprotein epitopes could bind antigens in P<sub>2</sub>J, and yet all those MoAbs that detected protein epitopes present in Ghanaian strains of *S. haematobium* could do so. Indeed, the diagnostic potential of some *Schistosoma* genus-specific glycoproteins which appear in infected human urine have been reported (De Jonge *et al.*, 1989; Deelder, Qian, Kremsner, Acosta, Rabello, Enyong, van Etten, Krijger, Rotmans, Fillie, De Jonge, Agnew and van Lieshout, 1994; and De Clercq *et al.*, 1995). It is, therefore, difficult to draw conclusions with the present data about the ability of Sh3/15.28 to detect urinary antigens.

The MoAb Sh3/44.3 appears to be *S. haematobium* strain specific as it could detect the antigen in only the Egyptian strain of the parasite. It is, therefore, not likely to be suitable for diagnosis of schistosomiasis. However, it may be potentially useful in studies intended to characterize *S. haematobium* strains.

Six other MoAbs; Sh1/71.7, Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10 had wider *Schistosoma* genus-specific reactivity. Sh3/34.10, Sh4/14.3, Sh5/32.30 and Sh5/34.10 in particular may be useful in the development of diagnostic assays to detect schistosome-specific antigens in urine of patients when species-specific identification is not critical, since they could detect the respective antigens in infected human urine. These four genus-specific MoAbs may also be useful in studies towards the search for protective antigens since they all bound protein antigens. This is because, as pointed out by Hillyer and Serrano (1982), the most suitable vaccine candidate antigens for protection against schistosomiasis are

the cross-reactive proteins. It should, therefore, be necessary to determine the protective nature of the protein antigens detected by Sh3/34.10, Sh4/14.3, Sh5/32.30 and Sh5/34.10.

Two other MoAbs, Sh3/15.13 and Sh4/16.45 bound protein antigens that appeared in the urine of *S. haematobium* infected individuals. However, they both cross-reacted with antigens in *S. haematobium* and *S. japonicum*, thereby suggesting that they may be limited in diagnostic applications. Nevertheless, it may be argued that the non-overlapping nature of the distribution of the two parasites justifies the exploitation of the MoAbs for diagnosis.

All the other MoAbs detected glycoprotein antigens in both *S. haematobium* and *S. japonicum*. Furthermore, the antigenic determinants bound by all of them except Sh3/45.17 were found to be glycoproteins present in the eggs. This suggests that the antigens may be unsuitable for protection, since it is generally known that immunological responses to glycoprotein egg antigens are largely responsible for the pathology in schistosomiasis (Erasmus, 1987).

## **CHAPTER 5**

### **REACTIVITY OF ANTI-SCHISTOSOMA HAEMATOBIIUM MONOCLONAL ANTIBODIES IN THE WESTERN IMMUNOBLOT ASSAY**

## 5.1 Summary

Nineteen anti-*S. haematobium* monoclonal antibodies (MoAbs) were characterized using the Western immunoblot assay with the aim of determining the molecular weights of the antigens detected. MoAb reactivity was found to be influenced by the biochemical nature of the antigens detected, the antibody concentration and the amount of current used in electrophoresis. MoAbs that detected glycoproteins antigens (except those with generally low reactivity) reacted when diluted in 2% skimmed milk, whilst those that detected relatively low molecular weight polypeptide antigens (MW<40) reacted at high concentration particularly when undiluted.

Out of the nineteen MoAbs investigated, seventeen of them including nine which bound glycoprotein epitopes (Sh1/71.7, Sh3/12.1, Sh3/12.31, Sh3/14.13, Sh3/15.28, Sh3/32.41, Sh3/44.31, Sh3/45.17 and Sh3/45.55) and eight which bound polypeptides (Sh2/15.F, Sh3/15.13, Sh3/34.10, Sh3/44.3, Sh4/14.3, Sh4/16.45, Sh5/32.30 and Sh5/34.10) reacted under varying conditions of complete denaturing, whilst the remaining two (Sh3/38.2 and Sh3/44.5) did not react under any of the denaturing and non-denaturing conditions tested. Three glycoprotein specific MoAbs (Sh3/15.28, Sh3/32.41 and Sh3/45.17) showed diffuse banding which were resolved by increasing the electrophoresis current by 50%.

One *S. haematobium* species-specific MoAb, Sh2/15.F bound a 29 kDa peptide in soluble egg antigens of Ghanaian (SEA<sub>Gh</sub>), and Egyptian (SEA<sub>Egy</sub>), strains

of the parasite, while the other detected epitopes in 53, 56.7 and 66 kDa antigen bands in both SEA<sub>Ghv</sub> and SEA<sub>Egypt</sub>. On the other hand, the *S. haematobium* Egyptian strain-specific MoAb, Sh3/44.3, detected 36 and 40 kDa peptides in SEA<sub>Egypt</sub>. One out of six *Schistosoma* genus-specific MoAb and three antibodies cross-reacting with *S. haematobium* and *S. japonicum* bound epitopes located on an 80 kDa antigen band in SEA<sub>Egypt</sub>. Three other *S. haematobium* and *S. japonicum* cross-reacting antibodies (Sh3/14.13, Sh3/32.41 and Sh3/45.17) bound epitopes in the 56.7 and 66 kDa bands from SEA<sub>Ghv</sub> and SEA<sub>Egypt</sub>. Sh3/32.41 and Sh3/45.17 also bound an additional 53 kDa band from both antigen extracts. None of the MoAbs detected antigens in electrophoresed proteins from *S. haematobium* infected human urine.

## 5.2 Introduction

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection (Towbin, Staehelin and Gordon., 1979), and can be used to determine a number of important characteristics of protein antigens, such as, the presence and quantity of an antigen and its molecular weight (Grzych, Roussel-Velge and Capron, 1989). The technique has therefore been used widely to characterize antibodies to parasite antigens (Harns *et al.*, 1985; Capron *et al.*, 1987). The advantages of such characterization include the possibility of purification and sequencing of blotted protein antigens.

The search for protective antigens in schistosomiasis has been lengthy, partly because most antigens were initially identified with *S. mansoni* because of the relative ease with which this species is adopted to the laboratory (Bergquist, 1995). Work on the two other major human schistosomes (*S. haematobium* and *S. japonicum*) was insignificant until just recently that extensive work on *S. japonicum* resulted in a long list of cloned antigens (Davern, Wright, Herrman and Mitchell, 1991). Unfortunately, studies on *S. haematobium* antigens continues to lag behind (reviewed by Bergquist, 1995). Identification of any *S. haematobium* antigens with protective or diagnostic ability will, therefore, greatly enhance work towards control of schistosomiasis.

Nineteen MoAbs were recently generated against *S. haematobium* by Bosompem and colleagues (see Chapter 4). Earlier attempts to characterized six of them (Amanor *et al.*, 1996) revealed that potentially diagnostic antibodies that

detected *Schistosoma* genus-specific protein antigens could not function in the western immunoblot assay, thereby limiting their exploitation through peptide sequencing and gene cloning.

In Chapter 4 all the nineteen MoAbs were characterized and those with potential for diagnosis or protection identified. The aim of the experiments conducted in this chapter was to determine the molecular weights of the potentially useful antigens using the western immunoblot technique.

### 5.3 Materials and Methods

#### 5.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of schistosome proteins was performed with the ATTO Corporation Slab Gel Apparatus (Bunkyo- Ku, Tokyo, Japan), following the SDS-Tris-glycine discontinuous buffer system (Laemmli, 1970).

#### 5.3.2 Assembly of slab gel apparatus and preparation of resolution and stacking gels

The gel casting apparatus was composed of a clamp apparatus, a pair of glass plates wiped clean with absolute methanol, and a rubber gasket. The gels were cast in a mould formed by a plain glass plate and a notched glass plate put together and sealed with a U-shaped gasket. The assembled plates were kept vertically upright by 4 spring clamps.

Resolution acrylamide gradient gels (10-15%) and the stacking gel (3%) were prepared as follows:

##### Solution A1. 10% resolution gel (one gel)

29.2% (w/v) acrylamide,	
0.8% (w/v) N,N'-methylene bis-acrylamide	6ml
1.5M (Tris-aminomethane)-HCL pH 8.8,	
0.4% (w/v) sodium dodecyl sulphate (SDS)	4.5ml
10% (w/v) Ammonium persulphate (APS)	0.7ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.01ml
Distilled water	7.5ml



Solution A2. 7.5% resolution gel (one gel)

29.2% (w/v) acrylamide,	
0.8% (w/v) N,N'-methylene bis-acrylamide	4.5ml
1.5M (Tris-aminomethane)-HCL pH 8.8,	
0.4% (w/v) sodium dodecyl sulphate (SDS)	4.5ml
10% (w/v) Ammonium persulphate (APS)	0.7ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.01ml
Distilled water	9.0ml

Solution B. 15% resolution gel (one gel)

29.2% (w/v) acrylamide,	
0.8% (w/v) N,N'-methylene bis-acrylamide	9ml
1.5M (Tris-aminomethane)-HCL pH 8.8,	
0.4% (w/v) sodium dodecyl sulphate (SDS)	4.5ml
10% (w/v) Ammonium persulphate (APS)	0.07ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.01ml
Distilled water	4.5ml

Solution C. 3% stacking gel (one gel)

29.2% (w/v) acrylamide,	
0.8% (w/v) n,n'-methylene bis-acrylamide	1ml
1.5M (Tris- aminomethane)-HCL pH 6.8,	
0.4% (w/v) sodium dodecyl sulphate (SDS)	1.5ml
10% (w/v) Ammonium persulphate (APS)	0.018ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.006ml
Distilled water	3.6ml

10-15% or 7.5-15% resolution acrylamide gradient gels were prepared by slowly mixing solution A1 or A2 and B using an improvised gradient former. The

7.5-15% resolution gradient gels were used only when it was necessary to resolve high molecular weight bands (40-100 kDa) into sharper ones. Each solution was swirled to mix and poured into one of the two linked chambers of the gradient mixer. Solution B with the higher acrylamide concentration was poured into the chamber closer to the outlet. The tube joining the two chambers was opened and a magnetic stirrer placed in solution B started. An ATTO chromatographic peristaltic pump (ATTO, Kunkyo-ku, Tokyo, Japan) set at a flow rate of 3ml/min was used to deliver the gel mixture via rubber tubing into the space between the assembled glass plates. The gel former was immediately rinsed with distilled water to prevent polymerization of gel in the delivery tubes. The gel in between the glass slabs was overlaid with about 400 $\mu$ l of water saturated butanol using an adjustable pipette and left for approximately 1 hr to polymerize. After it had set, the overlaying water saturated butanol was drained off and the gel surface rinsed with distilled water which was also drained off with the aid of a tissue paper. Stacking gel solution (C) was poured on top of the separating gel and a comb carefully inserted into the stacking gel to cast the desired wells for sample application.

After the stacking gel had polymerized, the comb was removed and the cast gel units assembled in an ATTO Corporation Cell Electrophoresis apparatus. The upper electrophoretic chamber, at the cathode, was filled with 0.2 litres of running buffer (24.8mM Tris, 191.8mM Glycine and 3.47mM SDS) and the lower chamber, at the anode, filled with 0.6 litres of the running buffer.

### **5.3.3 Preparation of samples and electrophoretic run**

Soluble egg antigen extracts from Ghanaian strain(s) of *S. haematobium* (SEA<sub>Gh</sub>) and from an Egyptian strain (SEA<sub>Egy</sub>) and Ammonium sulphate precipitated proteins from the urine of *S. haematobium* infected persons (P<sub>2</sub>J) were adjusted for protein and diluted with sample buffer [25mM Tris, 192mM Glycine, 0.1%(w/v) SDS and 20%(v/v) methanol] at a ratio of 2:1 (sample to buffer) in complete denaturing experiments or with the same buffer but without SDS in non-denaturing experiments to give 10µg of protein per lane. Standard low molecular weight markers (Sigma, St. Louis MO, USA or Pharmacia Fine Chemicals, Upsala Sweden) were prepared as described by the manufacturer and used. The samples were either not boiled at all in non-denaturing and denaturing without boiling experiments or boiled for 5 min in complete denaturing experiments. The samples were centrifuged for about 10min at 9900 Xg to remove particulate matter before they were loaded onto the gel. A constant current of 10mA was supplied by an electrophoresis power supply (ATTO Corporation, Japan) until the bromophenol blue tracer dye reached the interface between the stacking and separating gels. The current was then increased to 15mA and maintained until the bromophenol blue marker had barely run out of the separating gel. The operating currents were each increased by 50% where sharper resolution of peptides bands was necessary.

#### 5.3.4 Staining, destaining and western immunoblot

A vertical strip of the gel containing the standard molecular weight markers and electrophoresed antigens was cut using a surgical blade, and transferred into a plastic tray containing staining solution [0.5%(w/v) coomassie blue, 10%(v/v) acetic acid and 50%(v/v) methanol] for 5 min. The stained gel was then transferred to a destaining solution containing 10%(v/v) methanol and 7%(v/v) acetic acid on a gentle rotor (Red-Rotor Model PR70, Hoeffler) with several changes of the solution until stained protein bands were clearly visible in the gel (Weber and Osborn, 1969). Separated schistosome antigens were transferred electrophoretically from unstained gels onto polyvinylidene difluoride (PVDF) or nitrocellulose sheets, as described by Towbin, Staechelin and Gordon (1979) and Burnette (1981). Briefly, 3 pieces of 3mm Whatman chromatography paper (Whatman, Maidstone, England) trimmed to the size of the gel were soaked with the anode transfer buffer pH 11 [0.3M Tris, 10%(v/v) methanol] and placed on top of the anode electrode of a semi-dry transfer electrophoresis transfer unit (MiliBlot<sup>TM</sup>-Graphite Electroblotter 1 Millipore Company, Incorporated, Seattle, USA). A sheet of PVDF (0.45mm pore size Millipore Corporation, Bedford, U.K.) or nitrocellulose filter (0.45mm pore size, Schleicher and Schuell, Inc., Keen, NH, USA) also trimmed to fit the gel, was briefly wetted with transfer buffer and carefully placed on top of the Whatman chromatography paper without trapping air bubbles. The gel was then placed on top of the PVDF or nitrocellulose paper. A second batch of 3 pieces of Whatman chromatography paper soaked in the transfer cathode buffer pH 8.8 [25mM Tris,

40mM Glycine, 20%(v/v) methanol] was added on top of the gel. The cathode electrode was then placed on top of the sandwiched gel and electrophoretic transfer was conducted at a constant current of 15mA overnight.

The blotted PVDF membrane was removed, cut into strips and immuno-assayed using the method described for the detection of schistosome antigens by dot-ELISA (section 4.3.4.2).

### **5.3.5 Monoclonal antibodies**

The nineteen MoAbs used in this study have been described previously in Chapter 4. Two of them (Sh2/15.F and Sh3/15.28, both of IgG1 isotype) were *S. haematobium* species-specific and Sh3/44.3 (IgM) was specific to the Egyptian strain of *S. haematobium*. Another six of the MoAbs [Sh1/71.7 (IgM), Sh3/34.10 (IgM), Sh3/38.2 (IgM), Sh4/14.3 (IgG1), Sh5/32.30 (IgM) and Sh5/34.10 (IgM)] were *Schistosoma* genus-specific, and the remaining ten [Sh3/12.1 (IgM), Sh3/12.31 (IgM), Sh3/14.13 (IgM), Sh3/15.13 (IgM), Sh3/32.41 (IgM), Sh3/44.5 (IgM), Sh3/44.31 (IgM), Sh3/45.17 (IgM), Sh3/44.55 (IgM) and Sh4/16.45 (IgG3)] cross-reacted with antigens in *S. haematobium* and *S. japonicum*.

## 5.4 Results

### 5.4.1 Conditions of reactivity of the Monoclonal Antibodies

As shown in Table 6, different MoAb dilutions were required for optimum reactivity in the western immunoblot assay. The MoAb working dilution appeared to depend on the biochemical nature of the epitopes detected (Table 6). Detecting protein epitopes generally required the use of undiluted (neat) MoAb irrespective of the method of purification of the antibody.

Out of the nineteen MoAbs investigated in this study, seventeen reacted under varying conditions of complete denaturing (Table 6). However, as shown in Table 6 the reactivity was found to be influenced by the biochemical nature of the antigens and the amount of current used in electrophoresis. Nine MoAbs (Sh1/71.7, Sh3/12.1, Sh3/12.31, Sh3/15.28, Sh3/32.41, Sh3/34.10, Sh3/45.17, Sh3/45.55 and Sh4/14.3) including seven that bound glycoprotein epitopes (Table 6) reacted when diluted. The remaining eight MoAbs (Sh2/15.F, Sh3/44.3, Sh3/14.13, Sh3/15.13, Sh3/44.31, Sh4/16.45, Sh5/32.30 and Sh5/34.10) including six that bound protein epitopes (Table 6) could only react when undiluted. The glycoprotein antigens detected by three MoAbs (Sh3/15.28, Sh3/32.41 and Sh3/45.17) that showed diffuse banding were resolved into sharper bands by increasing the electrophoresis current by 50% from 10 and 15mA to 15 and 22.5mA for the stacking and resolution gels, respectively.

Table 6

The molecular weights of the antigens detected by the anti-*Schistosoma haematobium* MoAbs as determined by Western Immunoblot analysis

Monoclonal antibody	Isotype	Molecular weight (kDa)	Biochemical nature	Western-blot titre	Western- blot condition*
Sh2/15.F	IgG1	(29) <sup>SEAGh</sup>	P	Neat	3
Sh3/15.13	IgM	(43) <sup>SEAGh</sup>	P	Neat	3
Sh3/34.10	IgM	(40) <sup>SEAGh</sup> (42) <sup>SEAEgy, SEAGh</sup>	P	1:1000*	1
Sh3/44.3	IgM	(36,40) <sup>SEAEgy</sup>	P	Neat	3
Sh4/14.3	IgG1	(29) <sup>SEAEgy, SEAGh</sup> (38) <sup>SEAGh</sup>	P	1:1000**	1
Sh4/16.45	IgG3	(38, 42) <sup>SEAGh</sup>	P	Neat	3
Sh5/32.30	IgM	(36) <sup>SEAEgy, SEAGh</sup>	P	Neat	3
Sh5/34.10	IgM	(36) <sup>SEAEgy, SEAGh</sup> (38) <sup>SEAGh</sup>	P	Neat	3
Sh1/71.7	IgM	(80) <sup>SEAEgy</sup>	C and P	1:500*	1
Sh3/12.1	IgM	(80) <sup>SEAEgy</sup>	C and P	1:500*	1
Sh3/12.31	IgM	(80) <sup>SEAEgy</sup>	C and P	1:500*	1
Sh3/14.13	IgM	(56.7, 66) <sup>SEAEgy, SEAGh</sup>	C and P	Neat	3
Sh3/15.28	IgG1	(53, 56.7, 66) <sup>SEAEgy, SEAGh</sup>	C and P	1:500**	2
Sh3/32.41	IgM	(36, 53, 56.7, 66) <sup>SEAEgy, SEAGh</sup>	C and P	1:500*	2
Sh3/38.2	IgM	(-)	C and P	ND	
Sh3/44.5	IgM	(-)	C and P	ND	
Sh3/44.31	IgM	(55) <sup>SEAEgy</sup>	C and P	Neat*	3
Sh3/45.17	IgM	(53, 56.7, 66) <sup>SEAEgy, SEAGh</sup>	C and P	1:500*	2
Sh3/45.55	IgM	(80) <sup>SEAEgy</sup>	C and P	1:500*	1

SEAEgy = Soluble egg antigens of an Egyptian strain of *S. haematobium*.

SEAGh = Soluble egg antigens of Ghanaian strain(s) of *S. haematobium*.

= Monoclonal antibody did not react in the western immunoblot assay.

ND = Not determined.

P = protein antigenic epitope.

C and P = Glycoprotein antigenic epitope.

Neat = Monoclonal antibody was used without dilution.

\*\* = Purified monoclonal antibody fraction obtained by gel chromatography.

\* = x20 concentration of culture supernatant by ammonium sulphate precipitation.

• = The western immunoblot conditions are described in detail in materials and methods.

1 = Complete denaturing with test monoclonal antibody diluted in 2% skimmed milk in Tris-buffer, pH 8.0.

2 = Same as (1) but with 50% increase in electrophoretic current.

3 = Complete denaturing with neat concentration of test monoclonal antibody.

#### 5.4.2 Molecular weights of Antigens bounds by the MoAbs

The aim of the series of experiments described here was to determine the molecular weights of the schistosome antigens bound by the MoAbs (Table 6).

The *S. haematobium* species-specific MoAb, Sh2/15.F, bound a 29 kDa peptide in soluble egg antigens of Ghanaian (SEA<sub>Gh</sub>), and Egyptian (SEA<sub>Egy</sub>), strains of the parasite, while the *S. haematobium* Egyptian strain-specific MoAb, Sh3/44.3, detected 36 and 40 kDa peptides in SEA<sub>Egy</sub>. One pan-schistosome MoAb (Sh1/71.7) and three antibodies cross-reacting with *S. haematobium* and *S. japonicum* bound epitopes located on an 80 kDa antigen band in SEA<sub>Egy</sub> (Table 6). One other *S. haematobium* species-specific MoAb Sh3/15.28 and three other *S. haematobium* and *S. japonicum* cross-reacting antibodies (Sh3/14.13, Sh3/32.41 and Sh3/45.17) bound epitopes in 56.7 and 66 kDa antigen bands in both SEA<sub>Gh</sub> and SEA<sub>Egy</sub>. Sh3/15.28, Sh3/32.41 and Sh3/45.17 also bound an additional 53 kDa band in both antigen extracts. None of the MoAbs could detect antigens in precipitated proteins from *S. haematobium* infected human urine (P<sub>2</sub>J) by the western immunoblot assay.



## 5.5 Discussion

Effective control of schistosomiasis through accurate diagnosis and/or protection by vaccination first require the identification and possibly purification of potentially diagnostic and protective schistosome antigens (De Jonge, Kremsner, Krijger, Schommer, Fillie, Kornelis, van Zeyl, van Dam, Feldmeier and Deelder, 1990; Bergquist, 1995). Such investigations are normally facilitated by analysis of crude parasite antigens using relevant MoAbs in the western immunoblot technique (Grzych, Roussel-Velge and Capron, 1989). The aim of the series of experiments described in this chapter was, therefore, to characterize the antigens detected by the anti-*S. haematobium* MoAbs described in chapter 4, using the western immunoblot assay.

The western immunoblot technique described in this study was successfully applied to the determination of the molecular weights of the antigens bound by seventeen out of the nineteen monoclonal antibodies studied.

A number of potentially useful schistosome species-specific antigens including a *S. haematobium* species-specific egg antigen of Mr 51 000 have been described (Norden and Strand, 1984a; Hillyer and Pacheco, 1986). In this study two *S. haematobium* species-specific epitopes detected by Sh2/15.F and Sh3/15.28 were found to be located on a 29 kDa band for Sh2/15.F and a series of bands (53, 56.7 and 66 kDa) for Sh3/15.28 in soluble egg antigens of both Ghanaian (SEA<sub>Gh</sub>) and Egyptian (SEA<sub>Egy</sub>) strains of the parasite. Also, a *S.*

*haematobium* Egyptian strain-specific MoAb, Sh3/44.3, was shown to detect an epitope on 36 and 40 kDa bands from SEA<sub>Egy</sub>. These differences in molecular weights may suggest that *S. haematobium* species-specificity is defined by several epitopes, and the Ghanaian and Egyptian strains of the parasite are distinguishable by specific MoAbs.

It has been reported that the most suitable vaccine candidate antigens for protection against schistosomiasis are the cross-reactive proteins (Hillyer and Serrano, 1982), and the western immunoblot technique is known to be suitable for identification, separation and purification of useful antigens from complex antigenic mixtures (Grzych, Roussel-Velge and Capron, 1989). The determination of the molecular weights of four cross-reactive peptides detected by four different pan-schistosome MoAbs used in this study should, therefore, enhance investigations into their potential for immunoprophylaxis.

In all, six of the eight MoAbs that detected protein antigens could react in the western immunoblot assay only when undiluted. This need for high antibody concentrations in the assay is attributable to several causes including alterations in epitope accessibility following adsorption of the antigens onto filter membranes (Towbin, Staechelin and Gordon, 1979). The failure of two MoAbs namely, Sh3/38.2 and Sh3/44.5 to bind antigens in the western immunoblot assay may be explained similarly. Also, Goes, Rocha, Gazzenelli and Doughty (1989) reported that the binding of some MoAbs to schistosome soluble egg antigens (SEA) may be greatly reduced when antigens were electrophoresed in the presence of the

reducing agent 2-mecaptoethanol, and Su and Prestwood (1990) reported that in the presence of sodium dodecyl sulphate and 2- mecaptoethanol, the subunits of certain antigens are dissociated (denatured) and their biological activities are irreversible lost. However, Sh3/38.2 and Sh3/44.5 also failed to react when tested under non-reducing conditions in this study.

Hamburger *et al.* (1982) pointed out that binding of SDS to the carbohydrate portion of certain large glycoproteins may cause anomalous migration in polyacrylamide gels and produce diffuse bands. In this study, three MoAbs, Sh3/15.28, Sh3/32.41 and Sh3/45.17 that bound glycoprotein antigens showed diffuse banding which were resolved into sharper bands by increasing the electrophoresis current.

In an earlier study, Bosompem *et al.* (1996a) showed that schistosome antigens in P<sub>2</sub>J were in association with human immunoglobulins and complement in immune complexes, and further reported that anti-SEA<sub>Egy</sub> polyclonal sera failed to detect antigens in P<sub>2</sub>J in the western immunoblot assay, even though the same sera could bind the antigens in P<sub>2</sub>J using micro-plate ELISA. They explained that the observation was not surprising. This was because even though SDS-PAGE analysis could lead to denaturation of antigens (Su and Prestwood, 1990), it was reported that polyclonal antisera against native proteins normally contain some clonal products which recognize the denatured antigens and therefore allow the Western immunoblot to function (Chaicumpa, Ruankunaporn, Kalambaheti, Limavongpranee, Kitikoon, Khusmith, Pungpak,

Chongsa-Nguan, and Sornmani, 1991). However, as explained by Arnon (1973), the reactivity of both immune serum and MoAbs to the denatured antigens may be greatly reduced or even abolished. Moreover, the conformation of the antigens after they are transferred to NC membrane and the interactive effects of complexed antigens, immunoglobulins and complement in the western blotting are not known.

In conclusion, the molecular weights of potentially diagnostic and protective *S. haematobium* antigens have been successfully determined in this study, thereby opening up more avenues for further exploitation of the antigens and the MoAbs that detect them.

## **CHAPTER 6**

### **GENERAL DISCUSSION AND CONCLUSIONS**

Since the identification of schistosomiasis as an important global disease, several control efforts have been made. These include attempts to eliminate the intermediate snail hosts and health education aimed at interrupting the parasite's life cycle. However, these efforts have so far achieved limited success (Bergquist, 1995). As a result, the disease is spreading particularly in the developing world (Savioli and Mott, 1989) because of increasing snail habitats created mainly by water resource development. Consequently, there has been a shift of emphasis from transmission control or eradication of the disease to morbidity control through the primary health care system (Mott, 1987; Awadalla, el-Mansoury and el Azzouni, 1992; Bergquist, 1995). In addition, it has been widely communicated in recent times that effective management of schistosomiasis requires the development of accurate diagnostic tools (Peters and Kazura, 1987; De Clercq *et al.*, 1995; Bosompem *et al.*, 1996a) and eventually the identification of protective antigens (De Jonge *et al.*, 1990; Kremsner *et al.*, 1994; Bergquist, 1995).

Improved diagnosis of human schistosomiasis require identification of new *Schistosoma*-genus and species-specific antigens. This is because routine diagnosis of the disease at present is by microscopic examination of urine and stool for the presence of schistosome eggs or by the detection of haematuria or proteinuria. These methods are, however, labour intensive and inaccurate (Webbe, 1982). The more sensitive diagnostic methods involving the detection of host anti-parasite antibodies also have several limitations including inability to

identify active infections. Recent studies have shown that detection of circulating parasite antigens offers better opportunities for diagnosis as well as for monitoring drug efficacy and for determining pathological manifestations in schistosomiasis (De Jonge *et al.*, 1989; Deelder *et al.*, 1994). However, only two promising antigens namely the circulatory anodic antigen (CAA) and the circulatory cathodic antigen (CCA) have been identified and are currently being investigated. According to Bergquist (1995) and De Clercq *et al.* (1995) the unavailability of more suitable diagnostic and protective antigens against schistosomiasis may be blamed in part on the difficulty of maintaining the life-cycle of *S. haematobium* in the laboratory, since it has considerably slowed down the identification of novel antigens from this parasite. In this thesis efforts were directed at characterizing nineteen MoAbs recently generated against *S. haematobium* in order to find out if any of them may be suitable for diagnosis or for protection against schistosomiasis.

The most promising of the available schistosomiasis diagnostic techniques in terms of field applicability are those that detect urinary antigens (De Jonge *et al.*, 1989; Kremsner *et al.*, 1994). This is because the use of urine has several advantages over the use of serum. For example, unlike blood urine (is a waste product which) is easily obtained without the use of needles or syringes. As a result the procedure for obtaining urine test specimen presents no danger to the patient. Nevertheless, neither the CAA nor CCA which also appear in the urine of patients is specific to any of the schistosomes. As a result, it is still not possible

to differentiate between urinary and intestinal schistosomiasis by urine assay, even though the two forms of the disease overlap in distribution in hyperendemic areas of Africa (WHO, 1985), thereby necessitating differential diagnosis. Indeed, the use of a common drug (praziquantel) for treatment of schistosomiasis may suggest that genus-specific assays are preferable. However, specific identification of infecting schistosome species has several advantages. These include useful information on: (1) the influence of schistosome species on disease prevalence, incidence and intensity of infection in hyperendemic areas; and (2) drug efficacy and possible emergence of drug resistant schistosome species or strains.

In chapter 4, one of two *S. haematobium* species-specific MoAbs was shown to be capable of detecting antigens in the urine of infected individuals. This MoAb (Sh2/15.F) was also shown to bind a protein epitope located on the surface membrane of *S. haematobium* miracidia (chapter 4) and to a 29 kDa polypeptide in the soluble egg antigens of both Ghanaian and Egyptian strains of the parasite (chapter 5). The implications of these findings are that this MoAb will be suitable for developing non-invasive *S. haematobium* species-specific assays for use in different geographical areas. Furthermore, the protein nature of the antigen indicates that it could also be exploited by recombinant DNA technology for more efficient utilization in highly standardized diagnostic assays.

The other *S. haematobium* species-specific MoAb (Sh3/15.28) bound a glycoprotein antigen which was not detected in the precipitated urine protein



fraction (P<sub>2</sub>J) and may therefore not be as useful as Sh2/15.F. However, the results appear to suggest that *S. haematobium* species-specificity is defined by at least three different epitopes, including the one described by Hillyer and Pacheco (1986).

The inability of Sh3/44.3 to detect antigens in the soluble egg antigen extract of the Ghanaian strains of *S. haematobium* (SEA<sub>Gh</sub>) even though it could bind the antigen in the egg extract of the Egyptian strain of the parasite (chapters 4 and 5), suggests that it may be a strain-specific MoAb. If this were so the MoAb (Sh3/44.3) may be potentially useful in studies intended to characterize schistosome strains, especially considering that the epitope detected is a protein (chapter 4) for which the encoding gene can be cloned and sequenced.

Even though the two *Schistosoma* genus-specific glycoprotein antigens (CAA and CCA) have been extensively investigated for use in diagnosis of schistosomiasis, Kremsner *et al.* (1994) reported that the introduction of schistosomal antigen detection into routine diagnosis still awaits the development of rapid dipstick assays. Such a development obviously require the use of MoAbs with potential applicability in membrane-based assays such as the dot-ELISA which is reported to be simple, specific, sensitive, field portable and reagent conservative (Pappas, 1988). In studies described in chapter 4, six *Schistosoma* genus-specific MoAbs were identified. Four of them (Sh3/34.10, Sh4/14.3, Sh5/32.30 and Sh5/34.10) bound protein epitopes and were also able to detect the

antigens in the urine of patients by the nitrocellulose membrane-based dot-ELISA, thereby indicating their suitability for development of field-applicable assays.

Stage-specific schistosome antigens have been reported to play an important role in “concomitant immunity” which leads to survival of the adult stage of the parasite in the host while the larval stages are destroyed (Cioli, Liberti and Festucci, 1987). Domingo and Warren (1968), Hamburger, Pelley and Warren (1976) and Carter and Colley (1976) showed that some of these stage-specific antigens were also species-specific in either *S. haematobium*, *S. mansoni* or *S. japonicum*. In studies described in Chapter 4 of this study, species- and stage-specific schistosome antigens were observed. It is, however, not known whether any of the antigens identified in this study has protective significance. It has also been reported that protein antigens that cross-react with the different schistosome species may be the best candidate antigens for use in vaccines against schistosomiasis (Hillyer and Serrano, 1982). The routine procedure, therefore, is to identify potential antigens and test them for their ability to protect against infection (Mott, 1987; Simpson *et al.*, 1987). This indicates that the four MoAbs that bound *Schistosoma* genus-specific protein antigens (chapter 4, 5) may be worth further investigations so as to determine their protective ability.

In conclusion, this study provides information on the suitability of some of the anti-*S. haematobium* MoAbs in the development of field-applicable specific diagnosis of urinary schistosomiasis, and diagnosis of *Schistosoma* genus infections

in general. It also provides evidence that some of the MoAbs could be useful in the search for candidate antigens for use in a schistosomiasis vaccine.

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