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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *SCHISTOSOMA HAEMATOBIUM* SOLUBLE EGG AND URINE-BASED ANTIGENS

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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *SCHISTOSOMA HAEMATOBIUM* SOLUBLE EGG AND URINE-BASED ANTIGENS

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By

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January, 1995

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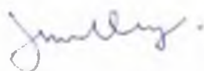
DECLARATION

I do hereby declare that except for references to other poeple'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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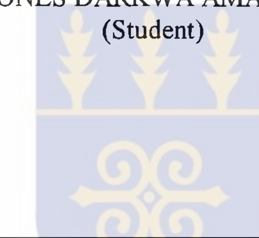

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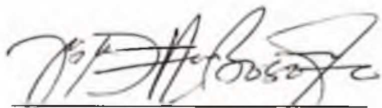

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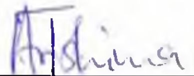

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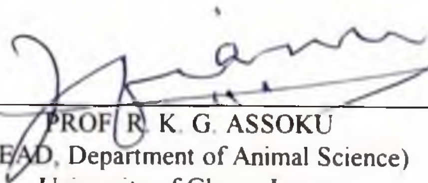

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DEDICATION

To my teachers

Prof. R.K.G. Assoku,

Dr. K. M. Bosompem
and
Dr. T. Arishima

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TABLE OF CONTENTS

Page No.

TITLE PAGE i

DECLARATION ii

DEDICATION iii

AKNOWLEDGMENTS iv

TABLE OF CONTENTS vi

LIST OF FIGURES x

LIST OF TABLES xi

ABBREVIATIONS xii

SUMMARY xiii

CHAPTER 1

INTRODUCTION

1.2 Objectives of the study 7

1.3 Justification 8

CHAPTER 2

LITERATURE REVIEW

2.1 Schistosomes and schistosomiasis 10

2.2 Classification and morphology of schistosomes 10

2.3 Distribution of Schistosomes and their intermediate hosts 12

2.3.1 The *S. haematobium* group 13

2.3.2 The *S. mansoni* group 14

2.3.3 The *S. japonicum* group 15

2.4 Schistosomiasis in Ghana 16

2.4.1 Population distribution of *S. haematobium* infection in Ghana 16

2.4.2 Population distribution of *S. mansoni* infection in Ghana 19

2.4.3 Distribution of the intermediate snail host of schistosomiasis in Ghana 19

2.5 The life cycle of schistosomes 21

2.5.1 The schistosome egg 22

University of Ghana http://ugspace.ug.edu.gh

2.5.2 The miracidium 23
2.5.3 The mother and daughter sporocysts 24
2.5.4 The Cercariae 25

2.6 Cercarial penetration and development of schistosomes in the definitive host 26

2.7 Consequences of schistosome infection - a general overview 27

2.8 Schistsome antigens 28
2.8.1 Schistosomula polypeptide surface antigens 29
2.8.2 Carbohydrate surface antigens 31
2.8.3 Schistosome egg antigens 32
2.8.4 Hepatotoxic egg antigens 34
2.8.5 Circulating schistosome antigens 35
2.9 Immunity to schistosomes 36

2.10 Diagnosis of schistosomiasis 39
2.10.1 The Parasitological diagnostic methods 40
2.10.1.1 Feacal examination for eggs of intestinal schistosomiasis 40
2.10.1.2 Direct faecal smear 40
2.10.1.3 The Formal-ether method 40
2.10.1.4 The Bell method 41
2.10.1.5 The Kato method 42
2.10.1.6 Examination for eggs of *S. haematobium* 42
2.10.1.7 Direct examination of urine 42
2.10.1.8 Urine filtration methods 43
2.10.1.9 Preserved urine samples 43
2.10.1.10 Demonstration of schistosome eggs in host tissue 44
2.10.2 Immunodiagnosis of schistosomiasis 44

2.11 Production of monoclonal antibodies (MoAbs) 48

2.12 Characterization of Monoclonal Antibodies 50

2.13 Usefulness of MoAbs in immunological studies 51

2.14 Vaccination against schistosomiasis 52

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Preparation of antigens 56
3.1.1 Fixation of miracidia with paraformaldehyde, acetone and ethanol 56

3.2 Myeloma Cell Lines and their Maintenance 57

3.3 Cell Fusion and Selection of Hybridomas 57

3.4 Screening, Cloning and Stabilation of Hybridomas 59

University of Ghana http://ugspace.ug.edu.gh

3.5 Propagation and Storage of Hybridomas 60

3.6 Purification of Monoclonal antibodies 62
3.6.1 Gel filtration 62

3.6.2 Ion exhcange chromatography 63

3.7 Preparation of enzyme antibody conjugates 63

3.8 Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) 65
3.8.1 Assembly of slab gel apparatus, and preparation of resolution and stacking gels 65
3.8.2 Preparation of samples and electrophoretic run 68

CHAPTER 4

STUDIES OF ANTI-*S. HAEMATOBIUM* MONOCLONAL ANTIBODIES. I: PRODUCTION

4.1 Introduction 70

4.2 Materials and methods 71
4.2.1 Mice 71

4.2.2 Preparation of parasite antigens 71
4.2.2.1 Extraction of crude schistosome worm, egg and hookworm egg antigens 71
Precipitation of proteins from *S. haematobium* infected human urine 72
4.2.3 Immunization of Mice for MoAb Production 72
4.2.3.1 Immunization with *S. haematobium* Soluble Egg Antigens (ShSEA) 72
4.2.3.2 Immunization with Antigens Extracted from the Urine of *S. haematobium* Infected Individuals 74
4.2.4 Screening of Immunized Mice for Antibody Activity 75
4.2.5 Antibody-detection Micro-plate ELISA 75
4.2.6 Dot-ELISA Procedure 76
4.2.8 Cell fusions, cloning and selection of hybridomas 77
4.2.9 Determination of Immunoglobulin Class and Subclass 77

4.3 Results 79
4.3.1 Cell fusion and selection of hybridomas 82
4.3.2 Anti-ShSEA MoAb Secreting Hybridomas 82
4.3.3 Anti- UP_2-IP MoAb Secreting Hybridomas 84
4.3.4 Cloning of MoAb Secreting Hybridomas and the Selection of Clones 84

4.4 Discussion 87
4.5 Summary 89

CHAPTER 5

STUDIES OF ANTI-*SCHISTOSOMA HAEMATOBIUM* MONOCLONAL ANTIBODIES.
II: CHARACTERISATION

University of Ghana http://ugspace.ug.edu.gh

5.1 Introduction 92

5.2 Materials and methods 94
5.2.1 Monoclonal antibodies 94
5.2.2 Western Blotting 94
5.2.3 Determination of the Biochemical Nature of Antigenic Epitopes 94
5.2.3.1 Detection of MoAbs specific for carbohydrate epitopes 94
5.2.3.2 Detection of MoAbs specific for protein epitopes 95
5.2.4 IFAT procedure 96
5.2.5 Dot-ELISA Procedure 97

5.3 Results 98
5.3.1 Anti-*S. haematobium* MoAbs selected for further characterization 98
5.3.2 Immunolocalization of *S. haematobium* antigens bound by the MoAbs 98
5.3.3 Reactivity of the selected MoAbs in the western immunoblot assay 98
5.3.4 Biochemical Nature of the Schistosome Antigenic Epitopes Bound by the MoAbs 101
5.3.5 Reactivity of the MoAbs with Antigens of Different Schistosome Species and Hookworm 101

5.4 Discussion 105

5.5 Summary5.4 108

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS 110

REFERENCES 116

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

Page No.

FIGURE 1 Extraction of UP_o P and UP_2-IP from *S. haematobium* infected human urine........ 73

FIGURE 2a Serum antibody responses of mice immunized with ShSEA as determined by reactivity with ShSEA........ 80

FIGURE 2b Serum antibody responses of mice immunized with UP_2-IP as determined with reactivity with UP_2-IP........ 81

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

Page No.

TABLE 1 Classification of schistosomes 11

TABLE 2 Summary of Cell fusions and hybridoma development experiments from spleen cells of BALB/c mice 83

TABLE 3 Selected MoAbs reactive with *S. haematobium* antigens as determined by micro-plate ELISA 86

TABLE 4 Immunolocalization of the *S. haematobium* antigens detected by the MoAbs by indirect fluorescent antibody test (IFAT) 99

TABLE 5 The molecular weights of the antigens detected by the *S. haematobium* reactive MoAbs as determined by Western Immunoblot analysis 100

TABLE 6 The nature of antigenic epitopes detected by the MoAbs as determined by periodate oxidation and proteinase-K digestion 102

TABLE 7 Reactivity of selected MoAbs with several antigens as determined by micro-plate ELISA and dot-ELISA 103

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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	Dymethylsulfoxide
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-hydrxyethyl-piperazine-N-2-ethane sulfonic acid
hr	Hour
HRPO	Horseradish peroxidase
HT-medium -	Hypoxanthine and thymidine medium
IFAT	Indirect immunoflourescenct 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	Nitrocellose
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

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SUMMARY

Diagnosis of schistosome infections is principally based on the demonstration of parasite eggs in excreta, or by haematuria and /or proteinuria. These methods are, however limited by inadequate sensitivity or specificity. Furthermore, even though microscopy is very specific, it is also tedious and time consuming.

The work reported in this thesis was conducted with the aim of producing monoclonal antibodies (MoAbs) against *Schistosoma haematobium* antigens, and to characterize them so as to determine if any of them would be useful in diagnosis, especially for detection of parasite antigens in the urine of infected persons. The availability of such MoAbs would pave the way for the development of more sensitive, specific and field-applicable immunological assays for diagnosis of urinary schistosomiasis caused by *S. haematobium*. The strategy employed in this study was to produce MoAbs, using *S. haematobium* soluble egg antigens (ShSEA) and infected human urine-based parasite antigens in protein extracts (UP_2-IP) as immunogens. Both antigens induced substantial serum antibody responses in immunized mice, with titres as high as 1:5,000 for ShSEA and 1:50,000 for UP_2-IP. The high immunogenicity of UP_2-IP was attributed to human immunoglobulins associated with parasite antigens in immune complexes. All MoAbs produced, using this antigen, were found to react with UP_2IP but not with ShSEA. Also, the MoAbs generated using ShSEA as immunogen did not react with UP_2-IP. However, one MoAb Sh5/32.30 reacted with both ShSEA

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and UP_2-IP. This MoAb was produced using both ShSEA and UP_2-IP as immunogen, but the final booster, just before cell fusion was made with ShSEA.

In all, six MoAbs were produced. These included two *S. haematobium* species-specific MoAbs (Sh2/15.F and Sh3/38.2), and four pan-schistosome MoAbs (Sh1/71.7, Sh3/15.28, Sh4/14.3 and Sh5/32.30) that cross-reacted with antigens in *S. haematobium*, *S. mansoni* and *S. japonicum* egg or adult worms. Three of the MoAbs were also found to detect *S. haematobium* antigens in urine of infected humans. Characterization of the antigens detected, using the indirect immunofluorescent test (IFAT), Western immunoblot analysis, proteinase-K digestion and periodate oxidation, showed that the two *S. haematobium* species-specific MoAbs bound different antigens (one protein and one glycoprotein), and at least three different antigens. The glycoprotein species-specific antigens had molecular weights (MW) of approximately 37kDa and 46kDa. It was also found that all the glycoprotein antigenic determinants detected were located on the surface membrane, as well as on intracytoplasmic organelles in *S. haematobium* miracidia, whilst the protein epitopes were located only on the surface membrane.

Of the six MoAbs that were produced and characterized, three of them bound protein epitopes, all of which were different from each other. One of the protein epitopes was detected by *S. haematobium* species-specific MoAb (Sh2/15.F), whilst the other two were detected by pan-schistosome MoAbs (Sh4/14.3 and Sh5/32.30). Interestingly, only the three protein antigens could be detected in the urine of *S.*

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haematobium infected persons. One of these protein epitopes, bound by Sh4/14.3, was shown to occur on different peptides of MW, 73kDa and 78kDa.

Cross-reactivity studies with soluble egg antigens of an Egyptian strain of *S. haematobium* revealed that only Sh2/15.F could not react with the north African parasite stain. This ability of most of the MoAbs to detect both Ghanaian and Egyptian strains of *S. haematobium* suggested that the MoAbs generated in this study might not only be useful for diagnosis of urinary schistosomiasis in Ghana, but also in other parts of Africa.

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CHAPTER 1

INTRODUCTION

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1.1 General introduction

Schistosomiasis is an economically important disease of man and livestock caused by trematode parasites of the genus *Schistosoma*. The disease is wide-spread and endemic throughout many parts of the world infested by the intermediate fresh water snail hosts belonging to the family Planorbidae. The affected areas include; Africa, Madagascar, S. America, India, Sri Lanka, South East Asia, China, Japan, Philipines, Taiwan, Indonesia and the Caribbean (Rollinson and Southgate, 1987), 75 countries in all. It is estimated that 250 million people are infected with schistosomes whilst another 600 million people are exposed to the risk of infection. In endemic rural areas of many developing countries, schistosomiasis is an important occupational hazard (Doumenge, Mott, Cheng, Villenave, Chapuis, Perrin and Reaud-Thomas, 1987).

Earliar reports indicated that, in Ghana, schistosomiasis was focal, occurring only in certain localized areas until the creation of the Volta dam in 1964 (Odei, 1964; Derban, 1983; Okoh, 1994). Today, schistosomiasis is known to occur in all regions of Ghana, especially among the riparian communities, some of which have registered prevalence rates as high as 100% for urinary schistosomiasis (Okoh, 1994). The creation of the Volta dams at Akosombo and Kpong, and the construction of dams and reservoires in various other parts of the country, have provided very siutable breeding sites for the Planorbid snail intermediate hosts, resulting in changes in the transmission patterns of the disease. The risk of transmission of bilharziasis continues to be

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aggravated as fishermen attracted by the Volta lake move from place to place while fishing and thereby contribute to the spread of the disease to previously uncontaminated areas. Both Odei (1975) and Okoh (1994) emphasized that this situation has made several water bodies in Ghana public health liabilities, thus undermining their economic importance.

The important schistosomes pathogenic to man are *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. mekongi*, and *S. intercalatum*. The disease in domestic animals is caused by *S. mattheei*, *S. curassoni*, *S. spindale*, *S. nasale*, *S. leiperi*, *S. indicum*, *S. bovis*, *S. incognitum* and *S. japonicum*. The first four schistosome species infect cattle, sheep and goats, whilst *S. leiperi* and *S. indicum* infect horses and camels respectively, in addition. *Schistosoma bovis* and *S. incognitum*, also called *S. suis*, are restricted to cattle and pigs respectively. *Schistosoma japonicum* is the only important schistosome pathogen of man which also infect domestic animals, namely, cattle, sheep and goats. On rare occasions, man may also be parasitised by the animal schistosomes, *S. bovis*, *S. curassoni*, *S. margrebowiei*, *S. mattheei* and *S. rodhainii* (Rollinson and Southgate, 1987).

In man, acute schistosomiasis may cause Katayama fever, anaemia, or fluid/electrolyte problems in severely toxaemic victims. A real threat of ectopic central nervous system involvement also exists during early infection (Lichtenberg, 1987). For these reasons, early anti-schistosomal treatment is important, as it helps to ameliorate

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the course of acute schistosomiasis. However, very few heavily infected patients have died from the acute disease.

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. It is known that a few egg patches at or near the ureterovesical junction can hinder urine flow. In general, pathological lesions may range from trivial to lethal (von Lichtenberg, 1987). On the other hand, chronic *S. mansoni* and *S. japonica*, are characterized by scattered granulomatous foci of the gut and liver, to full-blown bilharzial pipestem fibrosis of the liver. The risk of developing "bilharzial polyposis" and portal fibrosis remains benign until portal (venous) hypertension sets in.

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. Also, the determination of excess protein in urine (proteinuria) has been found to correlate well with *S. haematobium* infections, but it is less sensitive than some of the then known methods (Savioli and Mott, 1989).

The presence of blood or schistosome eggs in human stool specimen may indicate infection due to intestinal schistosomiasis be caused by either *S. mansoni*, *S. japonicum*, *S. intercalatum* or *S. mekongi*. Differential diagnosis is made possible by a

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consideration of the geographical distribution of the different schistosome species, and the morphology of the parasite eggs.

Diagnosis of schistosomiasis in man is, however, beset with several problems. Firstly, the identification of schistosome eggs in the urine or stool of infected persons is not sensitive enough, because of the great fluctuation of egg output and/or by the small numbers of eggs excreted. Secondly, the use of haematuria for the diagnosis of *S. haematobium* infections could be misleading since bloody urine could be the results of other conditions such as prostatic disease, genito-urinary tract infections and carcinoma of the genito-urinary tract (Goldsmith, 1985). Similarly, traces of blood in the stool of man, could be due to a variety of causes, including several gastro-intestinal tract infections, such as dysentery and hookworm infections (Savioli and Mott, 1989).

Schistosome infections in man can also be identified by serological tests based on the detection of host antibodies directed against schistosome antigens. Some of these tests have proven sensitive and specific. However, the presence of specific antibodies does not always indicate an active infection, since antibody titres remain positive for a long time after spontaneous or chemotherapeutic cure. Furthermore, antibody levels seldom show a good correlation with worm burden. Consequently tests have been developed to detect schistosome circulating antigens in the blood of infected hosts. The most promising of these tests is a monoclonal antibody (MoAb) based enzyme-linked immunosorbent assay (ELISA) which utilises two schistosome specific antigens: the circulating anodic antigen (CAA) and the circulating cathodic

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antigen (CCA) (Deelder, DeJonge, Boerman, Fillie, Hilberath, Rotmans, Gerritse and Schutte, 1989; Deelder, Miller, DeJonge and Krijger, 1990). However, none of these tests have been successfully adopted for application in routine diagnosis of schistosomiasis in the field.

The need for timely diagnosis and intervention of schistosomiasis in remote rural areas continues to call for the development of simple, accurate and field applicable assays. An attractive possibility in the case of human urinary schistosomiasis, would be to develop MoAb-based assays for detecting *S. haematobium* soluble egg antigens in patient urine. That this may be a feasible approach is evidenced by the works of Domingo and Warren (1968), Carter and Colley (1979) and Dunne and Doenhoeff (1983), in which the presence of immunogenic soluble egg antigens (SEAs) were demonstrated. Moreover, it has been shown by Inatomi (1962) that schistosome eggs possesses sub-microscopic egg-shell pores, and by De Jonge, Fillie, Hilberath, Krilger and Lengeleret (1989) that SEAs released into patient urine could be detected by MoAbs. Findings such as these led the WHO scientific working group (SWG) to recognise the advantage of supporting the incorporation of research on MoAbs into the overall strategy for schistosomiasis control (Bergquist, 1984).

Based on these observations, the work described in this thesis was conducted to produce and characterize monoclonal antibodies reactive to *S. haematobium* soluble egg antigens, with the objective that some of the antibodies may be useful in developing more specific and sensitive assays for urinary schistosomiasis.

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1.2 Objectives of the study

1.2.1 To produce monoclonal antibodies (MoAbs) against *S. haematobium* antigens.

1.2.2 To determine the specificity of the MoAbs through cross-reactivity studies with *S. haematobium*, *S. mansoni*, *S. japonicum* and *Necator americanus* (hookworm).

1.2.3 To investigate the ability of the MoAbs to detect urine-based *S. haematobium* antigens.

1.2.4 To characterize the antigens detected by the monoclonal antibodies using western immunoblot analysis and biochemical studies, and to localize them using immunocytochemical studies.

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1.3 Justification

The production and characterization of MoAbs that detect *S. haematobium* urine-based antigens, would provide useful information on the schistosome antigens identified, and facilitate the development of alternative diagnostic assays that employ MoAbs in the detection of parasite antigens in patient urine. Furthermore, some of the identified antigens may prove useful in studies relating identification of protective molecules and development of a vaccine against schistosomiasis.

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CHAPTER 2

LITERATURE REVIEW

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2.1 Schistosomes and schistosomiasis

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. The disease, bilharziasis was later named after him. Elucidation of the schistosome life cycle, however, was not made until 1913 when Miyairi and Suziki (cited by Rollinson and Southgate, 1987) showed that *S. japonicum* developed in the hydrobid, snail *Oncomelania hupensis nosophora*.

2.2 Classification and morphology of schistosomes

Schistosomes are trematodes which belong to the family Schistomatidae (Webbe, 1982). Members of this family are dioecious Digenea, parasitic in the blood-vascular system of vertebrates. A general feature of the family is that the mature female is more slender than the male and is normally carried in a ventral groove called the gynaecophoric canal, which is formed by ventrally flexed lateral outgrowth of the male body (Rollinson and Southgate, 1987). Of the twelve genera within the family (Table 1), seven are confined to birds and five to mammals, but only the genus *Schistosoma* is associated with man. Epidemiological studies have shown that the *Schistosoma* has achieved the greatest geographic distribution and diversification in terms of members of recognized species and different hosts parasitized (Rollinson and Southgate, 1987).

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

Rank	Taxa
Kingdom	Animalia
Subkingdom	Metazoa
Phylum	Arthropoda, Nematoda, Mesozoa, Platyhelminthes, Acanthocephala, Pentastomida, Annelida
Class	Aspidogastrea, Monogenea, Digenea, Cestoda, Turbullaria
Superorder	Epitheliocystida, Anepitheliocystidia
Order	Strigentida, Echinostomati
Family	Diplostomatidae, Bucephanidae, Schistosomatidae, Spirochidae, Cyclocoelidae, Strigeidae
Subfamily	Gigantobilharzinae, Schistosomainae, Bilharziellinae
Genus	Omitho-bilharzia, Micro-bilharzia, Oriento-bilharzia, Austro-bilharzia, Schisto-soma, Schistoso-matium, Bivetello-bilharzia, Hetero-bilharzia
Species	hippopotani, rodhaini, leiperi, matheei, japonicum, mansoni, haematobium, intercalatum, bovis, indicum, curassoni, margrebowiei, spindale, incognitum, mekongi, sinensium, nasale

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The genus *Schistosoma* differs from most digenetic trematodes in being dioecious, a consequence of heteromorphic chromosomes in the ovum (Rollinson and Southgate, 1987). Thus a population of schistosomes in the final host may be unisexual (male or female) or mixed, comprising both male and female worms. In nature, however, the latter situation is the most frequent, resulting in the pairing of males and females and the production of eggs (Erasmus, 1987).

The three principal schistosome species known to infect man are *Schitosoma haematobium, S. mansoni* and *S. japonicum*. *Schitosoma haematobium* causes urinary schistosomiasis which is also known as schistosomiasis haematobia or bilharziasis. *S. mansoni*, on the other hand, causes interstinal schistosomiasis also known as schistosomiasis mansoni, and likewise *S. japonicum* causes intestinal schistosomiasis referred to as schistosomiasis japonica or oriental schistosomiasis (Bergquist, 1987). The schistosomes are generally placed in 15 different groups which Kuntz (1955) referred to as species complexes.

2.3 Distribution of Schistosomes and their intermediate hosts

Prevalence rates of schistosomiasis vary widely between different areas, and even, between localities within a single endemic area. The highest rates are found near large bodies of fresh water, where up to 100% of the population may be infected. According to Bergquist, (1987) the highest infection rates are found in Brazil, Egypt and Ghana.

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2.3.1 *The S. haematobium group*

The distribution of *S. haematobium* is confined to Africa and some adjacent regions, extending through Arabia to the Khuzestan Province of Iran and to the Indian Ocean Islands of Madagascar and Mauritius (Rollinson and Southgate, 1987). There is evidence for geographical variation in many characters associated with this species. Thus 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 eggs in infected host organs (Wright and Knowles, 1972).

In general, *S. haematobium* in the tropical regions of Africa is transmitted by snails of the *Bulinus africanus* group. In the Mediterranean area and the South West of 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 for *S. haematobium*, 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).

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Related to *S. haematobium* are other African schistosomes which differ in the shape of their eggs and in their definitive hosts. These include the widely distributed *S. bovis* of cattle (Majid, Bushara, Saad, Hussien, Taylor, Dargie and Marshall, 1980); *S. matheei* of cattle, sheep and goats in South Africa (Rollinson and Southgate, 1987); *S. intercalatum* of man in Zaire; *S. curassoni* of ruminants and *S. margrebowiei* which infect a wide range of hosts (Rollinson and Southgate, 1987).

2.3.2 *The S. mansoni group*

Schistosoma mansoni is the most important parasite that causes intestinal schistosomiasis in man in both the New and Old Worlds. The parasite occurs in Oman, Saudi Arabia, Yemen, Poeple's Democratic Republic of Yemen, Libya and Madagascar, and it is 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 due to *S. mansoni* is found in Brazil, Surinam, and Venezuela (Rollinson and Southgate, 1987).

Of seventeen well-defined species of *Biomphalaria* in the Americas, only *B. glabrata* and *B. straminae* have been found naturally infected with *S. mansoni*. Other species such as *B. amazonica* (Correa and Paraense, 1971) and *B. peregrina* (Paraense and Correa, 1973) have been found to be susceptible to infection in the laboratory. Snails from different geographical areas tend to show variation in the levels of susceptibility to different strains of *S. mansoni* (Basch, 1976; Michelson and Dubois,

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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, whereas species such as *B. alexandria* appears to be susceptible only to the local *S. mansoni* from Egypt (Frandsen, 1978).

Other schistosome species found mainly in South East Asia are also placed in the same group as *S. mansoni*. They include *S. rhodaini* which infects rodents and dogs (Pitchford, 1977), *S. edwardiense* and *S. hippopotami* of hippopotamus (Pitchford and Viser, 1981), *S. indicum* of equines and a variety of other domestic animals on the Indian subcontinent (Montgomery, 1960), *S. spindale* of ruminants (Montgomery, 1960), *S nasale* of cattle , sheep, goats and buffaloes in India and surrounding regions (Dutt and Srivastava, 1968) and *S. incognitum* of pigs and humans in South East Asia (Ahluwala and Dutt, 1972).

2.3.3 *The S. japonicum group*

The disease caused by *S. japonicum* is widespread in the Far East where it is endemic in parts of China. *S. japonicum* also occurs on the Philippine Islands of Leyete, Samar, Mindanao, Bohol, Mindoro and Luzon. In Japan, the parasite is limited to three main areas, namely Kufu, Katayama and the basin of

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the Chikugo River. It is also found in parts of Taiwan and Indonesia (Rollinson and Southgate, 1987).

2.4 Schistosomiasis in Ghana

Urinary schistosomiasis was first reported in Ghana in the year 1895, whereas intestinal schistosomiasis was not identified until 1920 (Furu, 1987). However, until 1952, data on the distribution of schistosomiasis in Ghana was derived mainly from hospital records (Furu, 1987). Studies conducted after this period now form the basis for current available information. McCullough (1965), Paperna (1969) and Wen and Chu (1984) studied and reported on the distribution of the vector snails, as well as urinary and intestinal schistosomiasis. Paperna (1969) and Odei(1984) also considered the ecology of schistosomiasis transmission and the distribution of both aquatic weeds and the vector snails in the new man-made Volta Lake in Ghana. According to Furu (1987), both urinary and intestinal schistosomiasis were widely distributed in Ghana. Schistosomiasis haematobia was present in all the regions of the country whilst schistosomiasis mansoni was concentrated in the northern and southern quarters.

2.4.1 *Population distribution of S. haematobium infection in Ghana*

It was estimated in 1963 (Furu, 1987) that 15 to 20% of the inhabitants of Ghana were infected by *S. haematobium* at some time or other

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during their lives, often during childhood. The two most endemic areas were in the North-eastern and in the South-eastern parts of the country. High prevalence rates were reported in localities along the frontier with Cote d'Ivoire and the Central part of Ghana. The number of endemic areas identified was observed to increase steadily after 1965 (Furu, 1987). In most regions, prevalence rates in excess of 60% were recorded in the years 1970-1980.

Lake Volta, with its 5000 km of shoreline, was completely filled in 1968 and surveyed for the first time in the 1970s following the observation that *S. haematobium* infection was spreading (Furu, 1987). The surveys revealed high urinary schistosomiasis prevalence among peoples living near the shores of the new lake even though earlier studies carried out by Medical Field Unit had indicated that schistosomiasis was absent in many populations living traditionally on or close to the Volta river. Similarly, in areas where transmission had been present for some time, the number of foci was found to have increased (Tagoe, 1965; Bergquist, 1987). An extensive epidemiological survey carried out between 1959 and 1961 in the Upper Regions of Ghana showed that the eastern part was markedly more affected than the western part. The prevalence of *S. haematobium* infection was over 30% in 19 out of 20 districts in the eastern part, and less than 30% in 16 out of the 18 districts in the western part (Hunter, 1981). The zone most affected by urinary schistosomiasis had prevalence above 50%. This area was bounded in the east by the White Volta and in the west by the Sisili river.

Paperna (1969) reported that the Northern region of Ghana had low schistosomiasis prevalence rates mostly below 5% before the filling of the Volta

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Lake. However, after the formation of the lake, very high prevalence rates greater than 50% have been recorded in most places in the north. A complete assessment of the prevalence among the shore dwelling populations of Lake Volta carried out in 1982 also indicated that the eastern part of central Ghana had become an important endemic area (Klumpp, 1982). The prevalence varied between 38% and 96.7% among the peoples settled in these areas. Scott, Senker and England (1982) recorded an average prevalence rate already in excess of 83.9% among the shore-dwelling peoples of the lower reaches of the Afram branch, and among those living along the Pawmpawm branch of the lake.

In the area below the Akosombo dam and throughout the south east of Ghana, *S. haematobium* infection has become endemic (Bergquist, 1987 and Furu, 1987). In 1969, transmission of *S. haematobium* was significant around the marshes and ponds connected with the Volta river. Prevalence in excess of 75% was recorded in all localities surveyed (Paperna, 1968). The prevalence was inversely proportional to the distance from transmission sites. This observation was confirmed in the Volta river delta, as well as, in the Eastern Region, where prevalence of *S. haematobium* was low (Paperna, 1969). Paperna (1969) also reported that, the prevalence in the Tafo forest sector was higher in the primary schools of New Tafo, located near the main site of transmission than in those of Old Tafo, which were farther away. Teenagers in secondary schools in Old Tafo, however, had a higher prevalence than their counterparts in primary schools because of their more extensive water contact.

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Around the city of Accra, epidemiological surveys conducted in the years 1966-1967 revealed extremely variable prevalence rates which were low at Madina or Ofankor (13%), but high at Pokuase and Mayera (70%), and Ashiaman (84%). In the Accra region, the prevalence was 30% in 1966 and 84% in 1967.

2.4.2 *Population distribution of S. mansoni infection in Ghana*

Intestinal schistosomiasis due to *S. mansoni* is far less prevalent in Ghana than urinary schistosomiasis. In 1955, only the north-east of the country was known to be an endemic zone and the overall prevalence was only 2.4%. Ten years later McCullough and Ali (1965) reported five foci in the Upper Region, four of which were to the east of the Sisili river and one in the vicinity of the Black Volta. They also reported two foci in the south-west (Western Region), two others in the south-east (Volta Region) and one in the Ashanti Region. Most of the prevalence rates recorded were low, between 5% and 7%. Until 1965, *S. mansoni* had not been reported in the vicinity of the new Lake Volta. However, Wen and Chu (1984) identified four new foci in the area between the Akosombo dam and the mouth of the river Volta.

2.4.3 *Distribution of the intermediate snail host of schistosomiasis in Ghana*

The natural conditions for the development of the snail intermediate hosts of schistosomes are ideal over large parts of Ghana. *Bulinus globosus*, one of the principal snail hosts of urinary schistosomiasis, was present in three-quarters of

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the country before the filling of the Volta lake. This included both forest and savanna areas. Furu, (1987) observed that this snail was particularly common in the ponds and streams of the Volta plateau in the north-east, in the water collected on the Kwahu plateau in the south-west and in the Togo ranges as well as in the coastal plains from the Togolese frontier to Axim. On the other hand, *B. globosus* was practically absent from the region now occupied by Lake Volta. According to McCullough (1965), the porous nature of the mineral substrata accentuated the effects of the lack of water during the dry season and further limited the propagation of the snail. Since the creation of the lake, the snail has established itself only in the lower valley of Obosum in the Volta Region (McCullough, 1965).

The other intermediate snail host of *S. haematobium* found in Ghana *B. truncatus rohlfsi* was far more restricted in its distribution before the filling of the lake. This snail species was totally absent from the forest zone. It's presence was recorded in the Upper Region in the Wa area and between Bolgatanga and Bawku, in the valleys of the Black and White Volta; in the Northern region, from Tamale to Kete Krachi; in the Volta region, in the Dayi river valley, and lastly and most importantly, in the lower valley of the delta of the Volta river, from Akosombo to Agave, especially behind the Keta lagoon (Furu, 1987).

Biomphalaria pfeifferi, the intermediate snail host of *S. mansoni*, had a distribution similar to that of *B. globosus*. The snail proliferated both in the savanna of the Upper region and the forest zone of the south-west. Furthermore, *B. pfeifferi* was identified in the Togolese Ridge. This snail was, however, not found in the Volta Lake (Furu, 1987).

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Generally, the snail intermediate hosts of schistosomiasis referred to above are found in small water courses, flood-water ponds and the fresh water mashes of the Volta delta and the coastal plain. These snails are characteristically absent from the salt water mashes and fast-flowing rivers. *B. globosus* is found mostly in permanent marshes and slow-flowing water courses particularly in the forest zone and the coastal plain. *B. truncatus* on the other hand is found mostly in the area between the confluence of the Black and White Volta and the Volta delta.

The rapid growth of water plants, especially *Pistia stratoites, Spirodela polyrhiza and Ceratophyllum demersum*, in the Volta Lake has promoted the proliferation of *B. truncatus rohlfsi* (Paperna, 1969) and hence the spread of *S. haematobium*. *Ceratophyllum* is plentiful in the Afram and Dayi branches of the Volta Lake and along the Oti and White Volta rivers. It is found up to a depth of 4.5m normally in association with tree trunks left in situ in the inundation of the original Volta valley (Odei, 1975). *Pistia* and *Spirodela*, on the other hand, often form floating islands that drift around the lake. In so doing, these plants contribute to the spread of schistosomiasis through dispersion of the snails and their eggs.

2.5 The life cycle of schistosomes

Schistosomes undergo an alternation of generations with sexual reproduction taking place in the definitive host (man and other mammals) and asexual reproduction in the intermediate snail host. Schistosome eggs pass out of

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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 a compatible snail species which they penetrate few hours after hatching from the egg. Within the tissues of the snail, these organisms develop over a month into mother sporocysts, which then develop into thousands of fork-tailed free-swimming larvae called cercariae. The cercariae are released in daily bursts over a fairly long period that may last several months if the snail survives the infection. It is the cercariae which is capable of infecting man.

2.5.1 *The schistosome egg*

Schistosome eggs are generally oval in shape and non-operculate. They are characterized by a pointed expansion of the egg 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. Kussel (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 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, blood pressure, peristalsis and proteolytic enzymes secreted by the miracidia inside the

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egg 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.

2.5.2 *The miracidium*

The miracidium represents the first free larval stage in the life-cycle of schistosomes. This larval stage ensures transmission between the vertebrate and the snail which it actively penetrates. Electron microscopy studies by Pan (1965), Schutte (1974a) and Eklu-Natey, Wuest, Swiderski, Striebel and Huggel (1985) have thrown more light on the morphology of the miracidium. A newly released miracidium is generally pyriform, and varies between 150 and 180 µm in length and 70 and 80 µm in width. The body of this larval stage is covered by 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

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least 12 ciliated sensory organelles. The secretary 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. The mechanism by which miracidia seek to penetrate susceptible snail hosts have been investigated by several workers. For example, Jourdane and Theron (1987) reported that physical stimuli in the aquatic medium appear to favour movement of miracidia to the part of the biotope where the host snails are most likely to be found. The parasite larvae then home in by responding to chemical stimuli released from the snail host (Chernin, 1970; Sponholtz and Short, 1975).

Though it is generally accepted that penetration of miracidia into snail tissues occurs by a mechanical action, the role of histolytic secretions of the glandular cells of the miracidia could be implicated (Jourdane and Theron, 1987). In *S. mansoni*, *S. haematobium* and *S. intercalatum,* penetration occurs mostly via the foot of the snail (Jourdane and Xia, 1987).

2.5.3 *The mother and daughter sporocysts*

Following penetration of the snail tissues, the miracidium, undergoes morphological transformations which culminate in the formation of mother sporocysts. Schutte (1974b) studied the morphogenesis of the mother sporocysts

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of *S. mansoni* and reported that some of the early changes that occur with the miracidium upon penetration are the loss of epidermal ciliated cells, loss of the musculature and loss of the papillae. By the fourth day, the sporocyst is amoeba-like in shape. It gradually takes on the shape of a long sac which becomes folded and rolled back on itself and which is closely bound to snail tissues.

The germinal cells of the miracidium then undergo a series of multiplication and differentiation to form daughter sporocysts within the mother sporocysts. By the end of their differentiation the mother sporocysts look like vermiform larvae, measuring 150-250 μm in length (Jourdane and Theron, 1987).

Daughter sporocysts, leave the mother sporocyst by breaking through the tegumentary wall. Ten to seventeen days afterwards, the daughter sporocysts migrate towards the digestive gland of the snail host. This migration is either passive through the host's circulatory system (Becker, 1970; Meuleman, 1972; Schutte, 1974b) and/or active through the host's loose connective tissues (Pan, 1965).

2.5.4 *The Cercariae*

Germinal cells contained in the cavity of daughter sporocysts only start to differentiate into cercariae after the sporocysts have reached their permanent location in the digestive gland of the snail (Cheng and Bier, 1972). With the exception of *S. japonicum* for which cercarial production can stop for a period and restart, the production of cercaria in other species of schistosomes occur daily and sometimes for periods exceeding eight months (Jourdane and Theron, 1987).

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2.6 Cercarial penetration and development of schistosomes in the definitive host

Schistosome cercariae may remain infective for approximately 20 hours after emergence from the snail and can penetrate the unbroken skin of man and other mammals within minutes. 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. 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. 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. Approximately half of these eggs are excreted from the body, and the remainder remain trapped in the tissues (Warren, 1973).

2.7 Consequences of schistosome infection - a 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

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humans has been estimated to average 3.5-12 years with some worms surviving for 30 years or longer (Vermund, Bradley and Ruiz-Tiben, 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 in humans peaks around age 15-17 and then decreases with increasing age (Mott, 1987).

The schistosome parasites (adult worms) have an ability to evade the host's immune system, which probably, explains 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, direct immunosuppressive effects and the induction of humoral and cellular immunoregulatory mechanisms, including anti-idiotypic networks (Bloom, 1979; Colley and Colley, 1989).

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. 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

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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 is well established that the lesions that result from schistosome infections are largely caused by the parasite eggs, rather than the worms.

Erasmus (1987) observed that much of the pathology in schistosomiasis is the result of host immunological responses to the accumulation of parasite eggs in host tissues. Indeed with *S. haematobium* most of the eggs laid by the female worm in the host fail to reach the lumen of the gut or urinary tract. Instead, they get trapped in the wall of the viscus or swept on into the portal radicles or lung arterioles (Cheever, 1969).

2.8 Schistsome antigens

During a normal infection, the schistosome presents a complex array of antigens to its host, with major antigenic stimuli believed to come from the adult worm and the eggs (Kelly, 1987). Studies conducted by several workers including Kusel, Sher, Perez, Clegg and Smithers (1975), Haguya, Murrel, Taylor and Taylor (1979),

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Dissous, Dissous and Capron (1981), Arostein and Strand (1983) and Norden and Strand (1984) on antigens recognized by sera from patients infected with *S. mansoni, S. haematobium,* or *S. japonicum* revealed about 20-30 antigenic polypeptides of adult worms and about 20 glycoprotein egg antigens of each of the three schistosomes species. In the case of the glycoproteins, extensive cross-reactivity was observed. Thus, for example, sera from patients infected with *S. haematobium* precipitated all but three of the antigens recognized by *S. mansoni* infected sera. A slightly lower degree of cross-reaction was observed using sera from patients infected with *S. japonicum*.

2.8.1 *Schistosomula polypeptide surface antigens*

The existence of a set of polypeptide antigens on the surfaces of schistosomula has been demonstrated by radioiodination of intact live schistosomula, followed by immunoprecipitation with a variety of antisera (Dissous *et al*., 1981; Smithers, Simpson, Yi, Omer-Ali, Kelly and McLaren, 1987). However, Dissous, Gryzch and Capron (1982) confirmed earlier works by some including Dissous *et al.* (1981) and Smithers *et al.* (1987) that only a subset of the polypeptides available for surface labelling was recognized as antigenic by infected animals.

In various studies, Ramasay (1979), Snary, Smith and Clegg (1980) and Smithers *et al.* (1987) identified polypeptides of M_r less than 100,000 by lactoperoxidase-catalased iodination labelling. Simpson and Smithers (1985) also studied schistosomula surface antigens. They used immunoprecipitation techniques utilizing either lactoperoxidase- or iodogen-catalyzed radioiodination and showed that

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sera from chronically infected mice recognized 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, 92,000, 38,000-32,000, 20,000, 17,000 and 15,000. Additionally, both sets of sera precipitated an antigen of M_r 22,000 following lactoperoxidase but not iodogen-catalyzed iodination. Using another labelling technique, the diazonium salt of ^{125}I iodosulphanilic acid, Taylor, Haguya, and Vannier (1981) identified three major schistosomula surface antigens of M_r 15,000, 28,000 and 69,000.

Several schistosomulum surface antigens have also been identified using monoclonal antibodies which bind to the surface of live schistosomula as determined by immunofluorescence. Using such a monoclonal antibody, Harn, Mitsuyama, Haguenel and David (1985) precipitated an antigen of M_r 22,000 from solubilized schistosomula surface membrane-enriched fractions labelled with the Bolton-Hunter reagent. Dissous *et al.* (1982) also produced a monoclonal antibody which precipitated an antigen of approximate M_r 38,000. This antigen was shown to be recognized during chronic infections of both rodents and man.

Using iodogen-catalyzed labelling followed by immunoprecipitation with human immune sera, Simpson and Smithers (1985) identified the major surface antigens of *S. haematobium* schistosomulum to be of M_r 17,000 and a complex of M_r 24,000-30,000. These appeared species-specific since they were not recognized by human anti-*S. mansoni* serum. Sera from vaccinated mice or rabbits have also been successfully used to precipitate labelled *S. haematobium* antigens of M_r 94,000, 38,000, 26,000 and 10,500.

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2.8.2 *Carbohydrate surface antigens*

In studies with schistosome antigens the emphasis has been laid on protein or glycoprotein antigens. This is 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, 1981). 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 $M_r >$ 200,000, 38,000, 32,000 and 17,000. It is, however likely that some of these epitopes could also be present on other membrane components such as glycolipids (Kelly, 1987). Indeed Weiss, Magnami and Strand (1986) have demonstrated the presence of antigenic glycolipids in eggs, cercariae and adult worms of *S. mansoni*.

In studies with non-species-specific monoclonal antibodies that bound carbohydrate antigenic epitopes in glycoprotein antigens, Yi, Omer-Ali, Kelly,

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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. However, the immunogenic stimuli for concomitant immunity could 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 (Gryzch, Capron, Lambert, Dissous, Torres and Dissous, 1985; Yi *et al.*, 1986; Capron, Pearce, Balloul Gryzch, Dissous, Sondermeyer 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).

2.8.3 *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 (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

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eggs (Boros and Warren, 1970; Hang, Warren, and Boros, 1974). Warren (1972) showed that the soluble egg antigens (SEA) which is the supernatant of ultracentrifuged egg homogenate can elicit granulomatous hypersensitivity and other immunologic reactions characteristic of intact eggs. This crude SEA preparation was shown by Carter and Colley (1978, 1979) and 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; Boros *et al.*, 1977; Carter and Colley, 1979). Furthermore Cater 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_1, MSA_2 and MSA_3 and their molecular weights estimated by gel filtration to be 50, 450 and 80 kDa respectively.

Hamburger *et al.* (1976) investigated the specificity of these antigens using antigen-binding radioimmunoassay and showed that antibodies that reacted with MSA_2 and MSA_3 showed cross-reaction with cercarial, but not adult worm antigens of *S. mansoni*. However they both cross-reacted with SEA from *S.*

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japonicum and *S. haematobium*. In contrast, MSA_1 appeared to be both egg stage- and species-specific. Further investigations with MSA_1 suggested that it may be a major immunopathological egg antigen (Kelly, 1987).

Three glycoprotein antigens of *S. japonicum* were also identified by Tracy and Mahmoud (1982) 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, Boros, Hang, and Mahmoud (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 900,000 from SEA of *S. japonicum*. The antigen showed eosinophilic chemotactic activity which appeared to be dependent on the integrity of the carbohydrate moiety.

2.8.4 *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

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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 a non-glycosylated polypeptide of approximate M_r 22-26 kDa. This antigen appeared to be stage-specific and has proved a valuable immunodiagnostic reagent (Kelly, 1987).

2.8.5 *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 (Nantulya, Musoke, Rurangirwa and Moloo, 1984), 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).

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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 it is heat-stable and dialyzable. 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) who also showed the presence of CAA in the urine of infected hamsters and humans.

2.9 Immunity to schistosomes

Elucidation of the mechanisms involved in the immune response in schistosomiasis has been greatly facilitated by investigations with the animal model in the laboratory. This approach has been widely used because unlike

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other human parasites such as those that cause malaria and filariasis which have stricter host specificity, the principal human schistosomes are capable of infecting a range of laboratory animals (McLaren and Smithers, 1987). Some of the animals that have been extensively used are the rat, guinea pig, mouse and some primates. It is, however, necessary to mention that difficulties in maintaining the life cycle of *S. haematobium* under laboratory conditions has slowed down work with this parasite.

Despite the obvious differences with pathology and immunity to schistosomes in man, the animal experiments have played a pivotal role in the accumulation of data contributing to an understanding of human schistosomiasis. For example, it is now established that immunocompetent mammals may become immune to reinfection following either chronic infection or exposure to radiation-attenuated cercariae (Kelly, 1987). Furthermore, several antigens that play important roles in the acquired immunity have been identified.

Immunity induced following experimental infection and that obtained by the use of attenuated cercariae have been shown to differ in several ways. For example, resistance following exposure to normal cercariae develops about the time egg-laying commences and peak immunity is reached some 4-6 weeks later (Smithers and Terry, 1967), but with attenuated cercariae, immunity commences about two weeks following the death of larval schistosomula along their migratory pathway. It reaches a peak in about 5 weeks and remains indefinitely high. In the *S. mansoni*/mouse model, resistance has been shown to correlated with the presence of eggs in the tissues or with factors related to egg-associated pathology

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such as the degree of portal hypertension (Dean, Bukwoski,and Cheever, 1981). Nevertheless, intrahepatic transfer of adult worms into naive rhesus monkeys, baboons and mice has shown that immunity may be induced by the adult worm stage without a necessary prior exposure to cercariae or schistosomula (Smithers and Terry, 1967; Webbe, James, Nelson, Smithers and Terry, 1976; Peresan and Cioli, 1980). This immunity has been referred to as "concomitant immunity" because in this situation, schistosomula of challenge infection are destroyed whilst the adult worms of the primary infection remain unharmed (Smithers, Simpson, Yi, Omer-Ali and Kelly, 1987). In contrast, immunity following exposure to irradiated cercariae is called "vaccine immunity" 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 Doenhoff, 1982), whereas vaccine immunity is species-specific (Moloney and Webbe, 1987). Sher, Heiney, James and Asofsky (1982) demonstrated that vaccine immunity is based on both T-cell and B-cell responses. On the other hand, it has been suggested that concomitant immunity, is dependent on non-immunological factors, perhaps involving vascular changes in the liver which affect the circulatory patterns of challenge schistosomula (Dean *et al.*, 1981; Harrison, Bickle and

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Doenhoeff, 1982; Wilson, Coulson, and Metting, 1983). However, James and Cheever (1985) observed from their experiments that a major part of the resistance associated with infected mice is immunologically based. They showed that "P" strain mice which have a genetic defect in macrophage function develop poor levels of both concomitant and vaccine immunity despite the fact that infected "P" strain mice show normal levels of egg induced pathology. Also studies with a monoclonal antibody NIMP/R.41 which binds and destroys mouse neutrophils have shown that depletion of these cells abrogates dermal inflammatory response to schistosomula leading to the suppression of both vaccine and concomitant immunity.

2.10 Diagnosis of schistosomiasis

More simple, rapid, sensitive and reproducible diagnostic tests for schistosomiasis are needed 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, 1978). The currently available schistosomiasis diagnostic tests may be divided into three categories. Firstly, there are the parasitological methods which are based on microscopic demonstration of parasite eggs in host excreta or tissues. Secondly, there are serological assays based on the detection of anti-parasite antibodies or circulatory parasite antigens, and thirdly there are imaging techniques for demonstration of pathological changes resulting from the

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disease (Peters and Kazura, 1987).

2.10.1 The Parasitological diagnostic methods

2.10.1.1 *Feacal examination for eggs of intestinal schistosomiasis*

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

2.10.1.2 *Direct faecal smear*

Approximately 2mg of stool is emulsified following the addition of one or two drops of 0.9% saline solution on 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 the visualization of the parasite eggs. For these reasons, Peters and Kazura (1987) concluded that although the method is simple, it is mainly for identification of heavily infected persons.

2.10.1.3 *The Formol-ether method*

This method which leads to the preservation of specimen is also simple to perform (Garcia and Shimuzi, 1981). 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

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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. The method is semi-quantitative and, therefore, not suitable for accurate determination of intensity of infection.

2.10.1.4 *The Bell method*

The Bell method for the detection of schistosome eggs in faeces was the first to be extensively used in field studies (Bell, 1963; Teesdale and Amin, 1976; Jordan, Batholomew 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 mixture 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, distilled water is placed on the paper to improve the refractive index.

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2.10.1.5 *The Kato method*

This technique is generally useful for detecting the eggs of the intestinal schistosomes as well as of intestinal parasites in stool specimen. In the standard Kato method, a sample of stool is picked up with a wooden spatula and forced through a stainless steel screen to remove particulate and fibrous material. The specimen is then applied to fill a hole in a metal 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 microscopic examination for parasite eggs. This method is rapid and suitable for large epidemiological surveys in which sensitivity needed to detect light infections is not crucial.

2.10.1.6 *Examination for eggs of S. haematobium*

2.10.1.7 *Direct examination of 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 gravity.

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2.10.1.8 *Urine filtration methods*

With these techniques, urine is passed through a suitable membrane filter that traps schistosome eggs. The eggs are 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 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.

2.10.1.9 *Preserved urine samples*

In field situations where fresh urine samples cannot be processed immediately within a few hours, the excreted eggs may be preserved by adding an equal volume of 0.002% carbolfuchsine which consists of 100ml absolute ethanol, 50ml phenol, 850ml distilled water and 0.02 grammes of carbolfuchsine. 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 no. 1 Whatman filter paper attached to a vacuum apparatus. Air-dried filters are

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placed egg side up under a microscope and examined. It has been reported that concentration of preserved urine by centrifugation is more sensitive than by filtration.

2.10.1.10 *Demonstration of schistosome eggs in host tissue*

The absence of schistosome eggs in feacal specimen do not necessarily exclude the possibility of infection (Peters and Kazura, 1987). This is because eggs may not be found in the faeces but they may be present in various host tissues such as the rectal mucosa, liver, lung, 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 1-2nm snips of tissue which is subsequently sandwitched between two glass slides and examined for parasite eggs by microscopy.

2.10.2 Immunodiagnosis of schistosomiasis

Parasitological diagnosis by microscopic examination of stools or urine for parasite eggs has been the most widely accepted method of identifying individuals with schistosomiasis (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

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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, therefore, not surprising that the quest for simpler, more rapid, specific and sensitive quantitative field detection techniques for the diagnosis of schistosomiasis remains. An attractive possibility is the use of immunological diagnostic techniques (Sendo and Saito, 1991; WHO, 1992).

Virtually all the well-established immunological assays 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 applied to identification of schistosome species and the diagnosis of schistosomiasis (Kagan, 1968). however, very few of them have been advocated for large-scale use in diagnosis. Lack of sensitivity appears to be an important limitation in these applications. For example, complement fixation tests employing extracts of lyophilized worms were comparatively as specific as

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immunofluorescence utilizing adult worm sections, yet they could detect antibody in only 70% of infected cases investigated (Reviewed by Smithers and Doenhoff, 1982).

Several tests have also been developed for purposes of diagnosing schistosomiasis. One of the most important of these assays is the Circumoval Precipitin Test (COPT) introduced by Oliver-Gonzales (1954). The COPT which was established with fresh *S. mansoni* eggs was, made simpler by the use of lyophilized eggs by Tanaka, Matsuda, Blas and Nonsenas (1975). This technique was made even more applicable under field conditions in the tropics following the introduction of the use of air-dried schistosome eggs (Kamiya, 1983).

Both the ELISA and radioimmunoassy have improved the sensitivity of serodiagnostic procedures for schistosomiasis (Smithers and Doenhoff, 1982). However, ELISA has several advantages over radioimmunoassay. These advantages include the stability of antigen-antibody complexes and the need for no radioactive substances. 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 earlier infections. On the other hand, using the MSA_1 antigen obtained from *S. mansoni* eggs, it has been shown that the radioimmunoassay was stage-specific and more sensitive in detecting parasite-specific antibodies in chronic infections (Hamburger, Pelley and Warren, 1976; Hillyer and Pelley, 1980). Using antibody detection ELISA, McLaren, Long, Goodgame and Lilleywhite (1979) reported that 82-100% of intestinal schistosomiasis patients in St. Lucia could be diagnosed even

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though up to three different stool examination were required to detect some of those infections by parasitological means. It has, however, been pointed out that although antibody detection is the most sensitive serodiagnostic approach (Kelly, 1987), its field applicability is limited by the inability to differentiate active infections from previously cured ones (Nantulya, *et al.*, 1984). Despite its high sensitivity, antibody detection ELISA using crude parasite antigens is also limited by extensive cross-reactivity between *S. mansoni, S. japonicum* and *S. haematobium* (McLaren *et al*, 1987). To overcome this lack of specificity, efforts have been made to use more purified parasite antigens (McLaren, Lilleywhite, Dunne and Doenhoeff, 1981). Also, the 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) used polyclonal antisera in ELISA to detect CAA at very low concentrations in a two-site radioimmunoassay. More recently, DeJonge *et al.* (1989) used a MoAb produced against the CAA and showed that it could be used to detect the antigen in the urine of patients.

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Even though neither the CAA nor CCA are specific to any of the schistosome species, this new development has further demonstrated the applicability of MoAbs in diagnosis of schistosomiasis.

2.11 Production of monoclonal antibodies (MoAbs)

According to the clonal selection theory which is generally accepted by immunologists, a single matured B-lymphocyte produces only one kind of antibody with respect to epitope specificity (Roit, 1991). B-lymphocytes are, however, incapable of continuous growth *in vitro*. It was, therefore, not possible to obtain large quantities of these mono-specific antibodies even though their potential applications were obvious. This limitation remained important until Kohler and Milstein (1975) discovered and introduced a technique for the production of mono-specific antibodies (monoclonal antibodies). The principle involves fusing a normal antibody secreting B-lymphocyte with a tumogenic (myeloma) non-secreting plasma cell to obtain a "hybridoma" which possesses both the ability to secret antibodies and to grow continuously in culture.

The cell fusion procedure and rationale may be summarized as follows. A suitable mammal (such as a BALB/c mouse) is immunized with antigen (normally crude antigen preparations) containing epitopes to which it is desired to raise antibodies against. The spleen of the mammal is aseptically removed, minced and the splenocytes incubated with myeloma cells in the presence of a fusing agent such as polyethylene glycol. The myeloma cells are mutant cells selected for deficiency in the enzyme Hypoxanthine guanine phosphorybosyl transferase (HGPRT) or Thymidine Kinase

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(TK) based on their ability to grow in the presence of toxic drugs such as 8-azaguanine and 6-thioguanine, respectively. These drugs interfere with nucleic acid synthesis mediated by the respective enzymes (Goding, 1980). The lack of HGPRT, for example, provides the basis for selection of hybridomas after cell fusion. This selection is possible because cells synthesize nucleotides by two biosynthetic pathways. These are the *de novo* and salvage pathways. *De novo* synthesis is blocked by aminopterin, a folic acid antagonist. If the cell is, thus, grown in medium containing aminopterin, nucleic acid synthesis ceases. However, provided the enzyme HGPRT could be supplied, the cells can synthesis DNA by the salvage pathway which mainly involves the recycling of preformed nucleotides. Selection medium containing Aminopterin is, therefore, added with two nucleotides namely, Hypoxanthine and Thymidine, and abbreviated HAT.

Fusing $HGPRT^{+}$ spleen derived lymphocytes with $HGPRT^{-}$ myeloma cells results with three main cell types. These are fused $HGPRT^{+}$ hybridoma cells, unfused $HGPRT^{-}$ myeloma cells and unfused $HGPRT^{+}$ spleen cells. During HAT selection, the hybridoma cells survive because the presence of HGPRT enables salvage synthesis of DNA, whilst the unfused myeloma cells are killed because of lack of the enzyme. The unfused splenocytes also die with time because they are incapable of continuous growth in culture.

The selected hybridomas are then cloned to ensure that single hybridomas are separated. These proliferate into clones of antibody secreting cells secreting

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MoAbs (Goding, 1980; Lopes and Alves, 1984).

The mouse is usually the animal of choice as the donor of splenocytes and for the production of myeloma cells. All of the currently available mouse myeloma cells which are suitable for cell fusion are of BALB\c origin (Levy and Diley, 1984). Successful fusion of cells between different species have, nonetheless, been achieved.

Some of the myelomas available express their own heavy and light chains of immunoglobulins, example, P3-X63/AG8; while others express only one chain, example, NS1/1Ag4.1; and a third type of myelomas are non-producers, example, X63/AG8.653. As a general rule, the non-producer line with a good fusion performance is the best choice. Two of such mouse myeloma cell lines are in widespread use. They are X63.Ag.653 (Kearney, Radbruch, Liesegang and Rajowsky, 1979) and SP2/O.Ag14 (Shulman, Wilde and Kohler, 1978). Another cell line, known to have a good overall performance, is NS1/1 Ag4.1, a kappa chain producer (Kohler, Howe and Milstein, 1976).

2.12 Characterization of Monoclonal Antibodies

Being immunoglobulins, monoclonal antibodies (MoAbs) possess various characteristics (Ouchterlony, 1976). One of the first characteristic of MoAbs which one would want to determine is the immunoglobulin class or subclass. The range of immunoglobulin class or subclass depends on the species origin of the spleen and myeloma cells used for cell fusion. For mouse/mouse derived MoAbs, the likely immunoglobulin classes and subclasses are, IgM IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgE (Lopes and Alves, 1984).

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Another characteristic usually determined is the biochemical nature of the antigenic epitope which a MoAb binds. That is, whether protein, carbohydrate, lipid, glycoprotein, lipoprotein or glycolipid. This is important because of the varied immunologic implications of the various epitope types in an immunocompetent host (Gryzch *et al*., 1984).

Yet another important characteristic of MoAbs which gives them the advantages they have over polyclonal heterospecific antibodies, is their specificity with respect to antigenic epitopes. In most studies, this is the focus of attention (Goding, 1980; Lopes and Alves, 1984).

2.13 Usefulness of MoAbs in immunological studies

Conventional animal antisera contain the products of many different antibody-secreting clones, even when the animal has been immunized with a purified antigen. Such a heterogenous reagent has obvious limitations whenever greater specificity is required (Lopes and Alves, 1984). Fortunately, the introduction of monoclonal antibodies (MoAbs) has greatly improved the performance of immunological assays (Sikora and Smedley, 1984).

The extraordinary specificity of epitope detection provided by MoAbs has made it possible to develop standardizable immunodiagnostic reagents of high specificity. 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 in parasite

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populations; (3) for detection of cloned DNA in various vectors; and (4) for parasite typing and parasite detection (Goding, 1980). Monoclonal antibodies are thus convenient tools, not only for immunodiagnosis, but also for characterization and isolation of parasite antigens. One area in which this application has been particularly useful is the identification of immunoregulatory, and protective antigens for use in immunizations.

2.14 Vaccination against schistosomiasis

It is generally accepted that any breakthrough in the development of a vaccine for human schistosomiasis may require production of antigens by recombinant DNA technology or synthetic peptides constructed from knowledge of antigen gene sequences (Simpson, Chandler, Kelly, Walker, Knight and Smithers, 1987). The need therefore to employ recombinant DNA technology is necessitated by two requirements. Firstly, there is the need to ensure highly specific and controlled immunological intervention in which only antigens with a protective value are administered. Secondly, gene cloning and its associated technologies constitute the means for producing sufficient antigen for immunization.

Both carbohydrate and polypeptide epitopes expressed on the surfaces of newly transformed schistosomula have been reported to induce protective immunity in schistosomiasis. However, the carbohydrate epitopes are reported to be unsuitable as candidate antigens for vaccine production because of their

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similarity with schistosome egg antigens in terms of their ability to induce pathological responses. The polypeptide epitopes, on the other hand, are present in much lower densities but they appear to be more suitable for use in vaccine production. This is because they appear not to cross-react with the egg antigens, and therefore, they are less likely to initiate or enhance pathology (Hacket, Simpson, Knight, Ali, Payeres and Smithers, 1986). So far, three major polypeptide antigens of M_r 32,000, 25,000 and 20,000 which are expressed on the surface of both schistosomula and adult worms have been identified. These antigens which are presented to the host immune system during natural infections have been the focus of antigen gene cloning experiments (Simpson, Hackett, Kelly, Knight, Payeres and Omer-Ali, 1986). Moreover, a variety of cloned and expressed polypeptide antigens are now becoming available from several laboratories. Some of these antigens are well defined while others are simply labelled as antigenic peptides. It is likely that this trend of investigation may lead to the identification of novel protective antigens (Simpson *et al.*, 1987).

Lanar, Pearce, James and Sher (1986) identified and cloned a 97 kDa antigen from *S. mansoni* parasites. This internally localized antigen known as paramyosin has been found to resemble the α-helical protein (paramyosin) present 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

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against the venule wall and thus avoiding dislodgment by the blood flow.

Several other antigens have been discovered and are being considered in vaccine trials. These include SRP and 53 from *S. mansoni* schistosomula; 155, p28 GST and gp68 from *S. mansoni* adult worm; Sj26, from *S. japonicum*; JM8-36, an anti-idiotype antibody, and Fh(SmIII) from *Fasciola hepatica* adult worm (Colley and Colley, 1989).

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CHAPTER 3

GENERAL MATERIALS AND METHODS

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3.1 Preparation of antigens

3.1.1 *Fixation of miracidia with paraformaldehyde, acetone and ethanol*

Miracidia were hatched from *S. haematobium* eggs obtained from the urine of infected persons. Large volumes of urine (more than 3 litres) were left standing undisturbed overnight at 4°C and the supernatant decanted away. The sediment was collected into 50ml conical tubes and centrifuged at 500 *Xg* for 5 min. The sediments resuspended in 1% saline and washed by centrifugation at 500 *Xg* for 5 min. The final sediment was resuspended in distilled water and transfered 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 schistosme eggs hatched and the miracidia attracted to the light were concentrated in the small volume of exposed fluid. The organisms were harvested with a pasteur pipet into 50ml tubes and various fixatives diluted in PBS, pH 7.4 added to give final concentrations of 0.25% and 1% paraformaldehyde, 5% acetone, and 95% acetone. These preparations were kept at 4°C overnight to fix and stabilize membrane antigens after which the miracidia were washed thrice with PBS, pH 7.4 by centrifugation 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.

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3.2 Myeloma Cell Lines and their Maintenance

BALB/c-derived, 8-azaguanine-resistant parental myeloma cell lines were used. X63-AG8.653 were obtained from International Laboratory for Research on Animal Disease (ILRAD) while NS-1/1Ag 4.1 were obtained from Tokyo University, Japan. These two myeloma cell lines were adapted for *in vitro* propagation in Minimum essential Medium (MEM, Gibco) or in Iscove's Modified Dulbecco's Medium (IMDM, Gibco), all supplemented with 10%(v/v) heat-inactivated (56°C for 30 mins) Foetal Bovine Serum (FBS). The myeloma cells were cultivated in $25cm^2$ or 75cm2 sterile tissue culture flasks (Sumilon, Sumitomo Bakellite Company Ltd., Japan) in a CO_2 incubator set at 5% CO_2 and 37°C. The cells were allowed to grow to a concentration of about 1x 10^7/ml and maintained in culture by subculturing a third of the cell suspension with two-thirds volume of fresh growth medium into new flasks. This was to ensure that the cells remain in the logarithmic growth phase for at least 4 days prior to cell fusion (Pearson, Pinder, Roelants, Kar, Lundin, Mayor-Whitney and Hewett, 1980).

3.3 Cell Fusion and Selection of Hybridomas

Three to four days after the final booster with antigens, the highest responder mice were killed by terminal anaesthesia, with diethyl-ether and their spleens dissected out aseptically. Cell fusion was done according to the method described by Pearson *et al.* (1980) with some modifications. All the stages in this procedure were carried out under aseptic conditions in a Clean bench (Hitachi, Tokyo, Japan).

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The spleen from each mouse was separately minced with a curved pair of scissors and the splenocytes suspended in growth medium in a 15 ml conical tube. Clumps and membrane fragments were allowed to settle and the resulting cell suspension pipetted into another tube and washed once with serum-free medium by centrifugation at 290 *Xg* for 5 min. The cells were then counted using an improved Neubauer Counting Chamber (Hagayaki Works, Tokyo, Japan) and pipetted into a 50ml conical tube. The myeloma cells were similarly washed in serum-free medium and a volume of the cell suspension mixed with the spleen cells at a ratio of 1:5-10 myeloma to spleen cells. The mixed cell suspension was centrifuged at 290 *Xg* for 5 min and the supernatant completely removed. One millilitre of polyethylene glycol (PEG), warmed to 37°C, was added dropwise to the cells in a conical tube immersed in a beaker containing water at 37°C over a period of 1 min while gently mixing the cells by turning the tube in a circular manner. The cells were then gently resuspended by stirring with the same pipette for another 1 min. Soon after, 10ml of serum-free medium was added dropwise over a period of 3 min. The cell suspension was then centrifuged at 190 *Xg* for about 3 min and the supernatant removed. Care was taken to ensure that the total time from the beginning of the addition of the PEG to its removal did not exceed 8 min. The fused cell mixture was then resuspended in HAT medium consisting of 0.1mM hypoxanthine, 4 x 10^{-4}mM aminopterin, and 0.016 mM thymidine dissolved in fusion medium [growth medium modified by addition of 38.57mM $NaHCO_3$, 10mM N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid

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(HEPES) and 0.198mM L-glutamine]. The resuspended cells were distributed into two 24-well tissue culture plates, in 1ml of HAT medium per well. HAT-medium was replaced every 3 days until day 10 and changed to HT-medium consisting of 0.1mM hypoxanthine and 0.016mM thymidine dissolved in fusion medium. Usually between day 8 amd 14, wells with large colonies of hybrid cells, as determined by observation under an Inverted microscope (Nikon, 46212, Japan) were marked and the media allowed to turn acidic (yellow) and then tested for antibody activity. Hybrid cells from wells showing positive antibody activity were immediately cloned, and some stabilated as soon as possible. More supernatants were tested for antibody activity as hybrids grew. When selected hybrids were growing well, the HT-medium was replaced with normal growth medium.

3.4 Screening, Cloning and Stabilation of Hybridomas

Culture supernatants from wells containig hybridoma cell colonies were screened for antibody activity, using the antibody detection nitrocellulose membrane-based dot-ELISA or by micro-plate ELISA. Wells with detectable antibody activity as indicated by optical densities or intensity of staining on nitrocellulose membrane were selected for cloning. Some cells from these positive wells were transfered to $25cm^2$ tissue culture flasks and grown until $2x10^7$ cells could be frozen in liquid nitrogen. Cells were stabilated in growth medium containing 7.5%(v/v) Dimethylsulfoxide (DMSO). The remaining hybridomas were resuspended in 1% Trypan Blue in

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phosohate buffered saline, pH 7.4, which served as vital stain and live cells were counted in an improved Neubauer Counting Chamber. The hybridomas were then cloned by limiting dilution in growth medium. A cell suspension was made to give 1 cell/50µl of medium and 2 cells/50µl, and 50µl volumes of each cell concentration dispensed into the wells of different 96 well tissue culture plates previously incubated at 37°C overnight with 50µl/well of splenocytes and/or thymocytes from 2-4 week old BALB/c mice. The 96 well culture plates were incubated at 37°C in a humified atmosphere containing 5% CO_2 in air and left undisturbed for about 10 days. They were then examined for visible cell colonies by viewing the bottom of the wells against light or by observing under an inverted microscope. Wells with single visible cell colonies were marked and transferred into 0.5ml of growth medium in 48-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 allowed to become acidic before testing for antibody. Antibody positive culture fluids were concentrated 20 times, and used for determination of immunoglobulin class by double immunodifussion.

3.5 Propagation and Storage of Hybridomas

Cloned hybridoma cells secreting MoAbs of desired specificity were transferred from 100µl of growth medium in 96 well plates to 0.5ml of medium in 24 well plates, and the hybrids allowed to multiply before increasing the volume to 1ml. The hybridoma cells were grown in the 24 well plates with

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medium changes every three days until about 1 x 10^7 cells could be transferred into 25cm^2 tissue culture flasks. The hybrid cells were then transferred to 75cm^2 tissue culture flasks in 20-25ml of growth medium containing between 1 x 10^5 1 x 10^6 cells/ml and maintained by subculturing into other flasks when medium turned acidic. In order to produce more MoAbs, hybridoma cells were propagated in 1 to 2 litre glass flasks (Corning Glass Works, New York) in 500 to 2000ml medium. The cultures were then gassed with a mixture of 5% CO_2 in air and incubated at 37°C in an incubator (Model IF-41, Yamato Scientific Co. Ltd., Tokyo Japan). Culture fluids, containing MoAbs were harvested after centrifugation at 290 *Xg* for 5 min to remove the cells, and the supernatants stored frozen at -20°C. Some of the hybridoma cell cultures were maintained in the logarithmic phase of growth, for at least 4 days before preservation of the cells in liquid nitrogen. This was to ensure that healthy cells were preserved. Suspensions of cells in the logarithmic growth phase were centrifuged at 290 *Xg* for 5 min and the supernatant removed. The pelleted cells were resuspended in growth medium containing 7.5%(v/v) DMSO and 1ml aliquots pipetted into 2ml cryopreservation 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 specimen were subjected to a temperature gradient from room temperature to -140°C. The vials were then transferred into liquid nitrogen at -196°C.

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3.6 Purification of Monoclonal antibodies

MoAb containing culture spernatants stored at -20°C were retrieved and thawed at 37°C in a waterbath. The immunoglobulins were then precipitated by slow addition of an equal volume of saturated ammonium sulphate [$(NH_4)_2SO4$] while mixing. The precipitates formed were pelleted by centrifugation at 1,200 *Xg* for 30 mins and dissolved in minimum amount of distilled water. The antibody solutions were then transferred into dialysis membrane (Spectro Medical Industries Incorporated, USA) of molecular weight cut off 1,000 and dialysed overnight against Phosphate buffered saline (9mM NaH_2,PO_4, 0.9mM Na_2HPO_4, 15mM NaCl, pH 7.4) with one buffer change. Monoclonal antibody conentrated by this process was subjected to further purification.

3.6.1 *Gel filtration*

IgM monoclonal antibodies precipated and dialysed as described above were purified by gel filtration through a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column using Phosphate buffered saline, pH 7.4 as eluting buffer. Sephadex G-200 was prepared according to the manufacturer's instructions. Ten ml of dialysed antibody was applied onto the Sephadex G-200 column and fractionated. The eluate was collected in 5ml fractions and assayed for antibody activity using the antibody detection microplate ELISA. Fractions containing antibodies were pooled and concentrated using the Amicon concentration chamber (Amicon, Corporation, Ireland) with

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ultrafiltration disk membranes of molecular weight cut-off 10,000 (Sigma Chemical Company, USA).

3.6.2 *Ion exhcange chromatography*

Culture supernatants containing IgG MoAbs were also concentrated by the ammonium sulphate precipitation method and dialysed against PBS. IgG MoAbs were, however, purified by ion-exchange on diethyl aminoethyl cellulose (DE-52, Whatman, Kent, England). Sample was applied onto the column and washed with at least one column volume of dialysis buffer (PBS, pH 7.4). Bound MoAb was then eluted with a linear gradient of 15-300mM NaCl. 10ml fractions were collected in tubes and each assayed for antibody. Fractions with antibody activity were pooled and concentrated as described for IgM antibodies.

3.7 Preparation of enzyme antibody conjugates

Schistosoma haematobium reactive MoAbs previously purified by gel chromatography, were conjugated to horseradish peroxidase (HRPO) using the periodate method described by Wilson and Nakane (1987). The protein concentration of antibody fractions was estimated using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, USA). The desired amount of antibody IgG (MW 160 000) or IgM (MW 90 0000) in terms of protein to be conjugated was pipetted into a tube. HRPO enzyme (MW 40 000, Sigma Chemical Company, USA) was conjugated to antibody at a ratio of 1:1 (enzyme : antibody) by weight. Assuming an 80% coupling efficiency,

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the calculated value for enzyme was multiplied by 100 and divided by 80. For every **X** mg of IgG antibody and IgM antibody, therefore, **X** x 1/4 x 100/80 mg, or **X** x 1/22.5 x 100/80 mg of HRPO, respectively were required. For IgM antibody, this calculated value was multiplied by 5 to account for its pentameric structure. The required amount of HRPO was weighed and dissolved in 2ml of 50mM sodium acetate (CH_3COONa) buffer, pH 4.0. HRPO was oxidized using sodium metaperiodate ($NaIO_4$) at a ratio of 1:1 by weight. The weighed $NaIO_4$ was dissolved in 1ml of 50mM sodium acetate buffer before adding it dropwise to the enzyme solution to mix in a 50ml centrifuge tube covered with aluminium foil. The mixed solution was then incubated with gentle rocking for 15 min at room temperature after which 300µl of ethyleneglycol ($C_2H_6O_2$) were added and stirred. The mixture was then fractionated through a column packed with Sephadex G-25 equilibrated with 50mM sodium acetate buffer pH, 4.0 and eluted with the same buffer. The pH of the eluted enzyme was adjusted to pH 9.6 by adding 1ml of carbonate-bicarbonate buffer pH 9.6 and followed by dropwise addition of saturated sodium carbonate (Na_2CO_3). The pH of the antibody solution was likewise adjusted to pH 9.6. The enzyme solution was then added to the antibody solution and allowed to mix for 1 hr at room temperature in a tube covered with aluminium foil. This was followed by the addition of 300mg of Glycine ($NH_2.CH_2.COOH$) and adjustment of the pH of the resulting solution to pH 8.0 using 1M HCl.

The conjugate was kept at 4°C overnight, after which it was precipitated with 33%(v/v) saturated ammonium sulphate solution for IgG or 50% for IgM and then centrifuged for 2 min at 9,900 *Xg* and the pellet dissolved with glycine/Na_2EDTA

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buffer (0.4M glycine, 0.3M NaCl and 20mM Na_2EDTA, pH 8.0). 20mg of ovalbumin per mg of conjugate was added and mixed to dissolve, and the solution again centrifuged to remove particulate matter. The conjugate solution was then filtered with 0.45um membrane filter (Millipore Products Division, Bedford, MA, USA) followed by a 0.22um filter to remove polymerized conjugate. An equal volume of glycerol was finally added, mixed and stored at -20°C. Conjugate potency was determined by antigen detection micro-plate ELISA.

3.8 Sodium dodecyl sulphate-polyacrilamide 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 (Laemni, 1970).

3.8.1 *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 mold 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.

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Resolution acrylamide gradient gels (10-15%) were prepared as follows.

Solution A, 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) Ammoniun persulphate (APS)	0.07ml
N,N,N'-N'-tetramethylethylenediamine (TEMED)	0.01ml
Distilled water	4.5ml

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, Stacking gel (one gel)

29.2% (w/v) acrylamide,	
0.8%(w/v) N,N'-methylene bis-acrylamide	1ml

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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	0.006ml
Distilled water	3.6ml

A 10-15% resolution acrylamide gradient gel was prepared by slowly mixing solution A and B using an improvised gradient former. Each solutiion was swirled to mix and poured into one of the two linked chambers of the gradient mixer. A tube joining the chambers at the bases was closed with a clip to avoid premature mixing of the solutions. 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 stirer 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 glass plates. The gel former was immediately rinsed with distilled water to prevent polymerization of gel in the connecting tubes. The gel in between the glass slabs was overlaid with about 800µl of distilled water using an adjustable pipette and left to polymerize. After it had polymerized, the overlaying water was drained away with tissue. A stacking gel solution (C) was then poured on top of the separating gel and a comb carefully inserted to cast the desired wells for sample application.

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The stacking gel was allowed to polymerize overnight. 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 3.47 mM SDS) and the lower chamber, at the anode, filled with 0.6 litres of running buffer.

3.8.2 *Preparation of samples and electrophoretic run*

Crude antigens run on the gels were adjusted for protein and diluted with sample buffer [25mM Tris, 192mM Glycine, 0.1%(w/v) SDS and 20%(v/v) Methanol] in a 2:1 sample to buffer ratio, to give 15mg of protein per lane. The antigens included in this study were soluble egg antigens from Ghanaian strain(s) ($ShSEA_{Gh}$) *S. haematobium* and Egyptian strain(s) ($ShSEA_{Egy}$), *S. haematobium* adult worm crude antigen extract (ShW), *S. japonicum* soluble egg antigens (SjE) and adult worm crude antigen extract (SjW), *S. mansoni* adult worm crude antigen extract (SmW), precipitated proteins from *S. haematobium* infected patient urine (UP_2-IP) and (UP_o-IP), and normal control urine precipitates (NUX) and (NUJ). Standard low molecular weight markers (Sigma, USA) were prepared as described by the manufacturer and used. The samples were boiled for 5 min and centrifuged for about 2 min at 9,900 *Xg* to remove particulate matter before they were loaded unto the gel. A constant current of 15mA 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 25mA and maintained until the bromophenol blue marker had barely run out of the separating gel.

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CHAPTER 4

STUDIES OF ANTI-*S. HAEMATOBIUM* MONOCLONAL ANTIBODIES: I: PRODUCTION

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4.1 Introduction

Schistosomiasis is an economically important disease which affects about 250 million people worldwide, with a further 500-600 million others exposed to the risk of infection. The principal methods for identifying people with the disease is by the demonstration of parasite eggs in excreta or by haematuria or proteinuria. These methods are, however, not sensitive enough. Furthermore, haematuria and proteinuria could be due to causes other than schistosomiasis. These limitations have made the search for more sensitive and specific alternative schistosomiasis diagnostic methods inevitable.

One outcome of this search, has been the development of tests based on the detection of anti-schistosome antibodies or circulatory schistosome antigens in serum or urine (Sherif, 1962; Shoeb, Basma, Haseeb and Said El Din, 1968; Feldemeier, Stevens, Bridts, Dafalla and Buttner, 1983). None of these tests has, however, been able to replace the parasite egg detection method in routine field diagnosis of the disease. This is mainly because of the lack of adequate specificity and in some cases sensitivity.

The introduction of the technology for producing MoAbs by Kohler and Milstein (1975), has offered great opportunities for the development of more sensitive and more specific immunodiagnostic techniques (Bergquist, 1984; Sikora and Smedley, 1984). An attractive possibility, in the case of urinary schistosomiasis, is the development of assays which utilize MoAbs in the detection of parasite-specific antigens released into patient urine.

This study was, therefore, undertaken with the objective of producing

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MoAbs reactive with schistosome antigens, with the view that some of them may prove useful in the diagnosis of urinary schistosomiasis.

4.2 Materials and methods

4.2.1 *Mice*

Inbred male and female BALB/c mice aged between 12 and 16 weeks and nursling BALB/c mice 7-14 days old, were used for the production of MoAbs. All the mice used were obtained from the NMIMR colonies.

4.2.2 *Preparation of Parasite Antigens*

4.2.2.1 *Extraction of crude schistosome worm, egg and hookworm egg antigens*

Crude schistosome antigens were extracted using an extraction fluid consisting of phenylmethyl-sulfonylflouride (PMSF), N-α-P-Tosyl-L-Lysine chloromethyl ketone (TLCK) and N-Tosyl-L-phenylalamine chloromethyl Ketone (TPCK) all from Sigma Chemical Company, USA. These were reconstituted in absolute ethanol and diluted in Dulbecco's PBS pH, 7.4 to a final concentration of 1mM PMSF, 0.2mM TLCK and 0.05mM TPCK. Protease inhibitors, Leupeptine and trans-Epoxysuccinyl-leucylamido (4-guanidino) butane (E-64, Cambridge Research Biomedicals Incorporated, UK) were added to a final concentration of 10mg/ml.

Antigen extracts were prepared from *S. haemtobium, S. mansoni S. japonicum* eggs and worms as well as Hookworm eggs. Parasite eggs frozen at -20°C were

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retrieved and added with extraction fluid before thawing. After resuspension, each specimen was pipetted into a glass homogenizer (Kudoguki, Keiki, Tokyo, Japan) and disrupted on ice at 1200rpm for 5 min. Homogenized eggs or worms were collected into different eppendorf tubes and centrifuged at 290 *Xg* for 5 min and the supernatants collected as primary extracts. Fresh extraction medium was added to each pellet and mixed, and the extraction process repeated to obtain secondary and then tertiary extracts. One ml aliquots of the extracts were kept at -20°C until used in experiments designed to investigate MoAb specificity.

4.2.2.2 *Precipitation of proteins from S. haematobium infected human urine*

The protocol for the extraction of urine protein as illustrated in figure 1 was provided by Dr. Kwabena M. Bosompem of NMIMR.

4.2.3 *Immunization of Mice for MoAb Production*

4.2.3.1 *Immunization with S. haematobium soluble egg antigens (ShSEA)*

Soluble egg antigens from the eggs of an Egyptian strain of *S. haematobium* ($ShSEA_{Egy}$), were used for immunization of BALB/c mice. All immunogens were given by the intraperitoneal route. Initial immunizations were made with 30-50μg of schistosome proteins emulsified in Freund's complete adjuvant (Sigma, U.S.A.) supplemented with 1mg/ml of *Mycobacterium tuberculosis*, while subsequent

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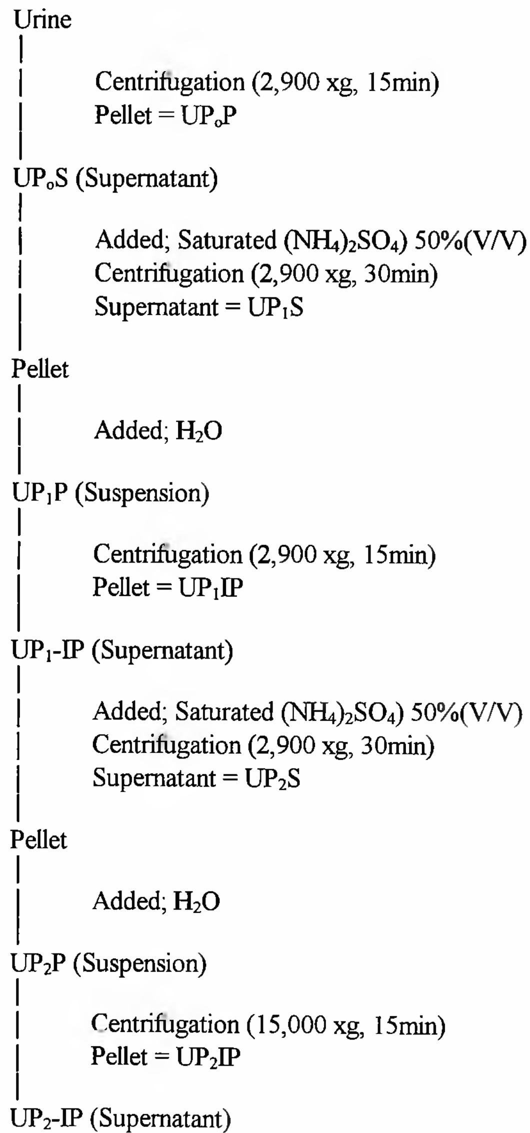
Figure 1: **Extraction of UP_oP and UP_2-IP from *S. haematobium* infected human urine (Bosompem, unpublished data)**



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(booster) immunizations were made with Freund's incomplete adjuvant. Booster immunizations consisting of similar concentrations of schistosome antigens were given 3 and 6 weeks after the initial immunization. About 10 days after the second booster, the mice were bled from the tail veins and tested for antibody response to $ShSEA_{Egy}$ by dot-ELISA or micro-plate-ELISA. Mice with high antibody titre (1:5000 or more) were each given a final intraperitoneal booster of 50μg of antigen. Pre-fusion sera were prepared from blood obtained by orbital bleeding 3-4 days after the final booster. Immediately after the bleeding, the mice were killed by terminal anaesthesia using diethyl-ether, and their spleens aseptically dissected out for cell fusion.

4.2.3.2 *Immunization with antigens extracted from the urine of S. haematobium infected individuals*

Proteins concentrated from *S. haematobium* infected human urine by ammonium sulphate precipitation were used to immunize male and female BALB/c mice. The mice were injected intraperitoneally with 100μl total inoculum consisting of 50μg of the antigen emulsified in Freund's complete adjuvant. First and second intraperitoneal booster immunizations were given respectively, 2 and 6 weeks later, using similar concentrations of antigen in Freund's incomplete adjuvant. Ten days after the second booster, immunized mice were bled and screened for antibody response as described in (section 4.3.4). The best responder mice with an antibody titre of 1:5000 or more were each given a final intraperitoneal booster of 50μg of the antigen preparation in Freund's incomplete adjuvant. Three to four days later, the mice were

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bled for pre-fusion serum, just before their spleens were aseptically removed for cell fusion.

4.2.4 *Screening of Immunized Mice for Antibody Activity*

The tip of the tail of each mouse was cut with a pair of scissors after cleaning with 70% ethanol and the tail gently squeezed from the base towards the tip. Drops of blood from the severed veins were aspirated using a 10µl Eppendorf pipette and then transferred each into an Eppendorf tube containing 490µl of Dulbecco's PBS pH 7.4 and mixed thoroughly. The tubes and their contents were then centrifuged at 9,900 *Xg* for 5 min and the supernatants pipetted into different tubes. The sedimented blood cells were discarded. Each supernatant was tested for antibody activity by micro-plate ELISA or dot-ELISA.

4.2.5 *Antibody-detection Micro-plate ELISA*

This procedure was used for screening immunized mice sera and hybridoma culture supernatants for antibody activity.

In this assay, polystyrene micro-plates (Sero-wel, Bibby Sterilin, UK) previously coated with 50µl/well of schistosome antigens were emptied and rinsed once before incubation with test samples for 15min at room temperature. Immunized mice sera were tested in two-fold dilutions beginning from 1:100 while 100µl of hybridoma culture supernatants were tested in duplicates without dilution. Normal mouse serum and pre-fusion serum were added as negative and positive controls, respectively. The plates were rinsed once to remove excess unbound antibody. Each

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well was then incubated with 50µl of goat anti-mouse HRPO conjugate diluted 1:500 in washing buffer and incubated for 15min at room temperature. Each plate was washed 3 times, each by 10min incubation with washing buffer to remove excess unbound conjugate. 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 in 50mM citric acid buffer, pH 4.0. The substrate was incubated for 30 min at room temperature. The colourless 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).

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4.2.6 *Dot-ELISA Procedure*

This assay was used to screen immunized mice sera and hybridoma culture supernatants for antibody. Schistosome egg or worm extracts were applied in 2µl dots unto Nitrocellulose membrane filters, 0.4µm pore size (Sigma Chemical Company, USA). The antigen "dotted" membranes were cut into strips and incubated for 1 hour with "blocking solution" containing 5%(w/v) skimmed milk in Tris-buffered saline (TBS) (50mM Tris and 150mM NaCl, pH 8.0). The strips were removed from blocking solution and incubated for 2 hours with primary antibody, ie., immunized mice sera diluted 1:250 with blocking solution or hybridoma culture supernatants diluted 2:3 in blocking solution. The strips were then washed in TBS and incubated for 1hr in

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goat anti-mouse HRPO-conjugated immunoglobulin diluted 1:500 in blocking solution, after which they were washed three times (10 min each). Finally the membrane strips were incubated for 3 min in a substrate solution containing 0.15%(v/v) Hydrogen peroxide and 0.05%(w/v) chromogen (3,3'-diaminobenzidine) in phosphate-Na_2EDTA buffer (10mM NaH_2PO_4, 10mM Na_2HPO_4, 10mM Na_2-EDTA). The strips were washed with distilled water and the substrate reaction stopped by the addition of a few drops of concentrated HCl. Positive reactions appeared as brown dots.

4.2.7 *Cell Fusions, Cloning and Selection of Hybridomas*

The procedures adopted for cell fusions, cloning and selection of monoclonal antibody secreting hybridomas have been described under sections 3.3 and 3.4 of Chapter 3.

4.2.8 *Determination of Immunoglobulin Class and Subclass*

The class and subclass specificity of the murine immunoglobulins (Igs) were determined using the double immunodifusion method described by Ouchterlony (1967). Commercially prepared antisera (Goat anti-mouse Ig against murine Ig isotypes: IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM) were purchased from (Sigma, USA) and used as recommended. A 1%(w/v) agarose (Bethesda Research Laboratories, USA) solution was prepared by melting solid agarose in Phosphate buffered saline (15mM NaCl, 1.1mM Na_2HPO_4 and 0.1mM KH_2PO_4, pH 7.4) Five millilitres of the molten agarose was poured unto a microscope slide and allowed to solidify. Wells were then

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cut into the solid gel in a circular arrangement surrounding a central well and each filled with approximately 15μl of reagent. Antisera were 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.

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4.3 Results

The BALB/c mice responded well to the *Schistosoma haematobium* antigen preparations that were used in the immunizations. In general, high antibody responses with titres far beyond 1:5,000 were obtained against the antigen preparations, as determined by the double-antibody sandwich micro-plate ELISA. Also, some of the sera could detect as low as 0.063μg of *S. haematobium* soluble egg antigens (ShSEA). Differences were, however, found in the ability of the two antigen preparations, namely, ShSEA and crude protein extracts from the urine of *S. haematobium* infected persons (UP_2-IP) to induce antibody responses.

Figures 2a and 2b illustrate the mean serum antibody responses of the mice immunized respectively with ShSEA and UP_2-IP as compared with serum reactivity of normal non-immunized mice. The individual curves in the figures show that antibody responses in mice immunized with UP_2-IP remained high with optical density greater than 0.2 even at antibody titres up to 1;51,200. On the other hand, antibody responses in mice immunized with ShSEA remained high above optical density 0.2 at antibody titres of 1:6000. Antibody titres of the response in the non-immunized mice, on the other hand, were very low in each case, and remained below 0.1 optical density at all the dilutions tested from 1:100 to 1:51,200.

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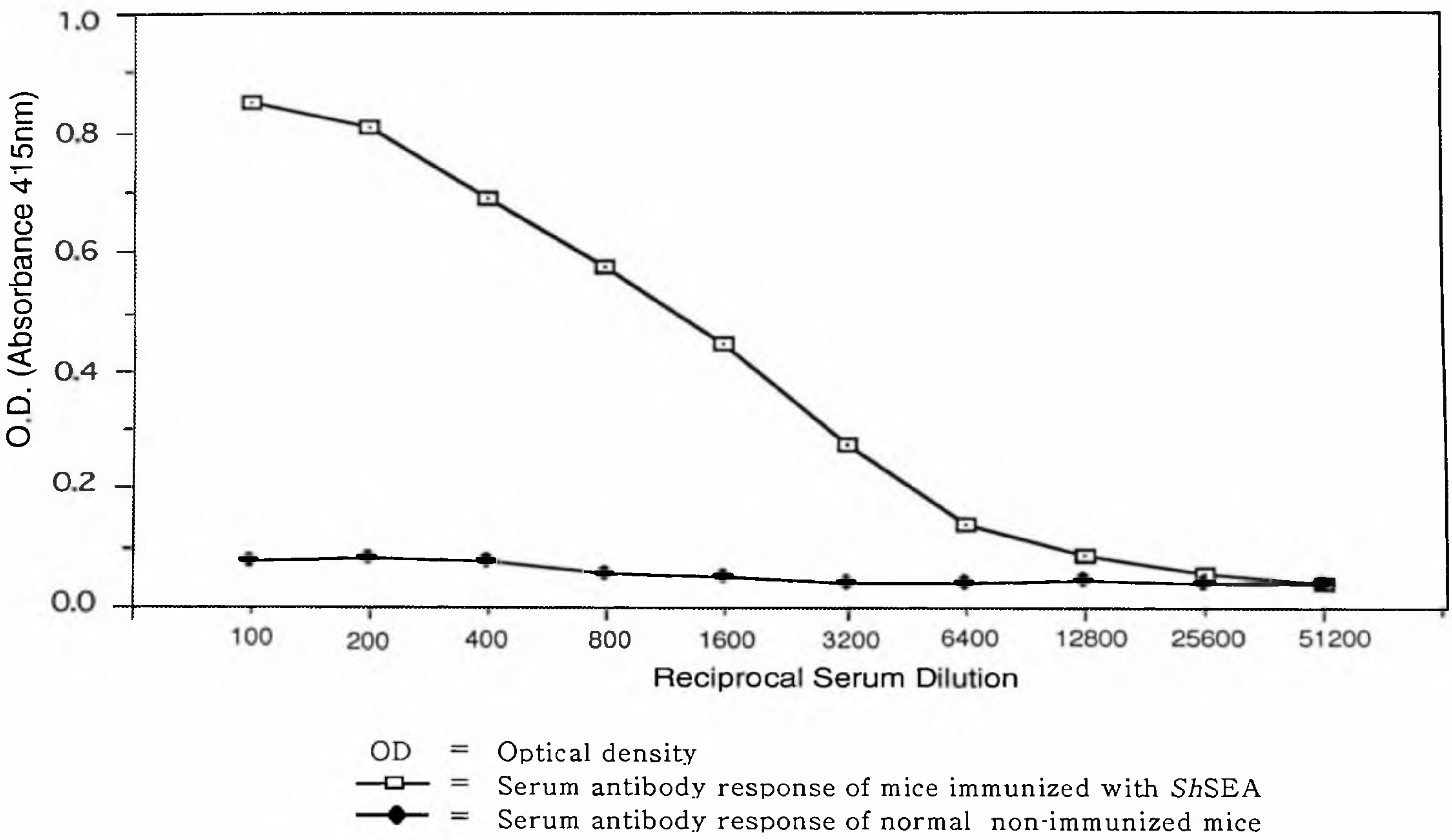
Figure 2a **Serum antibody responses of mice immunized against ShSEA and non-immunized BALB/c mice as determined by reactivity with ShSEA**



OD = Optical density

—□— = Serum antibody response of mice immunized with *Sh*SEA

—◆— = Serum antibody response of normal non-immunized mice

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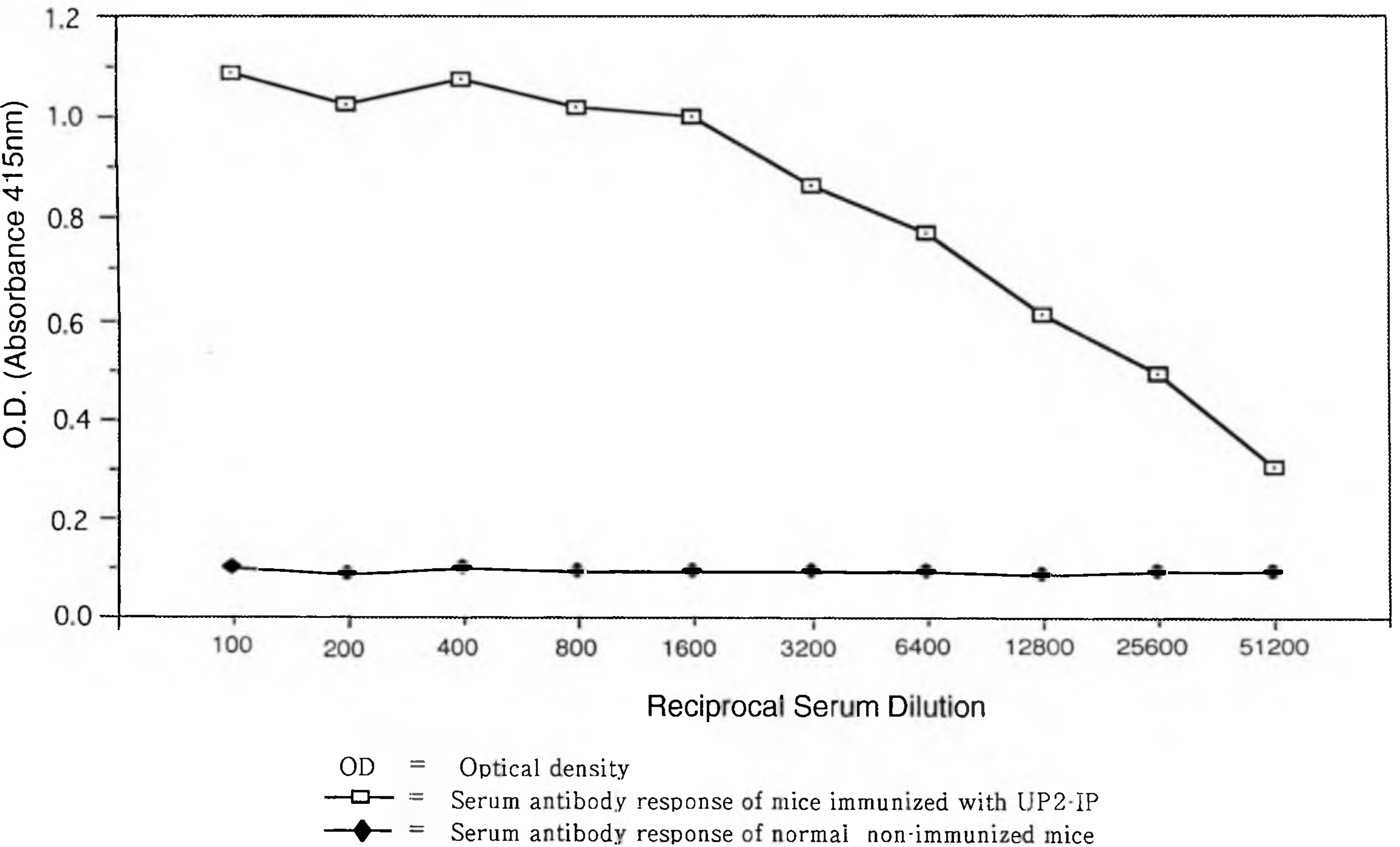
Figure2b Serum antibody responses of mice immunized against UP_2-IP and non-immunized BALB/c mice as determined by reactivity with UP_2-IP



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4.3.1 *Cell Fusion and Selection of Hybridomas*

Five independent cell fusions were made towards the production of MoAbs against *S. haematobium* antigens. Three of the fusions (Sh1, Sh3 and Sh4) were made with spleen cells from mice immunized with ShSEA, whilst another fusion (Sh2) was made with spleen cells from mice immunized with UP_2-IP. The last fusion (Sh5), however, was made with spleen cells from a mouse immunized with UP_2-IP except that the final booster, just before cell fusion, was made with ShSEA. Antibody secreting hybridomas from positive wells were cloned by limiting dilution and the classes of the antibodies determined by the Ouchterlony double immunodiffusion method.

4.3.2 *Anti-ShSEA MoAb Secreting Hybridomas*

The results of three independent cell fusions made towards the production of MoAbs against ShSEA are found in Table 2. From fusion Sh1, 56 out of 72 wells had hybridoma cell colonies. Nevertheless, only one well tested positive for antibodies reactive with ShSEA. Upon cell cloning, one stable hybridoma was isolated. Although a higher number of spleen cells was used for fusion Sh4 as compared to fusion Sh3 (Table 2), the latter fusion (Sh3) produced more hybridoma colonies, 60/90 wells as against 22/72 wells for fusion Sh4. Thirteen out of the 60 cell colonies in Sh3 secreted antibodies that reacted with ShSEA, whereas only 2 wells from Sh4 contained antibodies with reactivity against ShSEA positive for antibodies. A total of 11 stable hybridomas were obtained from these three cell fusions (Sh1, Sh3 and Sh4) (Table 2).

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Table 2: **Summary of cell fusions and hybridoma development experiments from spleen cells of BALB/c mice.**

Fusion	Origin of spleen cells	Immunogen	Myeloma parent	Spleen cell count for fusion	Numbers of wells showing growth of hybrids	Number (%) of antibody positive wells@	Number of wells with reactivity for SEA_{Egy}@	Number of wells with reactivity for UP_2S@	Number of selected hybrid clones secreting monoclonal antibody
Sh1	BALB/c	SEA_{Egy}	X63/AG8.653	$6.3x10^8$	56/72	1(1.8%)	1	0	1^s
Sh2		UP_2S	NSI/1Ag4.1	$5.3x10^7$	72/72	72(100%)	0	48	2^s,1*
Sh3		SEA_{Egy}	"	$9.3x10^5$	45/72	13(28.8%)	13	0	2^s
Sh4		SEA_{Egy}		$6.4x10^6$	22/72	2(9.1%)	2	0	1^s
Sh5		UP_2S/SEA_{Egy}		$5.8x10^6$	63/72	2(3.2%)	2	2	1^s

@ Supernatants were tested in double antibody sandwich ELISA using SEA_{Egy} or UP_2S coated micro-ELISA plates.
s Number of hybridoma cell clones selected on the basis of differences in immunoglobulin isotype.
* Number of hybridoma cells which aborted antibody secretion after cryopreservation.
UP_2S = Ammonium sulphate precipitated proteins from *S. haematobium* infected human urine.
SEA_{Egy} = Soluble egg antigens of an Egyptian strain of *S. haematobium*.

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4.3.3 *Anti- UP_2-IP MoAb Secreting Hybridomas*

Table 2 also shows the results of two cell fusions (Sh2 and Sh5) made towards the production of anti-*S. haematobium* MoAbs using infected human urine-based antigens (UP_2-IP). All the wells (48/48) in fusion Sh2 had hybridomas cell colonies that secreted antibodies against UP_2-IP. However, none of the antibodies reacted with ShSEA. Fusion Sh5 also produced many wells (42/48) (Table 2). However, only two of the colonies secreted antibodies, both of which reacted with ShSEA and UP_2-IP (Table 2).

4.3.4 *Cloning of MoAb Secreting Hybridomas and the Selection of Clones*

Antibody secreting hybridomas from positive wells were cloned by limiting dilution and positive clones selected on the basis of intensity of reaction for expansion. In all, 150 MoAb secreting hybridoma clones were obtained from fusion Sh1 well Sh1/71. A few (8) of the clones with strong reacting antibodies were, therefore, selected for expansion. Two wells Sh2/1 and Sh2/15 from fusion Sh2 were selected for cloning based on differences in the secreted immunoglobulin classes. The cloning efficiency was high in either case, exceeding 90 clones per 96 well plate. Also, majority (greater than 99%) of the clones secreted antibodies. However, the cell clones obtained from well Sh2/1 were not stable as they aborted antibody secretion following storage in liquid nitrogen. With fusion Sh3, two out of 13 wells identified with cell colonies were lost through bacterial contamination and were, therefore, not cloned. The cloning success rate was generally less than 50% for each of the 11 wells that were

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cloned. However, the percentage of clones secreting antibodies showed a wider variation. For example, out of 72 clones screened from well Sh3/6, there were no secretors, whereas 40 out of 45 clones obtained from well Sh3/12 secreted antibodies. A similar observation was made with fusion Sh5 in which one of the two wells cloned produced 31 out of 32 antibody positive clones, compared with only 2 positive clones obtained from the other well.

From the five fusions that were made, 6 hybridomas which secreted anti-ShSEA or anti-UP_2-IP MoAbs were derived. The isotypes and reactivity patterns of the six MoAbs are listed in Table 3. Five of these MoAbs [Sh1/71.7 (IgM), Sh3/15.28 (IgG_1), Sh3/38.2 (IgM), Sh4/14.3 (IgG_1) and Sh5/32.30 (IgM)] reacted with ShSEA, whereas Sh5/32.30 and Sh2/15.F (IgG_1) reacted with UP_2-IP.

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Table 3: **Selected MoAbs reactive with *S. haematobium* antigens as determined by micro-plate and dot-ELISA**

Monoclonal antibody	Isotype	ShSEA	UP_2-IP
Sh1/71.7	IgM	+	-
Sh2/15.F	IgG_1	-	+
Sh3/15.28	IgG_1	+	
Sh3/38.2	IgM	+	
Sh4/14.3	IgG_1	+	
Sh5/32.30	IgM	+	+

ShSEA = *S. haematobium* soluble egg antigens.
UP_2-IP= Ammonium sulphate precipitated proteins from urine of *S. haematobium* infected persons.
\+ = MoAb reacts with antigen.
= MoAb does not react with antigen.

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4.4 Discussion

The main aim of this study was to obtain MoAbs against *S. haematobium* antigens with the view that some of them might be useful in diagnosis with the probable detection of antigens in the urine of infected individuals.

The strategy was to immunize BALB/c mice with *S. haematobium* soluble egg antigens (ShSEA) and/or infected human urine-based parasite antigens so as to produce MoAbs, and to find out if any of them could detect antigens in the urine of infected individuals.

The feasibility of this approach is evidenced by the fact that schistosome antigens have been reported to appear in the urine of patients infected with *S. haematobium* (Sherif, 1962). Furthermore, Feldemeier *et al.* (1983) and Jassim *et al.* (1987) studied and reported the presence of immune complexes involving schistosome antigens and various human immunoglobulin isotypes in both urine and circulation, indicating that antigens involved are immunogenic. Also, Koech, Hirata, Shimada and Wambaya (1984) reported that human immunoglobulins (mainly IgGs) are usually found associated with *S. haematobium* eggs in the urine of infected individuals.

It has been shown in this work that human urine-based *S. haematobium* antigens in the protein fraction (UP_2-IP) are immunogenic and can be used for the generation of MoAbs.

The immune responses of the mice immunized with UP_2-IP compared with the responses in mice immunized with ShSEA showed that UP_2-IP is more immunogenic than ShSEA. This may be due to the presence of other more immunogenic

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components in UP_2-IP. The greater immunogenicity of UP_2-IP might also have resulted in the higher fusion success rate (48/48 wells) of the cell fusion made with spleen cells from mice immunized with only UP_2-IP. UP_2-IP gave MoAbs that reacted with only UP_2-IP (but not ShSEA), probably because immune response following immunization with UP_2-IP was induced by only the more immunogenic components in UP_2-IP. When the immunization strategy was, however, changed in the case of the mouse used for fusion F5, the MoAbs that were generated reacted with both UP_2-IP and ShSEA. The inability of the UP_2-IP derived MoAbs to react with ShSEA could be due to the presence of less immunogenic soluble egg antigens (SEAs) in UP_2-IP. With the immunization strategy changed to selectively booster the mice with ShSEA in Sh5, memory cells of the less immunogenic SEAs may have been activated to secret antibodies. This probably explains why the MoAb derived from fusion Sh5 detected antigens in ShSEA in addition to detecting antigens in UP_2-IP.

The MoAbs that reacted with only UP_2-IP may not have detected parasite antigens because of their inability to react with ShSEA. This cannot, however, be absolutely true since they could have detected circulating worm antigens that were stage-specific but do end up in the urine. MoAbs that reacted with both ShSEA and UP_2-IP are likely to be the most potentially useful for diagnosis, provided they are either *S. haematobium* or Schistosome specific.

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4.5 Summary

Mouse monoclonal antibodies (MoAbs) have been produced against *Schistosoma haematobium* antigens. To generate B-cell hybridomas, X63-AG.658 or NS-1AG4.1 myeloma cells were fused with splenic lymphocytes obtained from BALB/c mice previously immunized intraperitoneally with *S. haematobium* soluble egg antigens (ShSEA) and/or extracted proteins (UP_2-IP) from the urine of infected humans. Immunized mice sera and hybridoma culture supernatants were screened for antibody activity by micro-plate ELISA and dot-ELISA. Generally, the immunized mice responded well to the various immunogens with serum antibody titres greater than 1:5,000. However, UP_2-IP appeared to be more immunogenic as it maintained an optical density of 0.2 for serum dilution greater than 1:50,000 compared with 1:5,000 for ShSEA. The fusion success rate was higher when UP_2-IP was used as immunogen. All the immunizations with ShSEA resulted in the production MoAbs reactive with ShSEA but not UP_2-IP, and the converse was true for immunization with UP_2-IP. Nevertheless, when mice were immunized with UP_2-IP and selectively boosted with ShSEA, the MoAbs generated reacted with both antigens. Six MoAbs were established. Four of these [Sh1/71.7 (IgM), Sh3/15.28 (IgG_1), Sh3/38.2 (IgM) and Sh4/14.3 (IgG_1)] reacted with ShSEA while one [Sh2/15.F (IgG_1)] reacted with UP_2-IP. The last MoAb [Sh5/32.30 (IgM)] reacted with both ShSEA and UP_2-IP.

To characterise the MoAbs produced in order to establish their specificity, if any, to *S. haematobium* and whether any could be used for the detection of parasite

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antigens in the urine of infected persons, the following experiments were performed (Chapter 5).

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CHAPTER 5

STUDIES OF ANTI-*SCHISTOSOMA HAEMATOBIUM* MONOCLONAL ANTIBODIES:

II: CHARACTERISATION

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5.1 Introduction

Monoclonal antibodies (MoAbs) have improved immunodiagnosis of most parasitic infections and enhanced prospects for identification of protective antigens that may be useful in vaccines against diseases including schistosomiasis. In view of these, the World Health Organization's Scientific Working Group on schistosomiasis, recommended financial support and incorporation of research with MoAbs into the overall strategy for schistosomiasis control (Bergquist, 1984).

Many workers, including, Mitchell, Cruise, Garcia, Vadas and Munoz (1983), Cruise, Mitchell, Garcia, Tui, Hocking and Anders (1983), Nash, and Deelder (1985), Sidner, Carter and Colley (1987) and Yamashita, Watanabe, Hosaka, Minai, Saito and Sendo (1989) have produced and characterized MoAbs reactive with schistosome antigens. The most promising of these MoAbs were the ones that bound circulating schistosome antigens which are also excreted in the urine of patients. This is because they offered an opportunity to develop immunodiagnostic methods using either sera or urine as test specimen. Recently, Deelder *et al.* (1989) developed a highly specific and sensitive enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of a schistosome worm gut proteoglycan antigen called circulating anodic antigen (CAA) in *S. mansoni and S intercalatum* infected persons (DeJonge *et al.*, 1988, 1989). Also, DeJonge *et al.* (1989) used ELISA to detect CAA in the urine of *S. mansoni* and *S. haematobium* infected persons. The cross-reactive nature of CAA, however, makes it unsuitable for specific diagnosis of any of the schistosomiases.

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This study was, therefore, aimed at characterizing MoAbs produced against *S. haematobium* egg antigens ($ShSEA_{Egy}$) or patient urine-based antigens (UP_2-IP) so as to find out if any of them is *S. haematobium* species-specific, and whether any could be used in the detection of parasite antigens in the urine of infected persons. Such MoAbs, if found, could be useful in the development of more sensitive immunodiagnostic assays for *S. haematobium* infections.

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5.2 Materials and methods

5.2.1 *Monoclonal Antibodies*

The MoAbs used in this study were produced as described in Chapter 4. They are Sh1/71.7, Sh2/15.F, Sh3/15.28, Sh/38.2, Sh4/14.3 and Sh5/32.30.

5.2.2 *Western Blotting*

The procedures employed in characterizing the MoAbs using western blotting have been fully described in Chapter 3 sections 3.8, 3.8.1 and 3.8.2.

5.2.3 *Determination of the Biochemical Nature of Antigenic Epitopes*

5.2.3.1 *Detection of MoAbs 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 MoAbs produced were carbohydrate in nature. Each well of a 96-well micro-ELISA plate (Sero-wel, Sterilin, Hounslow, UK) was incubated overnight at 4°C with 1µg of ShSEA and 0.5µg UP_2-IP diluted in coating buffer (34.5mM $NaHCO_3$ and 15mM $NaCO_3$, pH 9.6) in a total volume of 100µl. The plates were washed once with washing buffer [0.136M NaCl, 9.7mM Na_2HPO_4, 1.4mM $KHPO_4$, 2.7mM KCl, 0.6mM $C_7H_5O_2Na$ and 0.5%(v/v) Tween 80, pH 7.4], and then rinsed with 50mM sodium acetate buffer, pH 4.5. Sets of wells were then incubated with (100µl/well) of three different concentrations of Sodium metaperiodate (0, 10 and 20mM) diluted in sodium acetate buffer for 1hr at room temperature in the dark. The plates were rinsed once again with sodium acetate buffer and incubated with

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100μl/well of 50mM Sodium borohydride for 30 min at room temperature, after which they were rinsed five more times with washing buffer. The wells were then incubated for 1hr at room temperature with 100μl/well of MoAbs diluted serially from 1:2 to 1:128 in washing buffer, after which they were washed with washing buffer to get rid of excess unbound antibody. Each well was then added with 100μl of goat anti-mouse HRPO-conjugate diluted at 1:500 in washing buffer and incubated for 15 min at room temperature. The plates were washed five times with washing buffer before being incubated with substrate solution [40mM 2,2'-azino bis (3-ethylbenzthiazoline 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 plates read at a wavelength of 410nm using a Dynatech MR 600 microplate reader (Dynatech Laboratories Inc., USA).

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5.2.3.2 *Detection of MoAbs specific for protein epitopes*

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

Proteinase-K (Sigma, USA) was diluted in Dulbecco's PBS, pH 7.4 to prepare a stock solution of 2mg/ml concentration. 40μl of 2mg/ml preparations of each antigen extract to be tested were pipetted into four Eppendorf tubes labelled A-D. Tubes labelled A, B, and C containing the same antigen were added with different concentrations of proteinase-K diluted in 40μl of PBS to give 1mg/ml, 0.5mg/ml and

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0.25mg/ml of enzyme in the three tubes respectively. Each of the tubes labelled D were added with 40μl of Dubelcco's PBS without proteinase-K to serve as controls. All the tubes were then incubated at 37°C in a waterbath for 1hr, after which 3μl of each sample was pipetted unto nitrocellulose membrane strips in dots. The strips were assayed in the dot-ELISA using *S. haematobium* reactive MoAbs described in chapter 4 as probes. To remove non-specific background, the antigen "dotted" strips were destained by incubation in destaining solution consisting of 5%(v/v) H_2O_2 in blocking solution as described by Bosompem (1993).

5.2.4 *IFAT Procedure*

The miracidia used were hatched and fixed as described in section 3.2.1. Indirect immunofluorescence was performed using a modification of the method described by van Meirvenne, Janssens and Magnus (1975). Ten microlitres of miracidia suspension were delivered using 50μl pipette into each of the wells on Teflon-coated multitest microscope slides (Cooper Wellcome, Fremhodegen, 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. The slides were removed and the wells incubated for 15 min at room temperature with 30μl of appropriately diluted MoAbs. Some of the wells were incubated with pre-fusion serum diluted 1:100 or with fresh growth medium to serve as positive and negative controls, respectively. Slides were then washed twice by immersion in fresh buffer for 2x10 min with one buffer change. FITC-labelled goat anti-mouse immunoglobulin

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prepared as described by Clark and Shepard (1963) and as modified by Katende, Musoke, Nantulya and Goddeeries (1987), was 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-bufered saline, 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.

5.2.5 *Dot-ELISA Procedure*

The dot-ELISA procedure has been described in Chapter 4, section 4.2.6

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5.3 Results

5.3.1 *Anti-S. haematobium MoAbs Selected for Further Characterization*

A list of the MoAbs generated against antigens in ShSEA and UP_2-IP is provided in Table 3 (Chapter 4). These MoAbs were further characterized as described below.

5.3.2 *Immunolocalization of S. haematobium Antigens Bound by the MoAbs*

Immunolocalization studies made by IFAT, revealed that some *S. haematobium* antigens bound by the MoAbs were located on the surface membrane of miracidia of Ghanaian strain(s) of the parasite. Other antigens bound by some of the MoAbs were found to be located intracytoplasmically. Table 4 summarises the results of these tests. All the 6 MoAbs bound antigens on the surface of miracidia while 3 MoAbs (Sh1/71.7, Sh3/15.28 and Sh3/38.2) bound internally located (intracytoplasmic) antigens in addition (Table 4).

5.3.3 *Reactivity of the Selected MoAbs in the Western Immunoblot Assay*

Characterization of the antigens bound by the selected MoAbs using the western immunoblot technique showed that only Sh3/15.28 and Sh4/14.3 could bind antigens with the procedure. As shown in Table 5, Sh3/15.28 bound two polypeptide antigens of approximate molecular weight (MW), 37 and 46 kDa in *S. haemtobium* soluble egg antigens of Egyptian strain(s) ($ShSEA_{Egy}$). On the other hand, Sh4/14.3 reacted with a 73 kDa polypeptide antigen in $ShSEA_{Egy}$, as well as in worm extracts of

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Table 4: **Immunolocalization of the *S. haematobium* antigens detected by the MoAbs by indirect fluorescent antibody test (IFAT)**

Monoclonal antibody	Reactivity with miracidia*		
	Isotype	Surface staining	Internal staining
Sh1/71.7	IgM	+	+
Sh2/15.F	IgG_1	+	
Sh3/15.28	IgG_1	+	+
Sh3/38.2	IgM	+	+
Sh4/14.3	IgG_1	+	
Sh5/32.30	IgM	+	-

+= stained.
-= not stained.
*= miracidia of Ghanaian strain(s) of *S. haematobium*.

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Table 5: The molecular weights of the antigens detected by the Schistoma haematobium reactive MoAbs as determined by Western Immunoblot analysis

Monoclonal antibody	Isotype	Molecular weight (kDa)
Sh1/71.7	IgM	-
Sh2/15.F	IgG_1	-
Sh3/15.28	IgG_1	$(37, 46)^{ShSEAEgy}$
Sh3/38.2	IgM	-
Sh4/14.4	IgG_1	$(73)^{ShSEAEgy,ShW,SjW}$ $(78)^{SjW}$
Sh5/32.30	IgM	-

$ShSEA_{Egy}$= Soluble egg antigens of an Egyptian strain of *S. haematobium.*
UP_2-IP= Precipitated proteins from urine of S. haematobium infected persons.
ShW= Worm antigen extracts of Ghanian strain(s) of *S. haematobium.*
SjW= Worm antigen extracts of *S. japonicum.*

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S. haematobium (ShW) and *S. japonicum* (SjW). In addition, Sh4/14.3 bound a 78 kDa polypeptide in SjW.

5.3.4 *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 6, periodate oxidation had no effect on the binding of three MoAbs namely, Sh2/15.F, Sh4/14.3 and Sh5/32.30. The binding of these same MoAbs was, however, completely abrogated by proteinase-K treatment (Table 6). Reactivity of the remaining three antibodies (Sh1/71.7, Sh3/15.28 and Sh5/38.2) was affected by both periodate oxidation and proteinase-K digestion.

5.3.5 *Reactivity of the MoAbs with Antigens of Different Schistosome Species and Hookworm*

The aim of the experiments described here was to determine the suitability of the selected MoAbs as diagnostic reagents based on their ability to specifically detect any of the schistosome species. Table 7, summarizes the reactivity of the MoAbs with antigens of different schistosome species and hookworm (*Necator americnus*) as determined by micro-plate ELISA and dot-ELISA.

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Table 6: The nature of antigenic epitopes detected by the MoAbs as determined by periodate oxidatiion and proteinase-K digestion

Monoclonal antibody	Isotype	Sensitivity to Periodate	Sensitivity to Proteinase-K	Nature of antigenic epitope
Sh1/71.7	IgM	+	+	*C and P
Sh2/15.F	IgG_1	-	+	P
Sh3/15.28	IgG_1	+	+	*C and P
Sh3/38.2	IgM	+	+	*C and P
Sh4/14.3	IgG_1	-	+	P
Sh5/32.30	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.

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Table 7: Reactivity of selected MoAb with several antigens as determined by micro-plate ELISA and dot-ELISA

Monoclonal antibody	Reaction with								
	$ShSEA_{Egy}$	$ShSEA_{Gh}$	ShW	SjE	SjW	SmW	UP_1-IP	UP_2-IP	HwE
Sh1/71.7	+(+)	-(+)	+(+)	+(+)	-(+)	-(+)	-(-)	-(-)	-(-)
Sh2/15.F	-(-)	-(+)	-(-)	-(-)	-(-)	-(-)	+(+)	+(+)	-(-)
Sh3/15.28	+(+)	-(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
Sh3/38.2	+(+)	-(+)	+(-)	+(+)	-(-)	-(+)	-(-)	-(-)	-(-)
Sh4/14.3	+(+)	-(+)	+(-)	-(-)	+(-)	+(+)	-(+)	-(+)	-(-)
Sh5/32.30	+(+)	+(+)	+(-)	+(+)	+(-)	+(-)	+(+)	+(+)	-(-)

$ShSEA_{Egy}$ = Soluble Egg Antigens from an Egyptian strain of *S. haematobium*.
UP_2-IP= Ammonium sulphate precipitated proteins from urine of *S. haematobium* infected persons.
UP_1-IP= Naturally precipitated proteins from urine of *S. haematobium* infected persons.
$ShSEA_{Gh}$= Egg antigen extracts of Ghanaian strain(s) of S. haematobium.
ShW= Worm Extracts of Ghanaian strain(s) of *S. haematobium*
SjE= *S. japonicum* soluble egg extract.
SjW= *S. japonicum* soluble worm antigen extract.
SmW= *S. mansoni* soluble worm antigen extract.
HwE= *Necator americanus* (hookworm) egg extract.
()= contains results of dot-ELISA
+= MoAb reacts with antigen.
-= MoAb does not react with antigen.

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With the exception of Sh2/15.F, all the MoAbs reacted with the soluble egg antigens of the Egyptian strain(s) ($ShSEA_{Egy}$) of *S. haematobium* in both assays. Nevertheless, only Sh5/32.30 reacted with $ShSEA_{Gh}$ (the Ghanian strain(s)) by the micro-plate assay, even though all the MoAbs reacted with this antigen using the dot-ELISA (Table 7). Three MoAbs namely Sh2/15.F, Sh4/14.3 and Sh5/32.30 could detect antigens in precipitated urine proteins (UP_2-IP and UP_o-UP) obtained from persons infected with Ghanaian strain(s) of *S. haematobium*. However, Sh4/14.3 could bind the antigen in urine only with dot-ELISA (Table 7). As shown in Table 7, Sh1/71.7 and Sh5/32.30 cross-reacted with antigens from *S. haematobium*, *S. mansoni* and *S. japonicum* worms or eggs except *S. mansoni* eggs which were not tested. On the other hand, Sh2/15.F and Sh3/15.28 reacted specifically with *S. haematobium* egg antigens. Sh3/38.2 and Sh4/14.3 cross-reacted with almost all the schistosome antigens tested, except SjW and SjE, respectively. Further investigations into the specificity of the MoAbs using hookworm egg antigens, revealed no cross-reactivity.

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5.4 Discussion

The aim of the work described in this chapter was to characterize the MoAbs produced and described earlier in Chapter 4. The purpose was to find out if any of them could be used for diagnosis of *S. haematobium* infections, especially by detection of parasite antigens in urine.

It was found in this study that the MoAb Sh2/15.F reacted with precipitated proteins obtained from the urine of persons infected with Ghanaian strain(s) of *S. haematobium*, but this MoAb failed to react with antigens in soluble egg extract of Egyptian strain(s) of the parasite. This result may suggest that Sh2/15.F bound to an antigen which is not shared between the Ghanaian and Egyptian strains of *S. haematobium*, or that the MoAb bound a non-parasite antigen in the precipitated urine proteins.

IFAT studies carried out to localize the antigens detected by the MoAbs, however, revealed that they all bound, at least, to antigens on the surface membrane of miracidia belonging to Ghanaian strain(s) of *S. haematobium*. The conclusion, therefore, is that Sh2/15.F may be detecting an antigen which is specific to the Ghanian strain(s) of *S. haematobium*, or that the antigen detected is not expressed in the egg stage of the Egyptian strain(s). In an earlier study, Hamburger *et al.* (1976) reported that a glycoprotein antigen coded MSA_1 was both egg-and species-specific to *S. mansoni*. With the cross-reacting analysis described in this work, the protein antigen bound by Sh2/15.F was shown to be specific to the egg stage of *S. hamatobium*. This observation coupled with the appearance of the antigen in the urine of infected persons, suggest that Sh2/15.F

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may be useful in the development of MoAb-based assays for specific diagnosis of urinary schistosomiasis. The protein nature of the antigen makes it even more suitable for use as a diagnostic antigen since it can be cloned and multiplied by recombinant DNA technology. 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 shown to be simple, specific, sensitive, field portable and reagent conservative (Pappass, 1988).

The inability of some of the MoAbs to detect antigens in both the micro-plate and dot-ELISA, was not surprising, since it had been reported that unlike plate-ELISA in which adsorption of soluble antigens to well surface may vary significantly (Burt, Carter, and Kricka, 1979; Kricka, Carter, Burt, Kennedy, Holder, Holliday, Telford and Wisdom, 1980), with the dot-ELISA technique the entire antigen applied is immobilized on the nitrocellulose membrane (Kumar, Band, Samantaray, Dang and Talwar, 1985). This means that differences in binding characteristics of the two different surfaces could lead to concealment of antigenic epitopes and hence failure of the MoAb to bind the antigen.

Another MoAb, Sh3/15.28 detected an antigen which is also *S. haematobium* species-specific and is present only in the egg stage. However, unlike the antigen bound by Sh2/15.F, the antigen detected by Sh3/15.28 was found to be a glycoprotein present in the egg lysate of both Ghanaian and Egyptian strains of *S. haematobium*, thus suggesting that Sh3/15.28 may be suitable for diagnosis of *S. haematobium* in different geographical areas. The applicability of Sh3/15.28 may, however, be limited by its inability to detect antigens in the urine of infected persons. Also, this

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glycoprotein may not be suitable for use in vaccination, because it is generally known that immunological responses to the glycoprotein egg antigens are largely responsible for the pathology in schistosomiasis (Erasmus, 1987).

Generally, the ability of some of the MoAbs to detect antigens in all the schistosome species tested, suggested that *Schistosoma* genus-specific antigens were being detected. Particularly important was the observation that Sh5/32.30 behaved as a pan-*Schistosoma* MoAb which was also able to detect antigens in infected human urine. This MoAbs is likely to be useful not only for general diagnosis of schistosoma infections, but also in studies towards the search for protective antigens. This is because it has been pointed out that the most suitable vaccine candidate antigens for schistosomiasis are the cross-reactive proteins (Hillyer and Serrano, 1982). It should, therefore, be interesting to study further the protein antigen detected by Sh5/32.30, especially, with respect to its specificity for the *Schistosoma* genus and its protective ability.

These findings indicate that, all the MoAbs included in this study are likely to be useful in diagnosis of urinary schistosomiasis caused by *S. haematobium*. In addition, some of the MoAbs could be useful in studies aimed at identifying *Schistosoma* genus-specific protective antigens for development of a vaccine against schistosomiasis.

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5.5 Summary

Six mouse monoclonal antibodies (MoAbs) [Sh1/71.7 (IgM), Sh2/15.F (IgG_1), Sh3/15.28 (IgG_1), Sh3/38.2 (IgM), Sh4/14.3 (IgG_1) and Sh5/32.30 (IgM)] produced against *S. haematobium* antigens were extensively characterized. IFAT studies showed that all the MoAbs stained the surface membranes of miracidia, yet three of them (Sh1/71.7, Sh3/15.28 and Sh3/38.2) stained internal cytoplasmic antigens in addition. Proteinase-K digestion and periodate oxidation studies showed that these three MoAbs bound glycoprotein antigenic determinants, whilst the remaining antibodies (Sh2/15.F, Sh4/14.3 and Sh5/32.30) detected protein epitopes. With the western immunoblot analysis, it was found that only Sh3/15.28 and Sh4/14.3 could bind antigens. Sh3/15.28 bound two antigens of MW 34 and 46kDa in soluble egg antigens from Egyptian strain(s) of *S. haematobium* ($ShSEA_{Egy}$), whilst Sh4/14.3 reacted with a 73kDa antigen in $ShSEA_{Egy}$, and in worm antigen extracts of *S. haematobium* (ShW) and *S. japonicum* (SjW), as well as a 78kDa antigen in SjW.

Cross-reactivity studies revealed that Sh3/15.28 and Sh2/15.F bound *S. haematobium* species-specific antigens. Both MoAbs also appeared to be stage specific as they could bind antigens in only the egg stage of the parasite. The remaining four antibodies cross-reacted with all the three major human schistosomes namely, *S. haematobium*, *S. mansoni* and *S. japonicum*. Three MoAbs, Sh2/15.F, Sh4/14.3 and Sh5/32.30 could detect *S. haematobium* antigens in infected human urine. None of the MoAbs cross-reacted with hookworm (*Necator americanus*) egg antigens.

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These findings indicate that the MoAbs generated in this study may facilitate the development of MoAb-based assays for specific diagnosis of schistosomiasis caused by *S. haematobium*.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

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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 (Manson, 1989). As a result, schistosomiasis prevalence is increasing, particularly in the developing world (Savioli and Mott, 1989), where the most successful control strategy, so far, involves the use of chemotherapy through primary health care (Mott, 1987; Bergquist, 1987; Manson, 1989; Awadalla, el-Mansoury and el-Azzouni, 1992). This chemotherapeutic approach requires identification of infected individuals in large populations and, therefore, demands very simple, sensitive, and specific diagnostic procedures.

Routine diagnosis of human schistosomiasis, at present is by microscopic examination of excreta (urine/stool) for the presence of schistosome eggs, or by the detection of haematuria or proteinuria. These methods are, however, either labour intensive or inaccurate (Webbe, 1982). It is, therefore, essential to develop more sensitive and specific field applicable schistosomiasis diagnostic methods. In this thesis, effort was directed at producing anti-*S. haematobium* MoAbs that could be used to diagnose urinary schistosomiasis through the detection of parasite antigens in the urine of patients.

Schistosome antigens have been reported to occur in the urine of patients infected with any of the three important schistosomes (*S. haematobium*, *S. mansoni* and *S. japonicum*) that infect man (Sherif, 1962; Shoeb *et al.*, 1968; Peters *et al.*, 1976; DeJonde *et al.*, 1989). Also, D`Amelio, Natu, Seminara, Palmisano and Aiuti

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(1981) and Feldmeier *et al.* (1983) reported the occurrence of immune complexes involving schistosome antigens and human immunoglobulins of various isotypes in the blood or urine of infected persons. In studies described in Chapter 4, anti-*S. haematobium* MoAbs were produced using proteins extracted from the urine of infected persons (Bosompem, unpublished data). This successful generation of anti-schistosome MoAbs with urine proteins as immunogen, has shown that despite widespread occurrence of immune complexes, urine is a good source of immunogen for raising MoAbs against urine-based parasite antigens. Furthermore, this work has shown that the non-schistosome proteins in infected human urine are very immunogenic, however, the chances of producing MoAbs against parasite antigens in urine could be greatly increased by selective immunization, using crude parasite antigens in the last booster just three days before cell fusion.

The potential usefulness of one of the two *S. haematobium* species-specific MoAbs as a diagnostic reagent was demonstrated through its reactivity with parasite strains from different geographical areas (Chapter 4). In those experiments, it was shown that some of the MoAbs were schistosome life-cycle stage-specific whilst others cross-reacted with other schistosome species. Perhaps one of the most interesting observations with these experiments was that some of the MoAbs behaved as pan-schistosome antibodies in that, they bound antigens in all the three important human schistosome species that were tested. Those MoAbs may, therefore, be useful in diagnosis of general *Schistosoma* infections. Similar observations were made by Hamburger *et al.* (1976) who characterized an *S. mansoni* species-specific antigen

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which was also egg stage-specific, and cross-reactive antigens that were expressed in *S. mansoni*, *S. haematobium* and *S. japonicum*.

"Concomitant immunity" in schistosomiasis has been interpreted to suggest the presence of stage-specific schistosome antigens which permit one stage of the parasite to survive in the host while another stage is destroyed (Cioli, Liberti and Festucii, 1987). Domingo, and Warren (1968), Hamburger et al. (1976) and Carter and Colley (1976) identified some of these antigens which were not only stage-specific but also species-specific in *S. haematobium*, *S. mansoni* and *S. japonicum*. In studies described in Chapter 5 of this study, species-and stage-specific schistosome antigens were observed. It is, however, not known whether any of the antigens identified has any such significance. It has also been reported that protective 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 some of the antigens bound by the MoAbs described in this thesis may be worth further investigations so as to determine their protective ability.

Using immunological procedures, urinary schistosomiasis may be diagnosed by serum or urine analysis (Deelder *et al.*, 1989; DeJonge *et al.*, 1989). However, 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

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patient. The availability of *S. haematobium* species-specific and pan-schistosome MoAbs (Chapter 5) that detect parasite antigens in the urine of infected humans, therefore, offers a good opportunity to develop field applicable MoAb-based assays for schistosomiasis. Furthermore, it is known that unlike parasite specific antibodies, circulatory antigens do disappear from circulation and urine within a few weeks after chemotherapeutic cure (DeJonge *et al.*, 1989). Detection of urine based parasite antigens, therefore, provides in addition to diagnosis a basis for determination of drug efficacy. Indeed, Deelder, *et al.* (1989) introduced a micro-plate ELISA for detecting a schistosome adult worm gut associated proteoglycan antigen called circulating anodic antigen (CAA) in the urine of patients. However, this CAA antigen was found to be non species-specific (Deelder *et al.*, 1990) and, therefore, not suitable for distinguishing between schistosome species infections in areas where they overlap. Thus, in Ghana for example, it had been necessary to differentiate between *S. haematobium* and *S. mansoni* infections in several localities.

The ability of some of the MoAbs to detect antigens with the dot-ELISA further indicated their suitability for field diagnosis of schistosomiasis. This is because the Nitrocellulose membrane-based dot-ELISA has been shown to be simple, specific, sensitive, field portable and reagent conservative (Pappas, 1988).

In conclusion, this study provides information on the suitability of MoAbs as diagnostic reagents for specific diagnosis of urinary schistosomiasis caused by *S. haematobium*, and *Schistosoma* genus infections in general. It also provides evidence

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that some of the MoAbs could be useful in the search for candidate antigens for use in a schistosomiasis vaccine.

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