The effects of anti-schistosome drugs on schistosomes and the immune responses of their hosts

by

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Abstract

The effects of Mirazid® (MZD), an extract of myrrh, on *Schistosoma mansoni* were investigated and compared to those of praziquantel (PZQ). The effects of both drugs on the soluble proteome and the immune responses to schistosome infections were also investigated. *In vitro* studies showed that MZD was more effective than PZQ against the schistosomula stage. However, its effects against adult worms were less than those of PZQ. Lengthy exposures to high concentrations of MZD were required for the drug to be effective. *In vivo* studies showed that MZD had no anti-schistosomal activity against *S. mansoni* in mice.

In vitro exposure of adult worms to either PZQ or MZD caused significant changes in the expression of some proteins. Interestingly, some vaccine candidates including paramyosin and actin were among the proteins that showed differential expression.

Treament of human *S. haematobium* infection with PZQ favoured the development of protective immune responses that render the individuals resistant to reinfection. Sera from putatively resistant individuals showed distinct recognition patterens of soluble worm antigen, after exposure to reinfection, compared to sera from susceptible individuals. Interestingly, many putative vaccine candidate antigens were recognised by antibody isotypes that are found in individuals with some degree of resistance to infection.

In conclusion, although the *in vitro* studies revealed some promising effects of MZD, the results of the *in vivo* studies do not support the use of this drug in the treatment of schistosome infections.

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Dedications

To my Dad,

to my Mum,

to my wife,

to my children, Ahmed and Mariam

and to my brothers and sisters

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Author's Declaration

I declare that this thesis is of my own composition and that the research described herein was performed entirely by myself except where expressly stated.

Osama F Sharaf
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List of abbreviations

2-D	Two dimensional gel
APS	Ammonium persulphate
BSA	Bovine serum albumin
Ca^{+2}	Calcium
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
Cm	Centimetre
ConA	Concanavalin A
DIGE	Differential in gel electrophoresis
D_L	Diffusion coefficient
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzume linked immunosorbent assay
ESMS	Electrospray mass spectrometry
EST	Expressed sequence tag
FCS	Foetal calf serum
FRAP	Fluorescent recovery after photobleaching
g	Gram
G3PGH	Glyceraldehydes 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GST	Glutathione-S-transferase
h	Hour
H&E	Haematoxylin and eosin
HPF	High power field
IAA	Iodacetamide alkylates
ICAM-1	Intercellular adhesion molecule 1 (ICAM-1),
IEF	Isoelectric focusing
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	interleukin

IM	intramuscular
IP	intraperitoneal
IP	Isoelectric point
IPG	Immobilised pH gradient
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
LFA-1	Lymphocyte function-associated antigen 1 (LFA-1),
LNs	Lymph nodes
Log	Logarithm
MALDI	Matrix-assisted laser desorption ionisation
MAP3	Multiple antigenic peptides
min	Minute
ml	Millilitre
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MZD	Mirazid
$Na\ N_3$	Sodium azide
NaCl	Sodium chloride
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMF	Peptide mass fingerprint
PZQ	Praziquantel
rIL	Recombinant interleukin
SC	Subcutaneous
SDS	Sodium dodecyl sulphate
SEA	Soluble egg antigens
SEM	Scanning electron microscopy
STAT-6	Signal transducer and activator of transcription 6
STEG	Anti-schistosomular tegument
STST-4	Signal transducer and activator of transcription 4
SWAP	Soluble adult worm antigen preparation
TEM	Transmission electron microscopy

TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
Th	T helper lymphocyte
TNF	Tumour necrosis factor
ToF	Time of flight
VLA-4	Very late antigen 4
VLA-6	Very late antigen 6
WHO	World Health Organisation
WWH	Whole worm homogenate

Chapter one

General Introduction

1.1 Introduction

Schistosomiasis is a chronic parasitic disease caused by a blood dwelling trematode (fluke) of the genus *Schistosoma.* It is a major parasitic disease in the tropics and subtropics, with more than 200 million people in 76 countries estimated to be infected and about 600 million people at risk of infection (Savioli *et al.*, 1997).

Schistosomiasis is strongly linked with human behaviour, as humans are both the victims of the disease and in most epidemiological settings the major source for the spread of the infection. Infection occurs on human contact with infected water containing the cercariae. Within minutes to hours of contact of the cercariae with the skin of a susceptible host, they penetrate the skin, shed their tails and complete their transformation into highly motile schistosomula. The schistosomula migrate into the micro-circulation and are carried to the heart, then to the lungs and after another 10 days or so, they reach the small veins of the portal circulation in the liver. There, they mature and the mature male and female worms pair. The female worms start to lay eggs 4-6 weeks after the initial penetration of the cercariae through the skin (Strickland and Abdel-Wahab, 1991). There is strong evidence that coupling with male worms is essential for the development, sexual maturation and subsequent egg production by female worms. However, the stimulus for their development and maturation is not yet determined (Ribeiro-Paes and Rodrigues, 1997).

The pathology and clinical symptoms of schistosomiasis are mainly related to the immunological reactions stimulated by the deposition of eggs which pass from the blood into the tissues of the host. Soluble egg antigens originating from the developing miracidium in the egg diffuse out through ultra-microscopic pores in the egg shell and

initiate the granulomatous reaction, a manifestation of a delayed hypersensitivity response mediated by T-cells (Warren, 1972).

The control of human schistosomiasis is principally dependent upon the use of chemotherapy. The major objectives of chemotherapy are to eradicate or control human infection thus alleviating the symptoms of the disease by preventing further deposition of eggs and reducing the number of eggs passed by patients into the environment. The reduction of contamination of the environment with eggs could result in reduced miracidium-snail contact and thus lead to lower levels of transmission (El Garem, 1998). However, few clear examples of such reductions can be found in the literature (Ouma *et al.*, 1985). Fortunately, non-toxic, oral, schistosomicidal drugs are available to treat the infection. The drug of choice is praziquantel (Fallon *et al.*, 1992). Reinfection after treatment and the possible appearance of resistant strains ensures schistosomiasis remains a major public health problem (Ismail *et al.*, 1996). In the last few years two 'new' drugs including artemesinin derivatives and Mirazid®, reported to have the potential for treating human schistosomiasis have been investigated.

1.2 Parasite

1.2.1 Historical notes

The ancient Egyptians recorded human infection with schistosomes in the papyrus of Kuhn with symbolic depiction of endemic haematuria around 1900 years BC. In 1910, Ruffer described ova in the kidneys of Egyptian mummies who lived around 1250-1090 years BC (El Garem, 1998).

In Egypt, Theodore Bilharz described the worm that caused Egyptian endemic haematuria in 1851. He named the parasite *Distomum haematobium*. In 1858, Weinland

proposed the name *Schistosoma*. At that time it was thought that one species was responsible for both the intestinal and vesicular forms of schistosomiasis. In 1905 Sir Patrick Manson postulated that intestinal and vesicular schistosomiasis are distinct diseases, caused by distinct species of worms. The complete life cycle of the parasite and the role of the snail intermediate host were defined in 1913 by Miyairi and confirmed experimentally by Leiper (a graduate of the University of Glasgow) in 1915 (Smith and Roberts, 1989; Strickland and Ramirez, 2000).

1.2.2 Details of the life cycle of schistosomes

Adult worms mate in the small vasculature of the liver and migrate against the venous blood flow to the venous plexus. The female progresses further alone to the narrowest parts of the veins and lays her eggs. The favoured eventual location of the adult worm and consequently of egg deposition varies according to species; *S. haematobium* is concentrated in the blood vessels around the bladder and *S. mansoni* in the inferior mesenteric vessels of the large intestine (Strickland and Ramirez, 2000).

In order for the parasites' life cycle to be completed, eggs must pass from their site of deposition in the vasculature through the tissues and into the lumen of the intestine or the bladder. This is achieved in part by virtue of their spines and the secretion of histiolytic enzymes. In mice, about 50% of the deposited ova succeed in reaching the external environment *via* urine and stools of the host while the remaining eggs are retained in the tissues. If eggs reach fresh water, they hatch and motile, relatively short-lived miracidia emerge and swim ceaselessly in water where they may encounter their next and intermediate snail host (El Garem, 1998).

In the vicinity of a snail host, miracidia are stimulated to swim more rapidly and change direction much more frequently, thus increasing their chances of encountering the snail.

During penetration of the snail, the miracidium sheds its epithelium and begins development into a mother sporocyst, normally near the point of entrance. After about 2 weeks, the mother sporocyst produces daughter sporocysts, which migrate to other organs within the snail. The mother sporocyst continues producing daughter sporocysts for up to 6 to 7 weeks (Smith and Roberts, 1989).

Cercariae metamorphose from sporocysts and start to emerge from the snail into the water about 4 weeks after snail infection with miracidia. The cercariae can survive up to 3 days under controlled laboratory conditions although their infectivity begins to diminish by 8-12 hours. Once cercariae come in contact with the skin of a suitable host, they penetrate the skin and transform into schistosomula. Schistosomula enter the vascular system directly (less commonly, indirectly through the lymphatics). They migrate through the right heart to the lungs, and then *via* the left heart and the systemic circulation to the splanchnic vasculature of the hepatic portal system, eventually reaching the sinuses of the liver to mature in about 4 to 6 weeks to complete the cycle (Sturrock, 2001).

1.2.3 Morphology

Morphology of cercariae and schistosomula

Schistosome cercariae are furcocercus with a head (the immature body of the adult worm) 175 to 240μm long by 55 to 100μm wide and a locomotory organ or tail 175 to 250μm long by 35 to 50μm wide. The head has an anterior organ, often called the oral sucker, and a prominent, muscular ventral sucker or acetabulum (see figure 1.1d) The body is bounded by a syncytial tegument with an external lipid bilayers which, in

electron micrographs, appears trilaminate and covered with an external amorphous and carbohydrate rich "glycocalyx" (Smith and Roberts, 1989; Sturrock, 2001).

During cercarial penetration the tail is shed and the body, now called a schistosomulum, enters the skin of the definitive host (see figure 1.1e). The conversion from the cercariae to schistosomula is a crucial and potentially vulnerable point of the life cycle. This transformation process involves many structural and physiological changes, the most important of which is the loss of the outer water-proof glycocalyx and its replacement with an outer lipid bilayer, providing the organism with a heptalaminate membrane. Furthermore, acquisition of host antigens and other manifestations of immunological survival mechanisms become evident at this point (Basch, 1991).

Morphology of adult worms

Adult worms are from 12 to 26mm in length and from 0.3 to 0.6mm in width. The adult male worm is shorter and thicker than the longer, slender female. The male has a longitudinal body cleft, the gynaecophoric canal or schist (hence their names), in which the female lies during most of her life. Both sexes have weakly developed oral suckers perforated by the mouth. The ventral sucker, situated antero-ventrally, is more strongly developed in male worms than female worms (see figure 1.1g). The entire body is covered with a tegument which is a continous syncytium bounded with an external heptalaminate membrane.

Beneath the tegument, there are circular and longitudinal muscles coordinated by a network of neural fibres to permit contractions and other movements. The alimentary canal passes from the oral cavity into an oesophagus, dividing immediately anterior to the ventral sucker into two lateral gut caeca that reunite behind the gonads to form a single, blind posterior gut caecum. There is no anus and the contents of the gut are

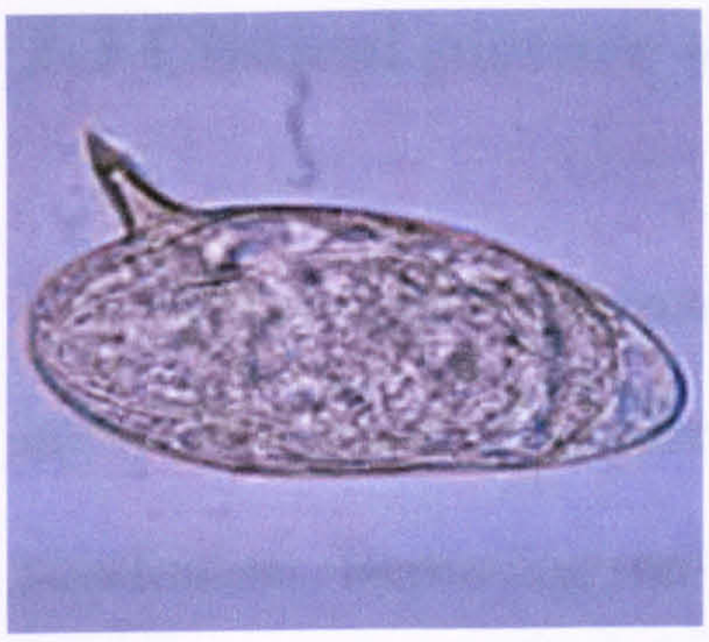
periodically regurgitated through the mouth. The vomitus contains both parasite digestive enzymes and the products of digestion, largely breakdown products of erythrocytes.

The reproductive organs of schistosomes are unremarkable in that male reproductive system consist of several testes (6 to 9 in *S. mansoni*), a number of *vasa deferentia* joining to form a *vesicula seminalis*, an ejaculatory duct, and a genital pore situated posterior to the vetral sucker. The female reproductive system is composed of an elongated ovary, an oviduct, vitelline glands, shell glands, and an ootype in the central canal that passes forward to the uterus. The straight or slightly tortuous tubule-shaped uterus leads to the genital pore just behind the ventral sucker.

The excretory system is composed of fine tubules which link the protonephridial flame cells embedded in the mesenchymatous cells to the posterior excretory bladder, allowing excretion of excess water and soluble metabolic waste products (Strickland and Ramirez, 2000; Sturrock, 2001).

Morphology of the eggs

The eggs of all schistosome that have so far been described are non-operculate. They are either ovoid or spherical and range in size from 60 to 400μm in length. They are immature when laid but mature rapidly during their 6-10 days' passage to the exterior (see figure 1.1a). The mature egg contains a single miracidium, a ciliated larval stage. The shell, which is either clear or yellow in colour, is composed of sclerotin-tanned protein and is perforated by numerous micropores through which can pass enzymes produced by the enclosed miracidium. The egg of *S. mansoni* is oval in shape with prominent lateral spine. It measures 115 to 175μm in length and 45 to 70μm in width (Strickland and Ramirez, 2000; Sturrock, 2001).



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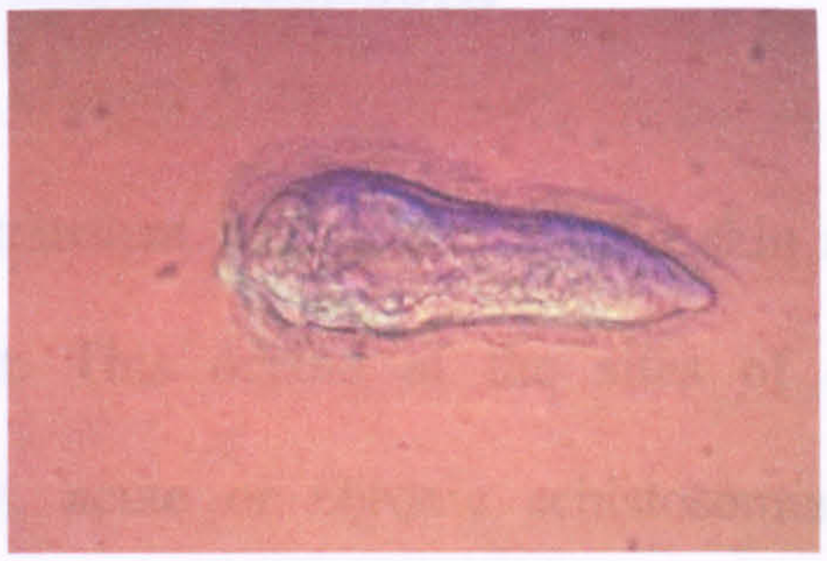

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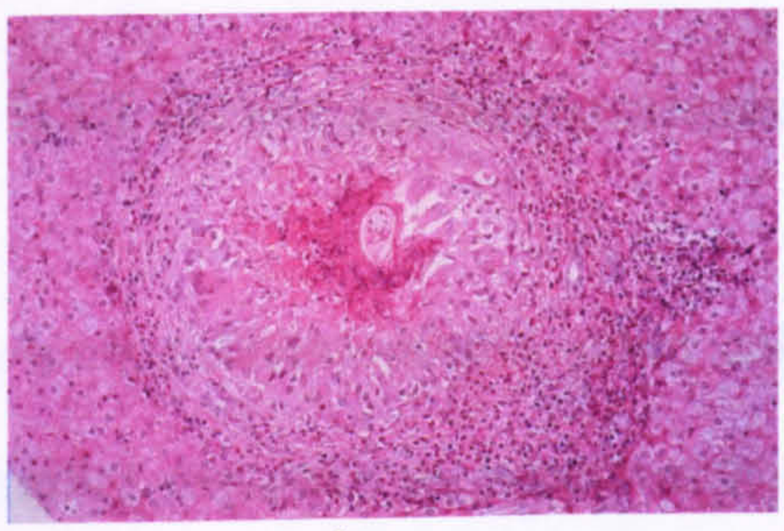

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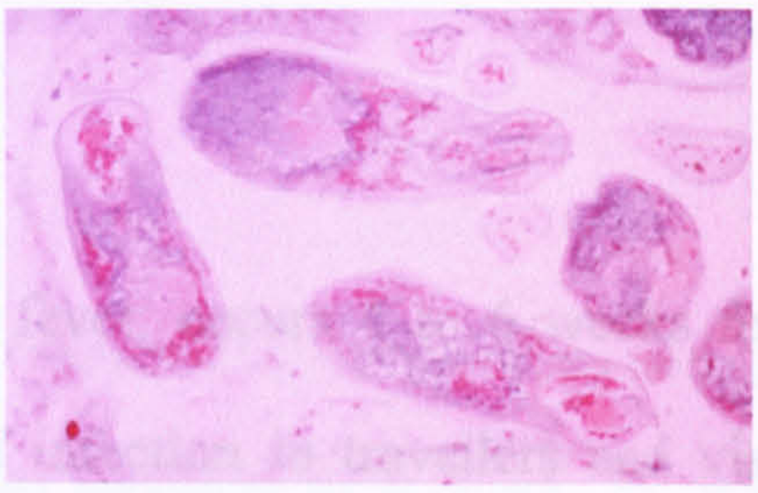

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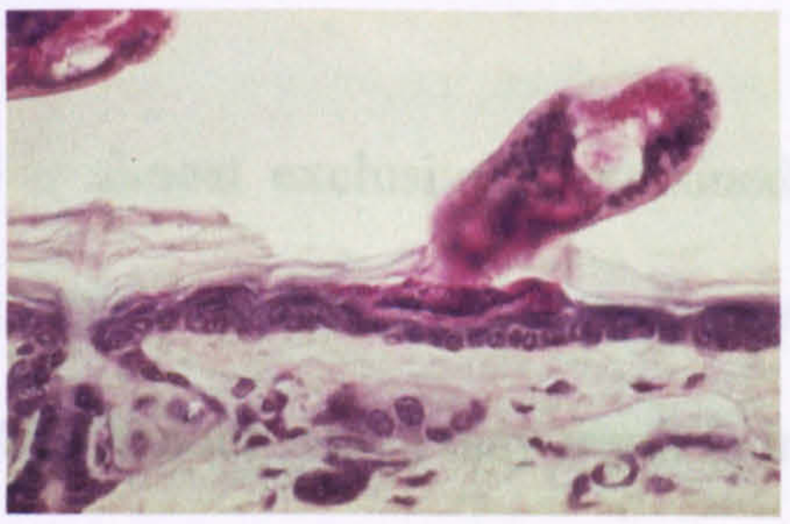

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Figure 1.1 Life cycle of *S. mansoni*.

(a) Eggs of *S mansoni*, (b) miracidium, (c) Intra-molluscan stages, (d) Cercaria, (e) schistosomulum, (f) maturing worms in the mesenteric blood vessels, (g) Adult worm pairs and (h) eggs surrounded by granuloma.
(Courtesy of The Wellcome Trust. International Health Image Collection.)

1.3 Clinical picture of schistosomiasis mansoni

The first evidence of infection may be a transient itching and a mild skin rash that develops immediately after water contact. This occurs at the sites of cercarial penetration. Depending on several factors, acute or chronic schistosomiasis may develop.

1.3.1 Acute schistosomiasis

Acute schistosomiasis, often called Katayama fever, occurs 4 to 6 weeks following initial exposure to infection. It is a feature of infection in travellers and visitors to endemic areas and is less commonly found in the indigenous peoples of endemic areas. Katayama fever usually starts at the time adult worms begin to mature and with the onset of egg deposition. It is presented as a systemic allergic illness characterised by fever, chills, weakness, weight loss, cough, arthralgias, marked eosinophilia and often, abdominal pain and bloody diarrhoea (Neva and Brown, 1994).

1.3.2 Chronic schistosomiasis

The pathogenesis of chronic schistosomiasis is almost exclusively explained by the hosts' granulomatous response to schistosome eggs deposited in tissues. Deposition of eggs in the intestinal wall results in granulomatous lesions which may result in intestinal polyposes, haemorrhages, enteropathies, fibrosis and strictures. The liver is another favoured site for the trapping of *S. mansoni* eggs during infection. The chronic granulomatous response produces a peri-sinusoidal inflammation and fibrosis called Symmers' clay pipe-stem fibrosis. The pathogenesis of severe schistosomiasis results from the slow, cumulative blockage of portal flow that results in portal hypertension,

porto-systemic collateral circulation, oesophageal varices and upper gastrointestinal bleeding which is the most common morbid consequence of hepatic schistosomiasis and is responsible for the majority of deaths due to schistosomiasis. Interestingly, the liver functions remain normal until the very late stages of the disease. Eggs can embolise in other places including the central nervous system resulting in granuloma formations at such sites (Neva and Brown, 1994; Strickland and Ramirez, 2000; King, 2001).

1.4 Diagnosis of *S. mansoni*

In order to diagnose schistosome infection, to determine the strain of schistosomes and to determine the severity of infection, several methods have been developed and exploited.

1.4.1 Parasitological diagnosis

Detection of ova and their morphological identification is the traditional method of diagnosis. It is a cheap, direct and specific means of diagnosis but there is a possibility of missing light infections that are characterised by low egg output. The most widely used protocols are those based on the Kato-Katz thick faecal smear (Kato and Miura, 1954; Katz *et al.*, 1972). Other methods including stool concentration techniques and rectal snip examination are also used. For instance, a separation technique based upon the greater density of viable schistosome eggs relative to faecal material has been described and has been found to be more sensitive in detecting light infections than the Kato-Katz thick smear method (Eberl *et al.*, 2002).

1.4.2 Detection of parasite antigens

The presence of detectable quantities of parasite-derived antigens in the circulation and excreta of infected hosts has prompted considerable research on their potential for immunodiagnosis of schistosomiasis. Several circulating antigens have been employed including circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), named according to their migratory behaviour in immuno-electrophoresis. A variety of assay methods have been used to detect these antigens. Parasite antigen detection methods have the advantage of being schistosome specific. However, they can miss light infections and, because of the shared carbohydrate epitopes between schistosome species, they cannot be used for differentiation between schistosome species (Doenhoff *et al.*, 2004).

1.4.3 Detection of antibodies

This method is based on the detection of anti-schistosomal antibodies produced by the host in response to exposure to infection. Both humans and animals normally respond to a range of different parasite antigens. Several crude and purified antigens from different life cycle stages have been used in antibody detection assays. The most widely used assay method is the enzyme-linked immunosorbent assay (ELISA). This method is normally less specific than the antigen detection method, it is of limited value in monitoring the curative effects of chemotherapy and it is not correlated with the intensity of infection. However, the use of purified antigens in such assays has greatly improved these factors (Hamilton *et al.*, 1998; Doenhoff *et al.*, 2004). For instance, the use of the CEF6 fraction of soluble egg antigens, which consists of two antigens (a 30kDa ω-1 and a 36-41kDa α-1), has been tested for the diagnosis of schistosomiasis

with sensitivity and specificity values of 91% and 90%, respectively (Mott and Dixon, 1982).

1.4.4 Detection of parasite DNA

In the last few years, a polymerase chain reaction has been developed for the diagnosis of human *S. mansoni* infections. It exploits a highly repeated schistosome DNA sequence that can be detected in the sera and faeces of infected individuals. The method is very specific and very sensitive. It is normally used effectively in situations when there is good laboratory infrastructure (Pontes *et al.*, 2002).

1.5 Liver granuloma in *S. mansoni* infection

After egg deposition, soluble egg antigens (SEA) are released from the developing miracidium. These antigens induce host sensitisation and recruitment of lymphocytes, eosinophils, macrophages, giant cells and fibroblasts which constitute the host granulomatous response (Boros and Warren, 1970). The granulomatous response serves to wall-off the eggs and prevent the release of potentially toxic molecules and help to destroy the ova (Doenhoff *et al.*, 1986). The mechanism of granuloma formation is T lymphocyte-dependent (Harrison and Doenhoff, 1983) and the $CD4^+$ T helper subset is key (Mathew and Boros, 1986).

While the schistosome granuloma formation and development is largely dependent on Th2-immune response, the role of Th1-immune response cannot be neglected especially in the early phases of granuloma formation. With the beginning of egg deposition and early phases of granuloma formation in murine schistosomiasis, at weeks 5 to 6, high levels of IFN-γ and low levels of IL-4 and IL-5 have been reported implicating a role of Th1-immune response. As deposition of eggs in tissues continues, the immune response

is shifted toward a Th2-type immune response and by 8 weeks after infection, when granuloma attain their maximal sizes, high levels of IL-4, IL-5 and minimal levels of IFN-γ are detected. These results indicate that the egg-induced granuloma is mainly a Th2-type immune response (Pearce *et al.*, 1991; Lukacs and Boros, 1992). The role of the Th2 response in granuloma formation was confirmed with the work carried out by Kaplan and colleagues (1998). They used (signal transducer and activator of transcription 4) STAT4- and STAT6-deficient mice, which lack Th1 and Th2 type responses, respectively. Lymphocytes from both naive and infected STAT6-deficient mice produced minimal levels of Th2 cell cytokines but showed enhanced production of IFN-γ following schistosome egg injection. This shift away from a Th2 cell-mediated immune response was coupled with the development of pulmonary and hepatic granulomas that were greatly decreased in size compared with those in control littermates. Hepatic granulomas in STAT6-deficient mice were composed predominantly of mononuclear cells with only a sparse appearance of eosinophils. The smaller size of the hepatic granulomas was accompanied by decreased amounts of liver hydroxyproline indicating reduced collagen deposition. In contrast, lymphocytes from infected STAT4-deficient mice produced Th2 cell cytokines in amounts comparable to those produced by control littermates, but low levels of IFN-γ. The granuloma size and amount of collagen deposition in the liver were equivalent to those seen in control littermates. These studies demonstrate that Th2 cells are required for the full development of the granulomas and fibrosis (Kaplan *et al.*, 1998).

While much is known about the roles of cytokines in granuloma formation, new information is being generated. For instance, repeated injections of rIL-2 or rIL-4 in chronically infected mice restored the down-regulated liver granulomas to their peak size (Mathew *et al.*, 1990; Yamashita and Boros, 1992). Injections of anti-IL-2 (Cheever *et al.*, 1992) and anti-IL-4 monoclonal antibodies (Yamashita and Boros,

1992) suppressed the granulomas in acute murine infection. These results together confirm the inflammatory role of IL-2 and IL-4 in schistosomiasis.

Using IL-4, IL-13 and IL-4/IL-13-deficient mice infected with *S. mansoni*, Fallon and colleagues demonstrated that IL-13-deficient mice showed significantly enhanced survival following infection, which correlated with reduced hepatic fibrosis. In contrast, increased mortality was manifest in IL-4-deficient and IL-4/13-deficient mice, and this correlated with hepatocyte damage and intestinal pathology. This hepatic damage was caused as a result of a skewed Th1 type response. They concluded that IL-13 is detrimental to survival following infection, whereas IL-4 is beneficial (Fallon *et al.*, 2000a; Fallon *et al.*, 2000b). Furthermore, IL-13 stimulated collagen production in fibroblasts, and procollagen I and procollagen III mRNA expressions were decreased in mice treated with an IL-13 inhibitor, soluble IL-13 R alpha 2-Fc fusion protein. The reduction in fibrosis observed in IL-4-deficient mice was much less pronounced than that in soluble IL-13 R alpha 2-Fc treated mice. These results confirm that IL-13 is the dominant Th2-type cytokine regulating fibrosis (Chiaramonte *et al.*, 1999).

Furthermore, administration of recombinant tumour necrosis factor-alpha (rTNF-α) to chronically infected mice enhanced liver granuloma size and injection of anti- TNF-α to acutely infected animals suppressed their granulomas (Joseph and Boros, 1993). In human *S. mansoni* infection, TNF-α was associated with aggravation of hepatic periportal fibrosis while IFN-γ and IL-10 was associated with protection against fibrosis (Henri *et al.*, 2002; Booth *et al.*, 2004).

Recombinant interleukin (IL)-12 administered together with schistosome eggs partly prevented pulmonary granuloma formation on subsequent intravenous challenge with eggs, partly inhibited hepatic granuloma formation and dramatically reduced the tissue

fibrosis in murine *S. mansoni* infection. The effect of IL-12 was attributed to increased production of IFN-γ. These results revitalised the concept of the development of a vaccine preventing pathology rather than infection (Wynn *et al.*, 1994; Wynn *et al.*, 1995; Sher *et al.*, 1996; Qadir *et al.*, 2001).

Interleukin-10 seems to be the most important down-regulatory cytokine for granuloma formation and fibrosis. Repeated injections of anti-IL-10 mAb and studies of IL-4, IL-10, IL-12 in single and double knockout mice showed that IL-10 is an important endogenous down-regulator of Th2-type as well as Th1-type cytokine synthesis, preventing over expression of either of these immune response types. It was also shown that IL-10 regulates not only the intensity of hepatic inflammation, but also granuloma organization and cohesiveness (Wynn *et al.*, 1997; Boros and Whitfield, 1998; Sadler *et al.*, 2003). *In vitro*, IL-10 was shown to be an important cytokine regulating the *in vitro* granulomatous reactivity of PBMC from intestinal *S. mansoni* patients (Falcao *et al.*, 1998). In murine *S. mansoni* infection, IL-10 is generated by both the innate and adaptive immune response, with both sources regulating the development of type-2 immunity, immune-mediated pathology, and survival of the infected host. Most of the $CD4^+$ T cell-produced IL-10 was confined to a subset of T cells expressing CD25 (Hesse *et al.*, 2004).

The role of transforming growth factor-beta (TGF-β) in *S. mansoni* granuloma formation and fibrosis is controversial. On the one hand, treatment of cultured hepatic cells with TGF-β1 increased the level of type 1 procollagen mRNA and increased levels of TGF-β1 gene expression that precedes the increase in collagen synthesis was demonstrated (Czaja *et al.*, 1989). On the other hand, SEA-induced TGF-β correlated with diminished hepatic granuloma size during *S. mansoni* infection in the baboons, implying its participation in down-modulation of granuloma (Mola *et al.*, 1999).

Furthermore, diminished immuno-pathology in *S. mansoni* infection following intra-nasal administration of cholera toxin B subunit conjugated to an immunodominant peptide (amino acid 234-246) of Sm-P40 of *S. mansoni* eggs correlated with enhanced TGF-β production (Hernandez *et al.*, 2002). A similar immuno-modulating effect of TGF-β in *S. japonicum* hepatic fibrosis was reported (Zhu *et al.*, 2000).

Other molecules and chemokines may play a role in formation or modulation of hepatic granulomas. The co-stimulatory B7 molecule, on the surface of antigen-presenting cells, may bind to CD28 receptor on T cells and enhance their proliferation (Greenfield *et al.*, 1998). Intercellular adhesion molecule 1 (ICAM-1), lymphocyte function-associated antigen 1 (LFA-1), very late antigen 4 (VLA-4) and VLA-6 may play a role in initiation and maintenance of hepatic granulomas (Jacob *et al.*, 1997). Furthermore, mice infected with *S. mansoni* and deficient in the IgE gene developed smaller granulomas than the wild animals implicating a role for IgE in granuloma formation (King *et al.*, 1997). On the other hand, gene knock out experiments indicated a role for B lymphocytes and Fcε-R-bearing cells in down regulation of granulomas and fibrosis (Jankovic *et al.*, 1997; Jankovic *et al.*, 1998).

Of course the effects of single or combination of cytokines on the formation of the granulomas are relatively easy to investigate but it must be remembered that during granuloma formation in humans and animals, an array of different cytokines operate at different levels and at different times.

1.6 Immunity to schistosomiasis

1.6.1 Evidence of acquired immunity in humans to schistosomiasis

The strongest circumstantial evidence for immunity to schistosomiasis in humans is the pattern of infection intensity in human populations in relation to their age. The age-intensity curve assumes a convex shape with older individuals being less heavily infected than young children. This pattern has been described in areas endemic with *S. haematobium* (Wilkins *et al.*, 1984; King *et al.*, 1988) and *S. mansoni* (Gryseels, 1991). The peak intensity of infection was shifted from approximately 15 years of age, for areas of low transmission, to 10 years of age for areas of high transmission of *S. haematobium* infection in Zimbabwe suggesting a role for acquired immunity that is dependent on a cumulative experience of infection (Woolhouse *et al.*, 1991). Similarly, a negative correlation between the mean intensity of infection and the age of the peak of intensity has been described in *S. mansoni* infection in Kenya (Fulford *et al.*, 1992) and in Senegal (Marguerite *et al.*, 1999). In this context, chemotherapy accelerated the development of the acquired immune responses to *S. haematobium* infection (Mutapi *et al.*, 1998) and this has been speculated to be a result of increasing the exposure and release of previously concealed antigens of the damaged or dead parasites (Woolhouse and Hagan, 1999).

In order to dissect the age-related immunity to schistosomiasis and to overcome the problems encountered with human population studies, Butterworth and colleagues established the treatment-reinfection approach to studying *S. mansoni* in humans. They treated children with praziquantel to remove the existing worms and re-examined the children for infection 12 months after chemotherapy. The mean age of the resistant group, with no or few eggs in their stools, was two years greater than the susceptible

group, with high egg output in the stools. These results were interpreted as evidence that the resistance was acquired and age-dependent (Butterworth *et al.*, 1984; Butterworth *et al.*, 1985).

A similar approach was used for the study of human *S. haematobium* infection in the Gambia. Reinfection was found to depend on exposure and other factors (Hagan *et al.*, 1985). In a second study by the same group, the trend for reinfection to decrease with increasing age, after an allowance for variation in exposure, was highly significant. These observations suggested that subjects in this area slowly acquired an increasing degree of immunity to the acquisition of *S. haematobium* infection (Hagan *et al.*, 1987; Wilkins *et al.*, 1987). Recently, more detailed experiments have confirmed the role of acquired immunity in human schistosomiasis haematobium (Hagan *et al.*, 1991; Mutapi *et al.*, 2003) and schistosomiasis mansoni (Demeure *et al.*, 1993; Abebe *et al.*, 2001).

1.6.2 Evidence of humoral immunity:

Due to ethical considerations it is impossible to conduct exposure experiments to examine the immune responses against schistosomes infection in humans. As an alternative, several experimental animals as well as animal reservoir hosts have been exploited to study the immune response to schistosome infection. In mice, while *S. mansoni* irradiated cercariae protected B-cell deficient mice against re-infection with *S. mansoni*, the protection achieved was not as great as that achieved in the wild-type mice vaccinated with the same regime, implicating a role for B-cells in immunity to *S. mansoni* infection. The level of protection was restored when B-cell deficient mice received sera from singly vaccinated wild-type mice. Furthermore, multiple vaccinations of mice deficient in IFN-γ increased the levels of protection compared to those obtained with a single vaccination. The increased protection was attributed to an

increase in the antibody levels (Jankovic *et al.*, 1999b). Multiple vaccination of wild-type mice was associated with Th2 reactivity in the form of an increase in IL-4, IL-5 and IgG1 antibodies (Caulada-Benedetti *et al.*, 1991). Moreover, sera from multiply vaccinated mice conferred protection when transferred to naïve mice confirming the role of humoral immunity in protection against schistosomiasis in mice (Wynn *et al.*, 1996).

In rats, that are essentially non-permissive hosts, primary infection with *S. mansoni* induced a strong, immunologically mediated resistance to re-infection, with IgE directly implicated in this phenomenon (Capron and Capron, 1994). Treatment of rats with anti-IgE antibodies partially inhibited the expression of this protective immunity, supporting the role of IgE in protection against schistosomiasis in rats (Cetre *et al.*, 2000). In relation to this, serum from rats multiply immunised with radiation-attenuated cercariae conferred passive protection to naïve animals when it was given at time the challenge parasites were migrating through the lungs (Ford *et al.*, 1987).

In baboons, a single infection with 1000 *S. mansoni* cercariae and multiple infections with 100 cercariae per week for 10 weeks induced 59% and 80% protection to challenge infection, respectively. The animals in this experiment were cured with praziquantel prior to challenge with 1000 cercariae. The only immune correlate of protection was the levels of soluble adult worm antigen (SWAP)-specific IgE in serum at the time of challenge infection and/or 6 weeks later (Nyindo *et al.*, 1999). Furthermore, three and five vaccinations of vervet monkeys with radiation-attenuated *S. mansoni* cercariae resulted in 48% and 39% protection, respectively. The specific IgG levels peaked after three vaccinations and there was a clear correlation between the antibody levels at the time of challenge and the protection observed in individual vervets (Yole *et al.*, 1996).

In human populations, the first trial to correlate the reinfection levels after treatment with humoral responses was carried out by Butterworth and colleagues. They treated 129 Kenyan school children infected with *S. mansoni* and measured the reinfection levels and the parasite specific IgG and IgM. They found a negative correlation between reinfection intensities and both IgM anti-schistosomulum antibodies and IgM and IgG anti-egg antibodies. They concluded that these antibodies act as blocking antibodies that interfere with an effective immune response (Butterworth *et al.*, 1987).

The first clear correlation between antibodies and resistance came from the work of Hagan and colleagues on *S. haematobium* in endemic areas in the Gambia. A 40% sample of the age spectrum of the study village were included in this study and the prevalence of re-infection after treatment was found to be higher in children than in adults although some had the same level of exposure. IgG subclasses and IgE specific to adult worm and egg antigens were measured. It was found that IgE was highly correlated with resistance to re-infection and IgG4 was correlated with susceptibility to re-infection (Hagan *et al.*, 1991). Similarly, correlation between anti-adult worm IgE (Dunne *et al.*, 1992) and anti-larval IgE (Rihet *et al.*, 1991) and resistance to re-infection after treatment was reported in human *S. mansoni* infection.

In another treatment re-infection study of *S. mansoni*, IgE showed positive and IgG4, and IgG2 showed negative association with resistance to re-infection after chemotherapy. The opposite effects of IgE and IgG4 were undissociable in the analysis, indicating that these isotypes probably antagonise each other in protection (Demeure *et al.*, 1993). This antagonism was further supported by the work of Caldas and colleagues (2000). They reported an increase in IgE anti-SWAP and anti-schistosomular tegument (STEG) responses and a decrease in IgG4 anti-SEA and anti-STEG responses after treatment in the resistant group (Caldas *et al.*, 2000).

In a Zimbabwean study, anti-SEA IgE and anti-SWAP IgA negatively correlated with levels and intensity of *S. haematobium* infection. On the other hand, intensity of infection correlated positively with total IgG, IgG4 and IgM responses to SEA and with IgG4 and IgM responses to SWAP. These findings supported the concept of IgG4 and IgM as blocking antibodies. Interestingly, similar recognition patterns by IgE and IgG4 of the schistosomulum antigens was detected in immunoblotting suggesting a sort of competition between these antibodies for the same antigens (Ndhlovu *et al.*, 1996). A similar positive correlation between IgE and IgA and protective immunity against *S. haematobium* was reported in Egyptian individuals (Khalil *et al.*, 1999).

In *S. japonicum*, a positive correlation between anti-SWAP and anti-SEA IgG4 antibodies and susceptibility to reinfection and a negative correlation between anti-SWAP and anti-SEA IgE antibodies and intensity of reinfection were reported in a Chinese population (Li *et al.*, 2001).

In summary, the immunoepidemiological studies of human populations showed significant correlation between resistance to re-infection with schistosomes and some antibodies. Of particular interest are IgE, which correlated positively with resistance and IgG4, which correlated negatively with resistance to re-infection.

1.7 Treatment of human schistosomiasis

At present, praziquantel, oxamniquine and metrifonate appear on the World Health Organisation's list of essential drugs: metrifonate is employed for the treatment and control of infection with *S. haematobium*, oxamniquine for *S. mansoni* and praziquantel for all schistosome species infecting humans, including *S. japonicum* (Cioli *et al.*, 1995).

1.7.1 Metrifonate

Metrifonate is an organophosphorous compound. It is unstable in aqueous solutions and it spontaneously and non-enzymatically transforms into various compounds, of which the most interesting is dichlorvos which is the active molecule (Nordgren *et al.*, 1980).

By inhibition of schistosome acetylcholinesterase, metrifonate (or its breakdown product, dichlorvos) causes accumulation of acetylcholine and flaccid paralysis of worms. The paralyzed schistosomes lose their hold on the inner wall of blood vessels and are swept away in the bloodstream. As *S. haematobium* are located mostly in the veins of the vesical plexus, they are shifted to the lungs *via* the *vena cava* where they remain trapped and eventually die (Cioli *et al.*, 1995).

The generally accepted therapeutic regimen in human *S. haematobium* infections consists of 3 oral administrations of 7.5-10mg kg^{-1} at intervals of 14 days (Davis and Bailey, 1969). Cure rates have been reported to average between 40-60% after one dose, 50-80% after two doses, and over 90% after three doses (Snellen, 1981).

Metrifonate is a well-tolerated drug with low incidence of mild and transient side effects thought to be a consequence of cholinergic stimulation. These side effects include; fatigue, muscular weakness, tremor, sweating, salivation, fainting, abdominal colic, diarrhoea, nausea, vomiting and bronchospasm (Davis, 1993).

1.7.2 Oxamniquine

Oxamniquine is considered as an oxidative metabolite of the parent compounds; lucanthone and UK-3883. It is effective against *S. mansoni* and ineffective against *S. haematobium* and *S. japonicum*. It shows more activity against male worms than female

worms and little activity against the immature stages. Oxamniquine exerts its action most likely through the irreversible inhibition of nucleic acid synthesis in sensitive schistosomes (Cioli *et al.*, 1995). Furthermore, hycanthone and oxamniquine have anti-cholinergic effects that by blocking the acetylcholine receptors, will remove the inhibiting effect of acetylcholine. The resulting disturbance of motility disrupts peristalsis and feeding mechanisms to the point of causing a delayed death by starvation (Hillman *et al.*, 1978).

The usual therapeutic dose is a single oral dose of 15-20mg kg^{-1}, although a higher dose of up to 60mg kg^{-1} divided over 2-3 days may be needed in Egypt, South Africa and Zimbabwe (World Health Organisation, 1990). With the appropriate therapeutic dose, cure rates of over 80% and often over 90% have been reported. The drug has been used safely in all stages of the disease. Some late-stage and complicated forms have shown clinical improvement. Oxamniquine is well tolerated with side effects limited to a mild and transient dizziness, headache and occasional drowsiness. Transient neuropsychiatric disturbances have been recorded in a very small number of patients (Foster, 1987; Davis, 1993).

1.7.3 Praziquantel

Praziquantel, a pyrazino-isoquinoline derivative, is the drug of choice for the treatment of schistosomiasis because it has a high efficacy against all schistosome species and against cestodes; lacks serious short-term and long-term side effects; is administered as a single oral dose and is cheap (Cioli *et al.*, 1995; Kabatereine *et al.*, 2003).

The most important effects of praziquantel on schistosomes are tegumental damage in the form of vacuolisation at the base of tegumental syncytium and blebbings at the

surface (Becker *et al.*, 1980), muscular contraction and spastic paralysis (Pax *et al.*, 1978) and metabolic changes (Andrews, 1985).

Damage to the surface of the parasite could prime hosts immune responses by exposing antigens previously concealed from the hosts immune system (Harnett and Kusel, 1986; Brindley *et al.*, 1989; Woolhouse and Hagan, 1999). This is indirectly supported by the previous finding that the effects of praziquantel are dependent upon the hosts immune responses (Sabah *et al.*, 1985; Brindley and Sher, 1987). In this context, treatment with praziquantel has been shown to change the antibody isotype profile of treated children. The effect was dramatic, a single treatment with praziquantel had the capacity to transform the response of susceptible children to that of adults considered to be immune against infection (Mutapi *et al.*, 1998; Mutapi *et al.*, 2003).

The exact mode of action of praziquantel is not known, however, its effects can be interpreted as a direct or indirect consequence of Ca^{2+} redistribution among various worm tissues and the environment (Day *et al.*, 1992). In support of this, calcium channels have been described in schistosomes and they have been shown to be crucial for praziquantel action (Kohn *et al.*, 2001b; Kohn *et al.*, 2003).

The standard recommended dose of praziquantel is a single oral dose of 40mg kg^{-1} body weight for *S. mansoni, S. haematobium,* and *S. intercalatum.* In *S. japonicum* infections, a total dose of 60mg kg^{-1} is recommended, split in two or three doses in a single day. *S. mekongi* may require two treatments at 60mg kg^{-1} (Davis, 1993). Praziquantel is a safe drug with minimal side effects related to the gastrointestinal tract in the form of abdominal pain, nausea, vomiting, anorexia and diarrhoea. Other side effects including headache, dizziness, drowsiness, pruritus and fatigue may occur (Davis, 1993).

The cure rates recorded with these doses were 75-85% for *S. haematobium*, 63-85% for *S. mansoni*, 80-90% for *S. japonicum*, 89% for *S. intercalatum* and 60-80% for double infections with *S. mansoni* and *S. haematobium* (Wegner, 1984). However, a lower cure rate of 36-38% has been reported in *S. mansoni* infection in Senegal and strains of lower susceptibility to praziquantel have been isolated (Gryseels *et al.*, 1994). Other resistant strains have been isolated from Egypt (Ismail *et al.*, 1996). *In vitro* tests on these isolates and laboratory-selected isolates support the conclusion that a degree of resistance to PZQ can occur in *S. mansoni*, but the levels of drug resistance found so far are low (Doenhoff *et al.*, 2002). Furthermore, although praziquantel is very effective against cercaria, invasive stages and adult schistosomes, it has been shown to have a significantly lower efficacy against the immature stages of schistosomes, between 1 to 4 weeks after infection (Xiao *et al.*, 1985; Sabah *et al.*, 1986).

1.7.4 Ro11-3128 and Ro15-5458

Ro11-3128 and Ro15-5458 are benzodiazepine derivatives which showed antischistosomal effects. A single oral dose of 80mg kg^{-1} of Ro11-3128 showed 90% cure rates of mice or hamsters infected with either *S. mansoni* or *S. haematobium*. Ro11-3128 was active against all stages of schistosomes (Bickle and Andrews, 1985). However, in a pilot clinical study in humans, a subcurative dose caused severe and long-lasting sedation, ataxia and muscle relaxation (O'Boyle *et al.*, 1985). RO15-5458 was administered at two dose levels (25mg and 15mg kg^{-1} body-weight) to *S. mansoni* infected vervet monkeys. It dramatically reduced the faecal egg-output, worm burden and tissue egg load (Suliaman *et al.*, 1989). In *S. haematobium* infection, RO15-5458 was used, in comparison with PZQ, for treatment of hamsters at different stages of infection. RO15-5458 was more effective against 4-week infection than PZQ. After 8

and 12 weeks of infection both drugs showed remarkable anti-schistosome effects (Guirguis, 2003).

1.7.5 Artemisinin derivatives

Artemisinin is the active principle derived from the leaves of *Artemisia annua.* The main anti-schistosomal derivatives of artemesinin are artemether and artesunate (Utzinger *et al.*, 2003). Artemisinin derivatives are characteristically active against the immature stages, 1 to 3 weeks after infection, of *S. mansoni*, *S. japonicum* and *S. haematobium* with less effect on the very early invasive and the adult worms (Utzinger *et al.*, 2001b; Utzinger *et al.*, 2001c). Hence, artemether has been used as a prophylactic agent against *S. mansoni*, *S. japonicum* and *S. haematobium* with significant reduction in prevalence (Xiao *et al.*, 2002; N'Goran *et al.*, 2003).

Furthermore, artesunate has been tried with praziquantel as a combination chemotherapy for the control of *S. mansoni* and *S. haematobium* resulting in higher cure rates as compared to placebo, praziquantel alone or artesunate alone (De Clercq *et al.*, 2000; Borrmann *et al.*, 2001). The usual dose of artemether for human prophylaxis against schistosomiasis is 6mg kg^{-1} every 2 to 4 weeks for periods of up to 6 months. This regimen has reduced the neurotoxicity which is the main adverse effect of the drug (Utzinger *et al.*, 2003).

1.7.6 Myrrh (Mirazid®)

In the last few years, myrrh which is an oleo-gum resin, derived from the stem of *Commiphora molmol* is said to be effective against *S. haematobium* and *S. mansoni* with cure rates of up to 91.7% after single course of 10mg kg^{-1} every day orally for 3 days. The cure rate increased to 98% after a second course. The drug was well tolerated with

minimal side effects (Sheir *et al.*, 2001). As a consequence of that, Mirazid®, myrrh, has been licensed and released for the treatment of human schistosomiasis in Egypt. Nothing is known about its mode of action, however, the product data sheet indicates that it causes separation of adult worm pairs with a consequent shift of female worms to the liver where they are destroyed. It also indicated that Mirazid® improves the clinical picture of the treated individuals one week of the treatment. However, there has been significant debate in the scientific community about the true efficacy of Mirazid® with very low cure rates being reported (Fenwick *et al.*, 2003).

In summary, there is a consensus that praziquantel is central to the treatment of human schistosomiasis and it has been used on a large scale for a long duration. The limited emergence of schistosome strains with lower susceptibility to praziquantel and the fear of the spread of praziquantel-resistant strains have made it very important to find alternatives and to investigate any drug with potential activity against schistosomes.

The work in this thesis has been designed to:

- Investigate the *in vitro* anti-schistosomal activities of Mirazid®, compared to the standard drug praziquantel, against adult worms and schistosomula stages of *S. mansoni*.

- Investigate the *in vivo* anti-schistosomal activities of Mirazid®, compared to praziquantel, against *S. mansoni* in mice.

- Try to elucidate the mode of action of Mirazid® in terms of the effects on viability and the damage it causes.

- Investigate the effects of Mirazid® and praziquantel on the expression of different proteins of adult *S. mansoni*.

- Investigate the impact of Mirazid® and praziquantel on the immune response of mice in response to *S. mansoni* infection.

- Investigate the different recognition patterns of *S. haematobium* antigens in human infections before treatment, after treatment and after re-infection.

Chapter Two

A comparative study of the effects of Mirazid® and praziquantel on schistosomula and adult worms of *Schistosoma mansoni* and on murine immune responses to infection.

2.1 Introduction

2.1.1 *Schistosoma mansoni*: the tegument

Schistosomes infect their mammalian hosts by direct penetration of the skin. As cercariae penetrate the skin of a suitable host, they begin to undergo substantial changes. These changes include the loss of the outer glycocalyx and substitution of their outer trilaminate membrane with a heptalaminate membrane. These structural changes start to be evident 30 minutes after penetration and manifest as loss of the glycocalyx, decrease in the number of cercarial spherical bodies and appearance of vacuoles with membranous contents in tegumental matrix. However, the outer membrane of the 30-min schistosomula is trilaminate. In the 3-h schistosomula the outer membrane is almost heptalaminate and the tegument contains small membranous bodies and few discoid bodies (Hockley, 1973; Hockley and McLaren, 1973). In this context it has been suggested that the gross changes that occur during schistosomula transformation are accompanied with modification of the surface membrane lipid from a gel phase with restricted mobility to a liquid crystalline phase with increased motility (Foley *et al.*, 1988).

The changes in the parasite tegument continue as the parasites develop. When *S. mansoni* has fully matured the tegument of the adult male is approximately 4μm thick and is bound externally by a surface membrane. While the surface membrane is normally heptalaminate, some areas of membrane showing a trilaminate or even a multilaminate appearance can be identified using transmission electron microscopy. The surface membrane serves as an interface between the parasite and the surrounding environment. The tegument is bounded internally by a trilaminate basal membrane which separates the tegument from the underlying muscle layer. This 10nm thick basal

lamina is highly folded and penetrates deep into the cytoplasm of the tegument. The tegumental cytoplasm consists of an electron-dense granular matrix in which membranous bodies, discoid bodies and mitochondria may be recognised. The inclusion bodies are formed in sub-tegumental cells (cytons) located beneath the muscle layers and are transferred to the tegumental cytoplasm through tortuous cytoplasmic connections connecting the cells with the tegument (Hockley, 1973; Hockley and McLaren, 1973; McLaren, 1980). The role of the inclusion bodies is not clear, however, the membranous bodies fuse with the outer membrane and may contribute to its formation during penetration, while the discoid bodies may contribute to membrane maintenance and turnover (McLaren, 1980; Skelly and Shoemaker, 2001).

In the area posterior to the ventral sucker, the dorsal surface of the male worm is covered with large tubercles covered with spines. The tubercles are few in number in the lateral and posterior parts of the dorsal surface. In these regions and between the tubercles, the surface of the worm is ridged and in the depressions between the ridges there are numerous distinct pits. Small numbers of individual spines and sensory papillae are present between the ridges. A characteristic feature of male schistosomes and one from which their name derives is the cleft (*schist*) in the body (*soma*). This groove is the normal location for the female worm during pairing and is designated the gynaecophoric canal. The surface of the gynaecophoric canal lacks tubercles and is ridged and covered with many small, irregularly arranged spines. The surface topography of female worms is less dramatic than that of male worms. The surface of the female worm lacks tubercles and only a few spines are present mainly at the posterior end (Race *et al.*, 1971; Hockley, 1973).

The tegument of schistosomes, particularly the surface membrane, is of fundamental importance for parasite survival in the blood stream of its hosts. It plays a role in nutrient uptake and in evasion of the hosts' immune responses (Chappell, 1974; Levy

and Read, 1975; MacGregor and Kusel, 1989). The tegument is metabolically highly active and undergoes continuous turnover and replacement. The *in vitro* rate of turnover of the outer bilayer of adult *S. mansoni* has been studied and estimated to have a half-life of 2-3 hours (Wilson and Barnes, 1977).

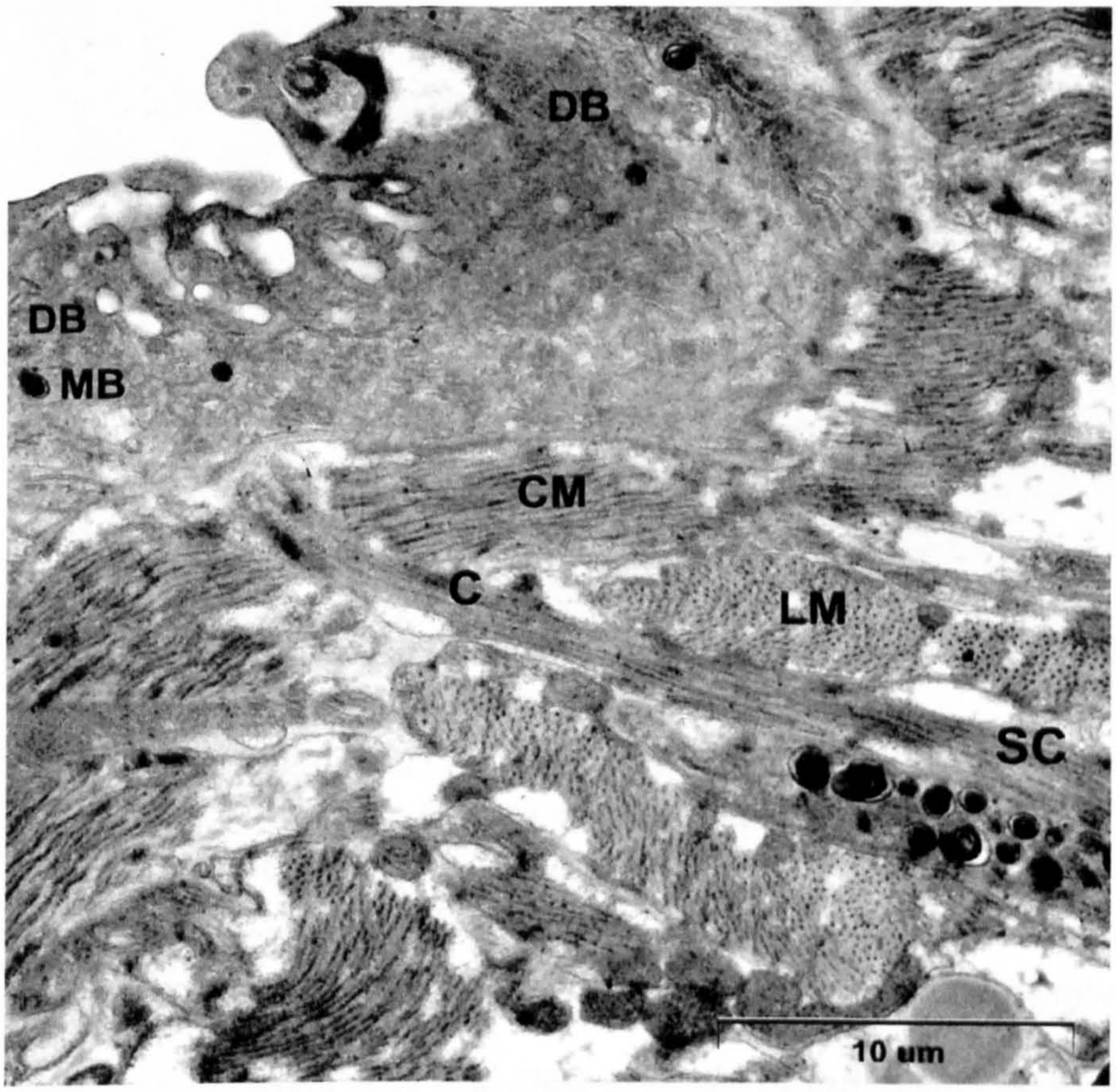

Figure 2.1 Ultrastructure of the male worm of *S. mansoni*.

The tegument of the schistosome contains inclusion bodies including discoid (DB) and membranous (MB) bodies. Beneath the tegument are the circular (CM) and the longitudinal muscles. The subtegumental cells (SC) are located beneath the muscle layers and they have cytoplasmic connections (C) with the tegument.

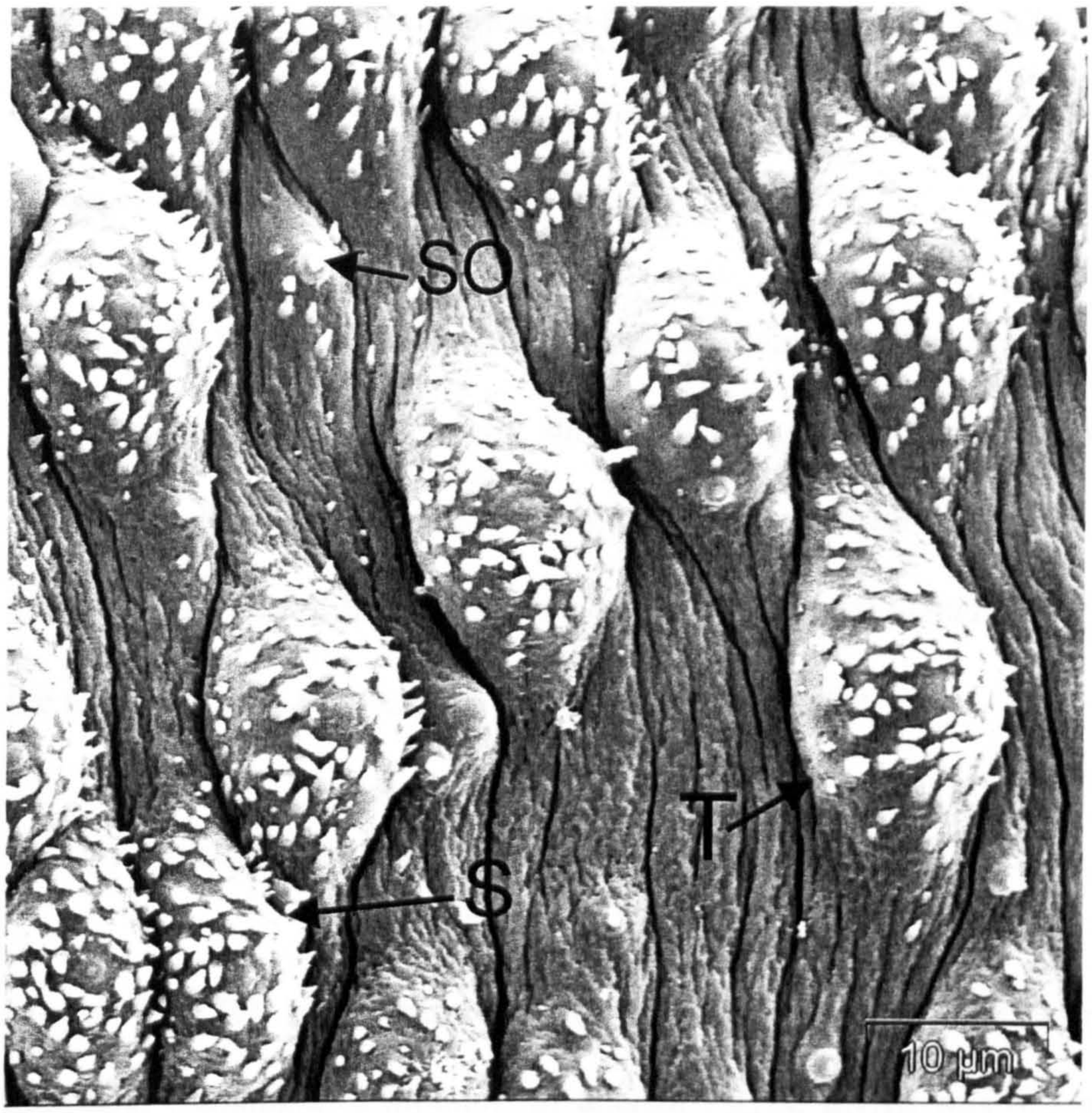

Figure 2.2 Surface topography of adult male *S. mansoni* worm.

The surface of adult male of *S. mansoni* has many tubercles (T) which are covered with spines (S). Between the tubercles the surface is ridged with a few scattered spines and sensory organs (SO).

The composition of the two bilayers of the outer tegumental membrane has been shown to be very different. The first clear evidence of differences between the layers was the selective requirement of the outer layer for uranyl acetate fixation while the inner layer could be fixed with ordinary fixatives (Hockley and McLaren, 1973). Freeze fracture studies of the tegument of *S. mansoni* have shown that the intra-membranous particles are more numerous in the outermost monolayer than the inner-most monolayer adjacent to the cytoplasm (McLaren *et al.*, 1978). Furthermore, Foley and colleagues (1986) have shown that lipid probes diffuse freely in the external monolayer of the outer tegument membrane of *S. mansoni* whereas in the inner layers they have a restricted diffusion (Foley *et al.*, 1986). This may be a consequence of the outer membrane being composed of micron-scale liquid crystalline-phase lipid domains that lack significant

amounts of trans-membrane proteins (Caulfield *et al.*, 1991) while the lateral diffusion of the inner bilayer is limited by the insertion of glycosylphosphatidylinositol-anchored glycoprotein antigens (Camacho *et al.*, 1995).

In addition to its critical role in the survival of the parasite, the tegument of schistosomes is also potentially important as a target for the action of many anti-schistosomal drugs, most importantly praziquantel (Andrews *et al.*, 1983; Cioli *et al.*, 1995). The effects of the anti-schistosomal agents, praziquantel (PZQ) and Mirazid®, on the tegument of *S. mansoni* are a major focus of this thesis.

2.1.2 Mirazid® as an anti-schistosomal drug

Mirazid® (MZD) is the trade name of myrrh which is an oleo-gum resin derived from the plant, *Commiphora molmol*. In ancient Egypt, myrrh was one of the constituents exploited to preserve mummies (Buckley and Evershed, 2001). In the recent years, there have been a number of studies on the biological activity of myrrh and as a result, many medicinal uses have been proposed for it. The anti-schistosomal activities of myrrh were first reported by Sheir and colleagues. They treated 204 Egyptian schistosomiasis patients, 171 infected with *S. mansoni*, 4 with *S. haematobium* and 29 with mixed *S. mansoni* and *S. haematobium*. They reported an average parasitologic cure rate of 91.7% after a single course of treatment and 98% after a second course of the drug. The drug was effective against both *S. mansoni* and *S. haematobium*. They also reported complete relief of symptoms in 90.7% of patients (Sheir *et al.*, 2001). In another study on human schistosomiasis, treatment with MZD at a dose of 600mg per day for six days was reported to have 97.4 and 96.2% cure rates against *S. haematobium* and *S. mansoni*, respectively (Abo-Madyan *et al.*, 2004). They also reported "a marvellous clinical cure without any side effects" after this treatment regime. Furthermore, MZD has been shown to have strong anti-schistosomal properties, *in vitro*, against *S. mansoni* adult

worms as determined by its ability to induce somatic muscle contractions and to cause tegumental damage of the parasite in the form of tegumental disruption, oedema and loss of the spines covering the tubercles (Hassan *et al.*, 2003).

In addition to its activity against schistosomes, MZD has been also reported to be effective against human fascioliasis at a dose of 12mg kg^{-1} of body weight daily for 6 consecutive days. All treated patients showed a pronounced improvement of their general conditions and symptoms and stopped passing eggs in their stools (Massoud *et al.*, 2001a). A similar fasciolicidal effect of MZD has been reported in sheep. A total dose of 600mg gave a cure rate of 83.3%, while a dose of 900 to 1200mg gave a 100% cure rate (Haridy *et al.*, 2003). MZD was also reported to be effective against human and sheep dicrocoeliasis dendriticum with a cure rate of 100% (Al-Mathal and Fouad, 2004).

Other reported properties of myrrh include; molluscicidal activities against *Biomphalaria alexandrina*, *Bulinus truncatus* and *Lymnaea cailliaudi* (Allam *et al.*, 2001; Massoud and Habib, 2003), larvicidal activity against *Culex pipiens* larvae (Massoud *et al.*, 2001b), anti-inflammatory effect (Kimura *et al.*, 2001), local anaesthetic, anti-bacterial and anti-fungal properties (Dolara *et al.*, 2000) and smooth muscle relaxing properties (Andersson *et al.*, 1997).

In Egypt, MZD has been licensed for use for the treatment of human schistosomiasis and fascioliasis and it has been said to be safe and well tolerated with few side effects (Sheir *et al.*, 2001; Massoud *et al.*, 2004). However, in other trials of treatment of human schistosomiasis, low cure rates have been reported and the previously reported high cure rate seems to be difficult to reproduce (Fenwick *et al.*, 2003). Furthermore, in a multicentre study for treatment of *S. mansoni* in mice indicated that MZD is not effective against adult schistosomes (Botros *et al.*, 2004). To clarify this situation, the

present study was designed to investigate the efficacy of MZD, compared to the standard drug PZQ, against *S. mansoni* both *in vitro* and *in vivo*.

2.2 Materials and methods

2.2.1 Drugs and reagents

Drugs

The extract of *Commiphora molmol* (myrrh) used in the experiments is produced by Pharco Pharmaceuticals (Egypt). It is sold as a "purified" extract in capsules of 300mg although details of the extraction process and subsequent processing are not available. It licensed and marketed as Mirazid® for the treatment of human schistosomiasis and fascioliasis in Egypt. Praziquantel was obtained as a powder from Bayer (Germany). Both MZD and PZQ were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 100mg ml^{-1}.

Reagents

Unless otherwise stated, all reagents used in the experiments were purchased from Sigma-Aldrich Company Ltd, Dorset, England. Details of the solutions are provided in the appendix.

In vitro experiments

2.2.2 Parasites

Recovery of adult *S. mansoni* worms

The Puerto Rican strain of *S. mansoni* is maintained in Professor John Kusel's laboratory (University of Glasgow) by routine infection of female TO mice. Adult worms were recovered by perfusion of 6-8 week-infected mice according to the method of Smithers and Terry (1965). Six to eight weeks after infection, mice were killed by an intra-peritoneal injection of 0.2ml (60mg ml^{-1}) of Sagatal. The abdominal and thoracic cavities of the mice were exposed by dissection and the mice were suspended against a vertical perspex sheet. An incision was made in the portal vein and heparinised RPMI-1640 medium was pumped into the heart using a 50ml disposable syringe. The worms passed through an incision in the portal vein and were collected on a sheet of muslin gauze and washed in RPMI-1640 before use in the experiments.

Mechanical transformation of schistosomula

The method used for mechanical transformation of schistosomula is a modification of that of Colley and Wikel (1974). *Schistosoma mansoni* cercariae freshly shed from *Biomphalaria galabrata* snails were transferred to a plastic universal. By chilling the cercariae on ice for 15 minutes, their motility is reduced and they rapidly sank to the bottom of the container. This facilitated their concentration in smaller volumes. Water was then removed until approximately 1ml remained and 9ml of pre-warmed (37°C) RPMI-1640 was added. The cercariae were then aspirated 10 times using a 10ml syringe and 21-gauge needle to shear off their tails. The schistosomula were washed twice in RPMI-1640 and left for 2 hours at 37°C to complete their transformation before use.

2.2.3 *In vitro* treatment and observations of adult worms of *S. mansoni*

Adult *S. mansoni* worms were incubated in complete RPMI-1640 medium (RPMI-1640 + 10% Foetal calf serum (FCS) + 2% penicillin/streptomycin + 2% Fungizone, all from Gibco Limited, Paisley, Scotland) with various concentrations (3, 6, 12, 25, 50, 100 and 200µg ml^{-1}) of either PZQ or MZD. Ten worm pairs, consisting of one male and one female, were incubated in a volume of 1ml in each well of a 24-well tissue culture plate (Microplate, IWAKI, Japan). As controls, worms were incubated in media containing 1% DMSO. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2. Damage, changes in shape, changes in motility and unpairing of adult worms were assessed using an inverted light microscope (Leitz DIAVERT®).

2.2.4 Assessment of the viability of schistosomula of *S. mansoni* after *in vitro* exposure to praziquantel or Mirazid®

Toluidine blue exclusion test

Schistosomula were incubated in 96-well tissue culture plates (Microplate, IWAKI, Japan). Two hundred schistosomula were incubated in 1ml of medium containing either MZD or PZQ at concentrations of 3, 6, 12, 25, 50, 100 and 200µgml^{-1}. As controls, schistosomula were incubated in media containing 1% DMSO. The viability and integrity of schistosomula were assessed by their ability to exclude the toluidine blue dye 1, 2, 4 and 8 hours after exposure. One percent of a methanolic solution of toluidine blue (Fisher Scientific UK LTD, Leics, UK) was spread over glass slides and allowed to dry. Schistosomula were then placed onto these slides and examined using a Leitz (DIALUX model 22EB) light microscope. Normal schistosomula remained unstained whereas damaged ones became stained with the dye (Dessein *et al.*, 1983).

Confirmatory evidence of drug effects on the viability of schistosomula of *S. mansoni* was obtained by using the Hoechst test.

Hoechst test

The effect of both drugs on the viability of schistosomula was further investigated using the Hoechst 33258 dye. Hoechst 33258 dye is relatively membrane impermeable and can access the cells through damaged membranes. It specifically binds to the DNA where it fluoresces (Modha *et al.*, 1997). Two hundred schistosomula were incubated for 4h in 1ml of medium containing either MZD or PZQ at concentrations of 50, 100 and 200μg ml^{-1}. As controls, schistosomula were incubated in media containing 1% DMSO. Hoechst 33258 was added (2μg ml^{-1}) during the last 20 minutes of incubation. Schistosomula were then mounted on slides and examined using a fluorescent microscope (Leitz Laborlux S with 3-1 PLEOMOPAK fluorescence illuminator). The damaged areas of the parasites gave a blue fluorescence.

2.2.5 Fluorescent Recovery After Photobleaching (FRAP)

Another method that has been used to investigate the effect of the drugs on the surface membrane of the schistosomes is FRAP. FRAP allows the assessment of membrane fluidity as an indirect indication of membrane function. For FRAP, the sample to be examined is probed with a fluorescent substance and then an attenuated laser beam is focused to a small spot on the sample. The initial fluorescence level inside the spot is measured and the laser is momentarily de-attenuated to bleach irreversibly the molecules within the spot. The recovery of fluorescence inside the spot is a consequence of the diffusion of unbleached molecules from outside the spot. FRAP thus provides two measures of diffusibility; the fraction (percentage recovery) of molecules

that are able to move and the diffusion coefficient (D_L) of that fraction (Modha *et al.*, 1997).

Incorporation of the fluorescent lipid analogue

Lipid motility on the surfaces of worms was examined by probing adult worms and schistosomula with a fluorescent lipid analogue, 5-N-octadecanoyl aminofluorescein (AF18) (Molecular Probes Europe BV, PoortGebouw, The Netherlands). *Schistosoma mansoni* adult males and schistosomula were incubated, as before, with either MZD or PZQ at concentrations of 200μg ml^{-1} (as a high drug concentration) and 20μg ml^{-1} (as a low drug concentration). As controls, parasites were incubated in media containing 1% DMSO. Twenty adult males or 200 schistosomula were incubated in 1ml of complete RPMI-1640 medium at 37°C and 5% CO_2. After 4 and 24 hours of incubation, 5μl of an ethanolic solution containing 10μg of the AF-18 probe were added to each well and the parasites were incubated for another 15 minutes. Then, parasites were washed 3 times with RPMI-1640 to remove any unbound probe. Worms were re-suspended in 100μl of RPMI, placed on a glass slide and immobilised by addition of 10μl of carbachol (10mg ml^{-1}). When myrrh was used, 100μl of carbachol (10mg ml^{-1}) was required to immobilise worms (see discussion).

Lipid mobility assessment

Measurement of the diffusion of lipid molecules in adult worm and schistosomula membranes was carried out according to the method outlined by Foley *et al.* (1986). Fluorescence was measured over a small and defined spot of 1.2μm diameter on the surface of the labelled parasite using an attenuated argon laser (LEXEL model 85) attached to a microscope (Ortho II). The power of the laser was increased for 70ms to irreversibly bleach a proportion of the fluorescent molecules in the spot, and the

recovery of fluorescence recorded using a Tektronix Digital real-time oscilloscope. The mobile fraction (percentage recovery), and the lateral diffusion coefficient of lipid molecules were measured in 5 spots on the tubercles of the dorsal tegument of each adult worm, just behind the head region. For measurements on schistosomula, a spot was selected in the central portion of the body.

2.2.6 Scanning Electron Microscopy (SEM)

Adult worms and schistosomula of *S. mansoni* were incubated with either MZD or PZQ at concentrations of 200μg ml^{-1} and 20μg ml^{-1} for MZD and 200, 20 and 5μg ml^{-1} for PZQ. As controls, parasites were incubated in media containing 1% DMSO (the drug solvent). Either twenty adult worms or 200 schistosomula were incubated in 1ml of complete RPMI-1640 medium at 37°C and 5% CO2. After 4, 24 and 72h of incubation, treated and control parasites were fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate for 1h and then washed 3 times for 5min each time with 0.1M phosphate buffer rinse (containing 2% sucrose). The samples were secondarily fixed in 1% osmium tetroxide for 1h, washed 3 times for 10 minutes each time with distilled water, fixed in 0.5% aqueous uranyl acetate, in the dark, for 1 hour and then washed again with distilled water. Then samples were dehydrated in ascending grades of alcohol, 30, 50, 70, 90 and 100% and finally in dried absolute alcohol, for 10 minutes each time. After critical point drying with liquid CO_2, the specimens were mounted on metal stubs with double-sided copper tape. They were coated with gold using a Polaron SC515 SEM coating system and examined using a Phillips SEM 500 electron microscope. The procedures were carried out as recommended by Hayat (1989).

2.2.7 Transmission electron microscopy (TEM) of adult *S. mansoni* worms after exposure to praziquantel or Mirazid®

Adult *S. mansoni* were incubated with either MZD or PZQ at concentrations of 200 and 20μg ml^{-1}. As controls, parasites were incubated in media containing 1% DMSO (the drug solvent). Twenty adult worms were incubated in 1ml of complete RPMI-1640 medium at 37°C and 5% CO_2. Four and 24h after incubations, treated and control parasites were fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate for 1h and then washed 3 times for 5min each time with 0.1M phosphate buffer rinse (with 2% sucrose). The samples were secondarily fixed in 1% osmium tetroxide for 1h, washed 3 times for 10min each time with distilled water, fixed in 0.5% aqueous uranyl acetate, in the dark, for 1h and then washed again with distilled water. Then samples were dehydrated in ascending grades of alcohol, 30, 50, 70, 90 and 100% and finally in dried absolute alcohol, for 10min each time. The specimens were washed with propylene oxide 3 times for 5min each time and further dehydrated by incubation in a 1:1 volume to volume mix of propylene and araldite. Specimens were left overnight on a rotator until the propylene oxide was evaporated leaving pure araldite, which was replaced by fresh araldite and left overnight. Samples were then embedded in moulds and left to polymerise at 60°C for 48h. Sections, 60-80nm thick, were cut using an ultramicrotome (Reichert Ultracut) and stained with 2% methanolic uranyl acetate for 5min followed by Reynold's lead citrate for another 5 minutes. Specimens were viewed using a TEM (Zeiss 902).

2.2.8 Calcium labelling with Fluo 3 AM

Fluo-3 AM cell permeant (Molecular Probes Europe BV, PoortGebouw, The Netherlands) is a fluorescent probe which fluoresces intensely when it binds calcium. Its

acetoxymethyl (AM) ester allows it to permeate the cell membrane. Once inside the cell, it is metabolised to an impermeable compound that leaks out only slowly. Fluo-3 AM is purchased as a powder in 50µg-vials. The contents of each vial were dissolved in 50µl of DMSO. Either twenty *S. mansoni* adult worms or 200 schistosomula were incubated in 1ml of complete RPMI-1640 medium to which 5µl containing 5µg of Fluo-3 AM was added. They were incubated for 2 hours at 37°C in 5% CO_2. MZD or PZQ was then added to the parasites to achieve a concentration of 200µg ml^{-1} for either drugs and incubation continued. As controls, parasites were incubated with 1% DMSO. After 15min, 30min, 1, 2 and 4h of exposure to the drugs, parasites were washed 3-4 times with RPMI-1640 to remove excess probe and drugs. The parasites were re-suspended in approximately 100µl of fresh RPMI medium and deposited within silicon grease squares on glass slips and covered with cover slides for quantitative measurements. All measurements were carried out using a fluorescent microscope (Leitz Laborlux S with 3-1 PLEOMOPAK fluorescence illuminator). Fluorescence was quantified using a Leitz MPV compact photometer with MPV-COMBI control electronics attached to a PC (Silicon Valley) installed with MPV-STAT software for data and statistical analysis (Leica).

The experiment was repeated but with the adult worms being exposed to the drugs prior to fluo-3 AM labelling. One and 4 hours after exposure to MZD or PZQ at 200µg ml^{-1}, parasites were washed with RPMI medium 3-4 times, to remove the drugs. They were then labelled with fluo-3 AM for 2h as before. The parasites were washed again with medium and quantitative fluorescent measurements were calculated as mentioned previously.

In vivo experiments

2.2.9 Design of the experiment

Thirty female TO mice were infected by subcutaneous injection of 150 cercariae (per mouse) of the Puerto Rican strain of *S. mansoni*. Six weeks after infection, they were divided into 3 groups; a control infected group (10 mice) left without any treatment, a MZD group (10 mice) treated with 400mg kg^{-1} of body weight daily dose of MZD given orally for 3 consecutive days and a PZQ group (10 mice) treated with a single dose of 400mg kg^{-1} of body weight of praziquantel given orally. A fourth group of control non-infected mice (5 mice) were included as control for cytokine assays (see figure 2.3). Two weeks after treatment, mice were perfused for recovery of adult worms. The whole liver of each mouse was dissected, weighed, a piece was dissolved in KOH to assess egg numbers in liver tissue and the rest of the liver was fixed for histopathology sectioning. Spleen and mesenteric lymph nodes (LNs) were dissected, washed with RPMI and processed for lymphoblastogenesis and cytokine assays. Further technical details of the methodologies are given below.

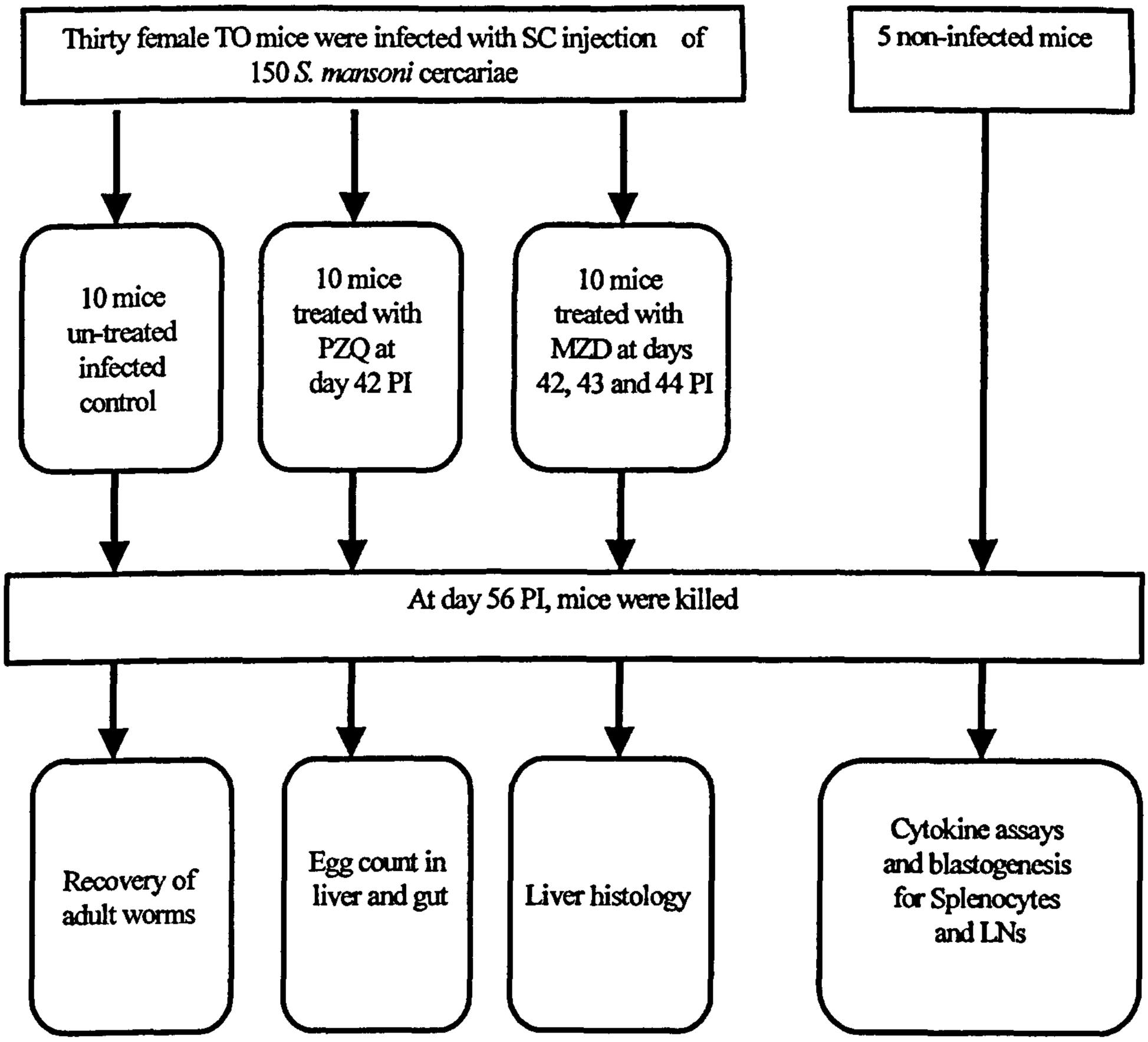

Figure 2.3 Design of the *in vivo* experiments.

Sub-cutaneous injection (SC), post infection (PI) and lymph nodes (LNs).

2.2.10 Recovery of adult *S. mansoni* worms

The process of liver perfusion and recovery of adult worms was carried out as mentioned previously (see section 2.2.2). The worms recovered from each mouse were collected in a universal container containing RPMI-1640 medium supplemented with 10% FCS. The worms were placed into a Petri dish and they were counted using a dissecting microscope. The worms of each group were then pooled (for the proteomic analysis) and washed in 40mM Tris solution and stored at –20°C until used.

2.2.11 Counting of eggs per gram of liver tissue

A piece from each liver was excised, weighed and placed in a labelled universal tube containing 10ml of 5% KOH solution. The tubes were incubated for 24h at 37°C. The content of each tube was mixed thoroughly with an equal volume of 10% formol saline solution and then centrifuged at 60g for 1min. The residues were re-suspended in 6ml of phosphate buffered saline (PBS) and mixed thoroughly. A 200µl was then placed on a glass slide and the eggs were counted. Duplicate counts were made and the mean number of eggs per gram of liver tissue was calculated.

2.2.12 Liver histology

The liver samples were fixed in neutral buffered formol saline over night at room temperature. They were washed under gently running tap water and then passed in ascending grades of alcohol (2 x 30min in 70%, 4 x 30min in 90% and 4 x 30min in 100% alcohol). The samples were incubated in tissue-clear solution (2 x 15min) and then in paraffin wax (2 x 2h). Samples were then embedded in cassettes using fresh paraffin wax. The specimens were trimmed, cooled on ice and then sectioned at 5µm using a microtome (Leica RM2035). The sections were floated on distilled water at 36°C, mounted on poly-l-lysine coated microscope slides and dried over night at 36°C.

For general tissue structure sections were stained by haematoxylin and eosin (H&E). The sections were de-waxed in tissue clear solution and then hydrated in decreasing concentrations of alcohol (100, 90 and 70%). The sections were washed in gently running tap water and then stained with Mayer's haematoxylin for 5min. They were washed in gently running tap water and then stained with eosin stain for 2min. The excess stain was washed in gently running tap water and then the sections were dehydrated in increasing concentrations of alcohol (70, 90 and 100%). The sections

were cleared again in tissue clear and then mounted in synthetic mounting media. The nuclei stain blue and all other structures stain pink/red.

The collagen fibres were stained with picro-sirius red F3B dye according to the method of Junqueira *et al.* (1979). Briefly, the sections were hydrated in water and stained with picro-sirius red for 1h. They were then washed in two changes of acidified water and dehydrated in 3 changes of absolute alcohol. The sections were cleared in tissue clear solution and mounted. In ordinary brightfield microscopy, collagen fibres stain red on a yellow background.

Eosinophils were localised using the picro-sirius red technique according to the method of Llewellyn (1970). The sections were hydrated in water and stained using Mayer's haematoxylin for 1min. They were washed gently under running water until the stain became blue. They were rinsed in alcohol and then transferred to picro-sirius red for 1-2h. The sections were washed thoroughly under gently running water. They were then dehydrated in absolute alcohol, cleared in tissue clear and mounted. Eosinophil granules stain bright red, nuclei stain blue and the background colourless. The eosinophils in the high power field (HPF) surrounding the egg of each granuloma was counted. Only granulomas surrounding a single egg were included.

2.2.13 Extraction of lymphocytes

The spleens and mesenteric lymph nodes (LNs) were dissected and collected in labelled, sterile universal tubes containing RPMI-1640. The spleens of each group of mice were pooled in a single tube. Similarly the LNs were pooled in another tube. The spleens and LNs were pressed with a sterile syringe plunger through a fine sieve in a Petri dish containing 15ml of RPMI-1640. The Petri dishes were tilted to allow cell clumps to settle and the supernatants were collected in 50ml-sterile tubes, supplemented to 30ml

with RPMI-1640 and centrifuged for 7min at 180g and 4°C. Residues from spleens were re-suspended in 6ml of RPMI and added gently to a universal tube containing 4ml of Lymphoprep (1.077 ± 0.001g/ml) (Nycomed Pharma AS, Oslo, Norway). Then they were centrifuged at 4°C for 40min at 220g. The lymphocytes were collected using a glass pipette and placed in a universal tube. The cells from the spleens and LNs were then washed twice for 7min each time at 180g at 18-25°C. After the last wash, cells were re-suspended in 1ml of RPMI-1640. A 10µl sample from each tube was diluted with 190µl of 2.5% solution of trypan blue. The cells were counted using an improved Neubauer haemocytometer. After calculating the total number of cells in each tube, they were diluted with RPMI-1640 to achieve a working concentration of 1x 10^7 cells/ml.

2.2.14 Lymphocyte proliferation assay

Lymphocytes from spleens and LNs of each group were cultured in 96-well tissue culture plates. Blastogenesis was assessed by incubation of lymphocytes with 5µg ml^{-1} of Concanavalin A (ConA) or 25µg ml^{-1} of either *S. mansoni* soluble worm antigens (SWAP) or soluble egg antigens (SEA) (150µl of complete RPMI-1640 + 25µl of cell suspension containing 2.5 x 10^5 cells + 25µl of antigen) with unstimulated cells as control (175µl of complete RPMI-1640 + 25µl of cell suspension). Assays were run in triplicate. The plates were incubated at 37°C, 5% CO_2 and humidity. After 5 days, 0.2µCi of radioactive-thymidine (Amersham Biosciences UK Ltd, Buckinghamshire, UK) was added to each well and further incubated for 18 hours. At the end of incubations, the plates were harvested and isotype incorporation by the cells measured using a liquid scintillation counter (Wallac 1450 Microbeta® Trilux).

2.2.15 Cytokine assays

Culture of lymphocytes and splenocytes with different antigens

Lymphocytes were cultured as described previously and the supernatants were collected after 5 days of incubation. Aliquots of 150µl of supernatant were collected from each well and stored in sterile tubes at –80°C until they were used.

Cytokine detection and estimation by Duoset® enzyme linked immuno-sorbent assay (ELISA)

DuoSet® kits for detection of IL-4, IL-10, IL-13 and INF-γ cytokines were purchased from R&D Systems Europe Ltd, Abingdon, UK. The protocol used was that defined by the supplier. The plates were coated with 100µl of capture antibodies, 4µg ml^{-1} for IL-4, IL-13 and IFN-γ and 2µg ml^{-1} for IL-10, at room temperature overnight. They were washed three times with wash buffer, 0.05% Tween 20 in PBS, pH 7.2. They were blocked with 200µl of block buffer (1% BSA, 5% sucrose in PBS with 0.05% Na N_3) sealed at room temperature for two hours and washed as before. 100µl of each sample and standard dilution were loaded per well. Samples were run in duplicate. Seven two-fold dilutions of the standard were used starting with 1000pg ml^{-1} for IL-4, 2000pg ml^{-1} for IL-10 and IFN-γ and 2500pg ml^{-1} for IL-13. They were sealed and incubated for 2 hours at room temperature. The plates were washed and 100µl of detection antibodies, 0.2µg ml^{-1} for IL-4 and IL-13 and 0.4µg ml^{-1} for IL-10 and IFN-γ were loaded and incubated for 2 hours at room temperature. Excess antibodies were washed away with wash buffer and a 100µl of streptavidin-HRP was loaded per well. Plates were then incubated for 20 minutes at room temperature, away from direct light. The plates were washed as before and a 100µl of substrate solution (1:1 mixture of H_2O_2 and

tetramethylbenzidine) was added to each well and were further incubated for another 20 minutes at room temperature, away from direct light. The reactions were then stopped with 50µl of 2N H_2SO_4. Optical densities were determined immediately using a microplate reader (DYNEX MRX Revelation®) set to 450nm with wavelength correction set to 540nm.

2.2.16 Statistical analysis

All statistical tests were carried out on the means using the analysis of variance (ANOVA) test with $P < 0.05$ as the criterion of significance. When there was a significant difference it was followed by the student t-tests for pair wise comparisons between different groups. The MINITAB software (version 12) were utilised for this purpose.

2.3 Results

Results of the *in vitro* experiments

2.3.1 Effects of Mirazid® and praziquantel on the viability of schistosomula of *S. mansoni*

Toluidine blue dye exclusion test

The effects of MZD on schistosomula were assessed using toluidine blue dye exclusion. Schistosomula were incubated with either MZD or PZQ at concentrations of 3, 6, 12, 25, 50, 100 and 200µg ml^{-1} and their ability to exclude toluidine blue dye were observed microscopically 1, 2, 4 and 8 hours of exposure to the drugs. Control schistosomula were incubated in medium with the solvent only (1% DMSO).

At all times, most of the control schistosomula retained their ability to exclude the dye and remained unstained. In the drug exposed groups, although most of the schistosomula exposed to either MZD or PZQ showed increased granularity and decreased motility, only stained parasites were regarded as damaged or dead. The characteristic feature of schistosomula exposed to PZQ was blebbing in the area around the mouth of the parasites. At lower concentrations, 3, 6, 12, 25μg ml^{-1}, neither drug had any visible effects on the ability of parasites to exclude the dye. Even after 8 hours of exposure, the numbers of stained parasites were not significantly different from that found in the controls. After 2h of exposure, only MZD at a concentration of 200μg ml^{-1} caused a significant decrease in the viability of schistosomula (P=0.037). After 4h of exposure, MZD at concentrations of 200, 100 and 50μg ml^{-1} had highly significant (P<0.01) effect on parasites viability (63, 25 and 10% of schistosomula were stained, respectively). This effect progressed with time and after 8h of exposure 91, 37 and 18% of schistosomula were stained, respectively (see figure 2.4).

After 4h of exposure to PZQ, only PZQ at a concentration of 200μg ml^{-1} caused a significant impairment (P=0.013) of the ability of schistosomula to exclude toluidine blue (11% of schistosomula were stained). After 8h of exposure to praziquantel at concentrations of 200μg ml^{-1} and 100μg ml^{-1} there was significant impairment (P<0.01 and P=0.032) of the ability of the parasites to exclude the dye (16% and 7% of schistosomula were stained).

These results indicate that MZD has a strong damaging effect presumably on the membranes of schistosomula, resulting in their inability to exclude toluidine blue dye. PZQ has a modest effect. With both drugs the effects are dose and time dependent with the highest effect evident after 8 hours of exposure to a concentration of 200μg ml^{-1}.

Hoechst test

The effect of MZD or PZQ on the viability of schistosomula of *S. mansoni* was further confirmed using the Hoechst 33258 dye. Schistosomula were incubated with either drug for 4h with the dye added during the last 20 minutes of incubation and then they were examined under the fluorescent microscope. The percentage of damaged parasites of each group was calculated.

Control schistosomula were unstained. After exposure to MZD at concentrations of 200μg ml^{-1} and 100μg ml^{-1}, 65% and 25% of schistosomula were fluorescent ($P<0.01$). After exposure to MZD at a concentration of 50μg ml^{-1}, 15% of schistosomula were fluorescent ($P=0.012$). After exposure to PZQ at concentrations of 200μg ml^{-1} and 100μg ml^{-1}, 19% ($P<0.01$) and 9% ($P=0.02$) of schistosomula were fluorescent.

These results (not shown) confirmed the results of the toluidine blue test that MZD is more effective than PZQ against the schistosomula stage of *S. mansoni*.

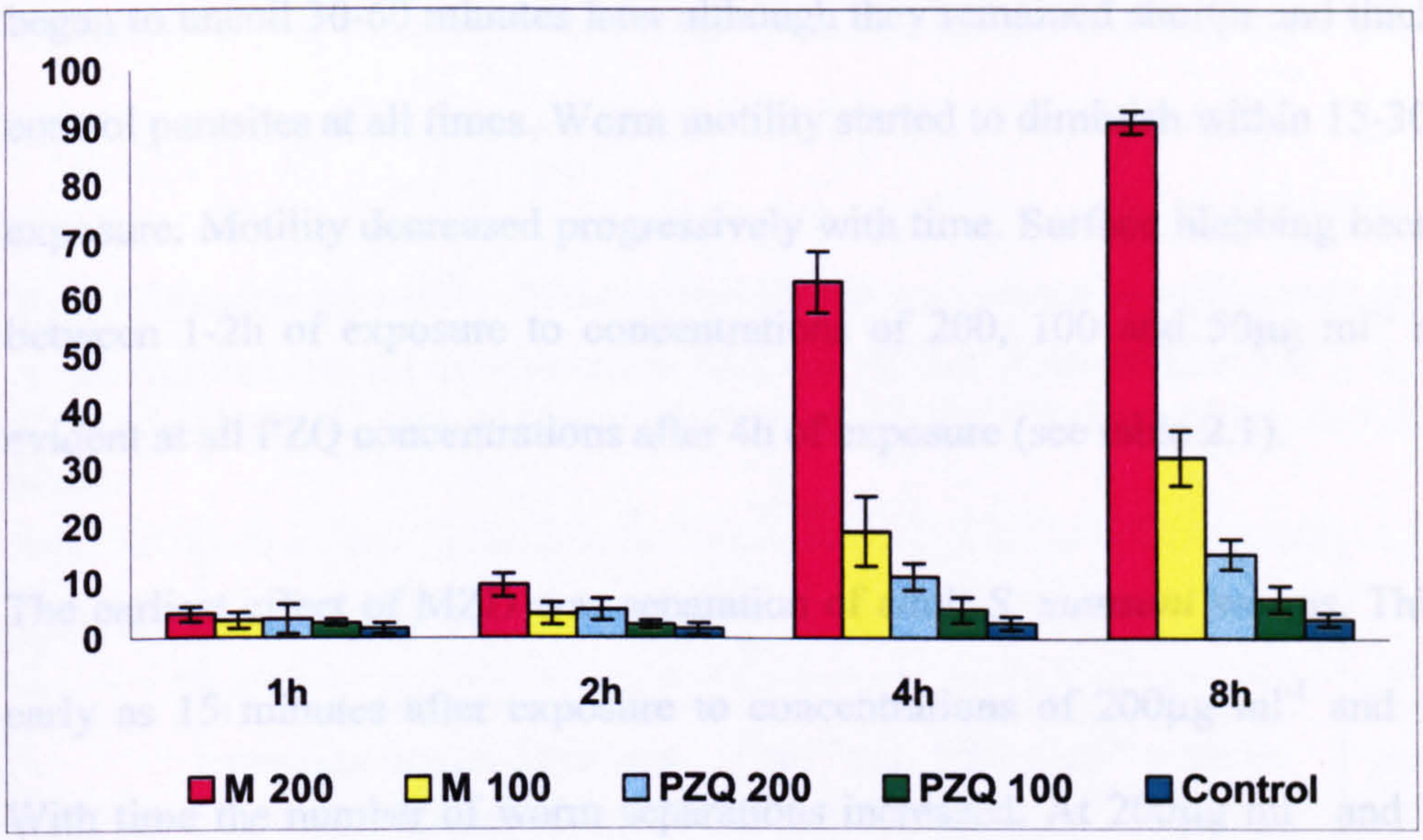

Figure 2.4 Effect of Mirazid® and praziquantel on the viability of schistosomula of *S. mansoni* assessed by the toluidine blue exclusion test.

Schistosomula of *S. mansoni* were incubated with either PZQ or MZD at concentrations of 100µg ml^{-1} and 200µg ml^{-1}. One, 2, 4 and 8 hours after exposure, their ability to exclude toluidine blue dye was determined. The columns represent the mean percentages of damaged (stained) schistosomula ± 95% confidence interval. MZD 200µg ml^{-1} (M 200), MZD 100µg ml^{-1} (M 100), PZQ 200µg ml^{-1} (PZQ 200), PZQ 100µg ml^{-1} (PZQ 100) and control schistosomula incubated with 1% DMSO (Control). All groups consisted of 500 schistosomula and these results represent the mean of five experiments.

2.3.2 Effects of Mirazid® and praziquantel on adult worms of *S. mansoni*

Adult worms of *S. mansoni* were cultured with either MZD or PZQ at concentrations of 3, 6, 12, 25, 50, 100 and 200µg ml^{-1}. Worms were observed under an inverted light microscope immediately after exposure and at 15min, 30min and at 1, 2, 4, 8, 24 and 48h after exposure. Control worms were incubated in medium with 1% DMSO. Control worms remained alive and moved normally. The female worms started to lay eggs between 18 and 24h of incubation.

The earliest changes, caused within seconds of exposure to PZQ, were muscular contractions and rapid coiling of worms, at all PZQ concentrations used. The worms contracted becoming shorter and thicker than the control parasites. The coiled worms

began to uncoil 30-60 minutes later although they remained shorter and thicker than the control parasites at all times. Worm motility started to diminish within 15-30 minutes of exposure. Motility decreased progressively with time. Surface blebbing became evident between 1-2h of exposure to concentrations of 200, 100 and 50μg ml^{-1} and became evident at all PZQ concentrations after 4h of exposure (see table 2.1).

The earliest effect of MZD was separation of adult *S. mansoni* worms. This started as early as 15 minutes after exposure to concentrations of 200μg ml^{-1} and 100μg ml^{-1}. With time the number of worm separations increased. At 200μg ml^{-1} and 100μg ml^{-1}, MZD caused an initial increase of motility and an increase in length of the parasites compared to the control group. This increase in motility was accompanied by increased regurgitation of intestinal contents of the worms, especially by the female worms, causing increased turbidity of the media. Worm motility returned to control levels after 3 to 4 hours of exposure. At a MZD concentration of 200μg ml^{-1}, worm motility began to decrease after 8 hours of exposure. This decrease in motility progressed with time. MZD at concentrations of 100μg ml^{-1} and 50μg ml^{-1} caused a decrease in worm motility 18 and 48h after exposure, respectively. At concentrations of 200μg ml^{-1} and 100μg ml^{-1}, surface blebbing became evident between 18-24h after exposure. At concentrations ranging between 3 and 25μg ml^{-1}, MZD seemed to have little effect on adult worms. They showed no obvious evidence of damage and the female worms started to lay eggs after 24h of incubation.

These results indicate that while PZQ is very effective against adult *S. mansoni* worms, MZD seems to be effective only at high concentrations and has obvious effects only after a considerable duration of exposure. However, at high concentrations MZD caused rapid separation of worm pairs but it is impossible from these data to determine whether the effect of MZD on pairing was due to a differential effect of the drug on male or female worms. Other evidence from SEM studies indicates that both male and female

worms are likely to be affected though specific changes in the gynaecophoric canal of male worms may be significant (see section 2.3.7).

Table 2.1 Observations of the effect of Mirazid® and praziquantel on adult worms of *S. mansoni.*

Adult worms were exposed to either MZD or PZQ at concentrations of 3, 6, 12, 25, 50, 100 and 200μg ml^{-1}. Worms were observed at 15min, 30min and at 1, 2, 4, 8, 24 and 48 hours.

TIME	PRAZIQUANTEL	MIRAZID	CONTROL
15min	Coiling of worms at all concentrations. This was apparent immediately after worm exposure although worms remained coiled at 15min.	Increased motility at 200μg ml^{-1} and 100μg ml^{-1} with an increase in worms' length. At 200μg ml^{-1} and 100μg ml^{-1} separation of paired worms.	Control worms were alive throughout the experiment. Worms showed no abnormal movements and began to lay eggs after 18-24 hours of incubation.
30min	Uncoiling of worms. Decreased worm motility. Worms thick and short.	At 200μg ml^{-1} and 100μg ml^{-1} further separation of paired worms.	
1h	Further decrease in worm motility.	At 200μg ml^{-1} and 100μg ml^{-1} separation of paired worms	
2h	Surface blebbing at concentrations of 200, 100 and 50μg ml^{-1}.	At 200μg ml^{-1} and 100μg ml^{-1} worm separation. Worms' motility returns back to normal.	
4h	Worms non-motile. Surface blebbing at all concentrations.	At 200μg ml^{-1} all worm pairs separated. At 100μg ml^{-1} worm separation increased.	
8h	Worm paralysed. More surface blebbing.	At 200μg ml^{-1} decreased motility.	
24h	Extensive surface blebbing and paralysis.	At 200μg ml^{-1} and 100μg ml^{-1} sluggish motility. At 200μg ml^{-1} and 100μg ml^{-1} surface blebbing.	
48h	Complete paralysis of worms. Severe surface blebbing.	At 200μg ml^{-1} worm paralysis. At 100μg ml^{-1} and 50μg ml^{-1} sluggish motility. At 200μg ml^{-1} and 100μg ml^{-1} surface blebbing increased.	

2.3.3 Unpairing effect of Mirazid® on adult *S. mansoni* worms

Based on the previous observations, the unpairing effect of MZD was further investigated. Paired adult *S. mansoni* worms were incubated with MZD at concentrations of 50, 100 and 200μg ml^{-1} and were observed using an inverted light microscope at 15min, 30min and at 1, 2, 3, 6 and 12h after exposure. Control worm pairs were incubated in medium with 1% DMSO.

Control worms remained paired and after 12 hours of incubation only 15% of them were separated. With MZD at 200μg ml^{-1} and 100μg ml^{-1} worm separation was evident as early as 15 minutes after exposure (28.9 and 13%, respectively). The separation of worm pairs was highly significant for both groups compared to the control group ($P<0.01$). After 30 minutes of exposure to MZD at 200μg ml^{-1} and 100μg ml^{-1} 40.2% and 17.6% of worm pairs were separated, respectively. One hour after exposure, MZD at 200μg ml^{-1} and 100μg ml^{-1} caused separation of 52 and 23.7% of worm pairs, respectively. Additional worm separation occurred gradually and 6h after exposure, all worms (100%) were separated at 200μg ml^{-1} and 47.9% were separated at 100μg ml^{-1}. After 12h of exposure to MZD at 100μg ml^{-1}, 59% of worm pairs were separated (see figure 2.5). With MZD at 50μg ml^{-1} worm separations were not significantly different from those of the control group throughout the duration of the experiment.

A similar experiment (data not shown) was conducted using PZQ. However, the rapidity with which PZQ acts on the parasites precludes observations of an effect on worm pairing over time. The worm unpairing effect of MZD appears to be at least in part dependent upon continued worm motility.

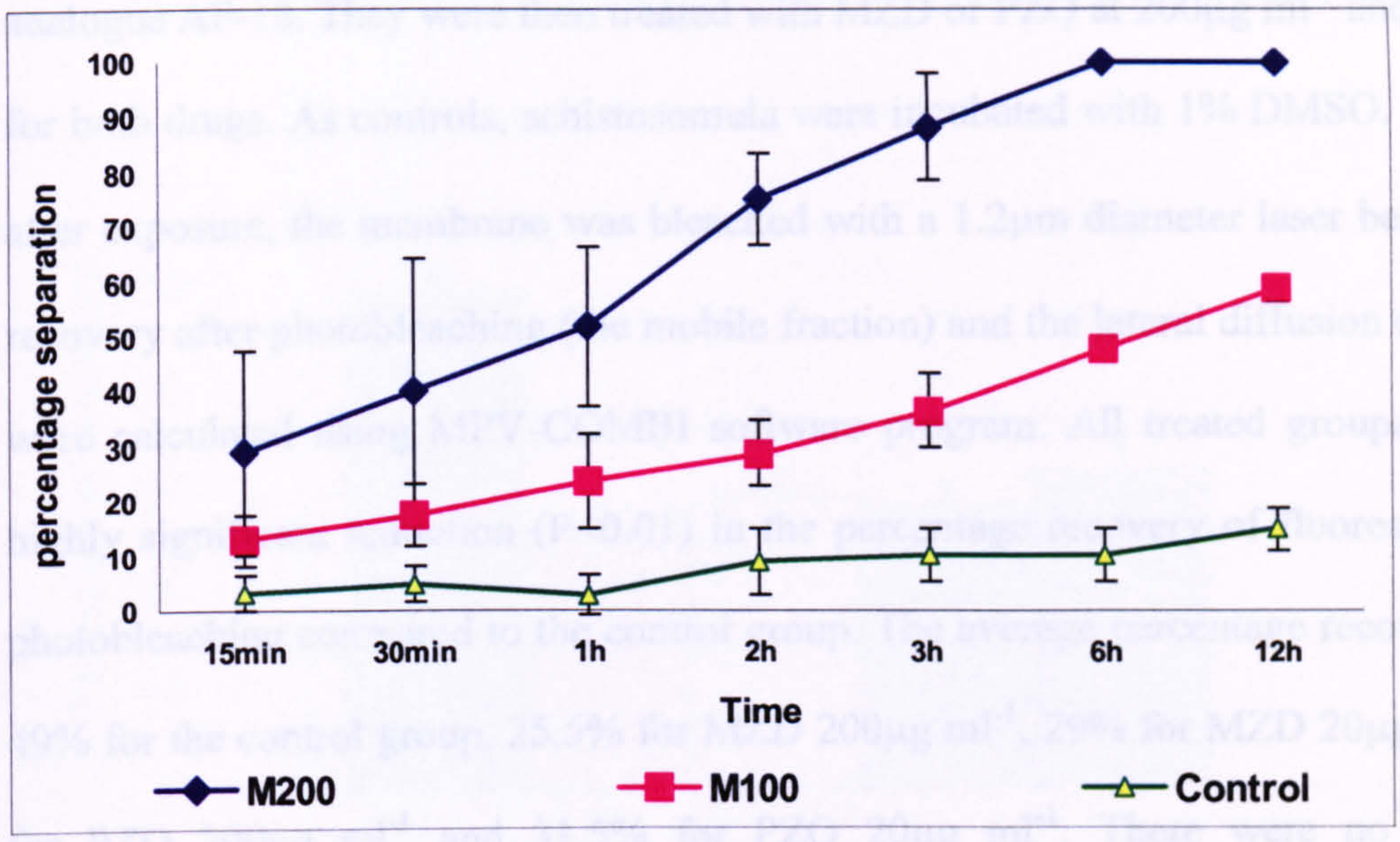

Figure 2.5 Unpairing effect of Mirazid® on *S. mansoni* adult worms.

Adult worm pairs were incubated with MZD at 100µg ml^{-1} or 200µg ml^{-1} and were observed for separation at 15min, 30min, 1, 2, 3, 6 and 12h after exposure. Figures represent the mean percentage of adult worm separation ± 95% confidence interval. MZD 200µg ml^{-1} (M 200), MZD 100µg ml^{-1} (M 100) and control worms incubated with 1% DMSO (Control). (Note; x-axis scale intervals are not proportional). All groups consisted of 30 worm pairs and these results represent the mean of five experiments.

2.3.4 Effects of Mirazid® and praziquantel on the membrane fluidity of schistosomula of *S. mansoni* as determined by fluorescent recovery after photobleaching (FRAP)

The outcome of a *S. mansoni* infection is dependent upon the host parasite relationship and the parasite surface is the primary interface for this interaction. It has been suggested that the outer layer of the membrane consists of a sheet of phospholipids and sterols that is shed continuously releasing potentially antigenic material and bound antibodies (Wilson and Barnes, 1977). The effect of MZD or PZQ on the fluidity of the outer membrane of schistosomula was investigated using the FRAP technique. Only two concentrations of the drugs were used, 200µg ml^{-1} as a high concentration which appeared to have such dramatic effects in the previous experiments and 20µg ml^{-1} as a low concentration which appeared to have no significant effect in the previous experiments. The membranes of schistosomula were labelled with a fluorescent lipid

analogue AF-18. They were then treated with MZD or PZQ at 200μg ml^{-1} and 20μg ml^{-1} for both drugs. As controls, schistosomula were incubated with 1% DMSO. Four hours after exposure, the membrane was bleached with a 1.2μm diameter laser beam and the recovery after photobleaching (the mobile fraction) and the lateral diffusion coefficients were calculated using MPV-COMBI software program. All treated groups showed a highly significant reduction ($P<0.01$) in the percentage recovery of fluorescence after photobleaching compared to the control group. The average percentage recoveries were 49% for the control group, 25.5% for MZD 200μg ml^{-1}, 29% for MZD 20μg ml^{-1}, 28% for PZQ 200μg ml^{-1} and 31.5% for PZQ 20μg ml^{-1}. There were no significant differences among the treated groups (see figure 2.6). The average lateral diffusion coefficients were $6.9 \pm 4.1 \times 10^{-8}$ for the control, $3 \pm 1.7 \times 10^{-8}$ for MZD 200μg ml^{-1}, $3.3 \pm 2.1 \times 10^{-8}$ for MZD 20μg ml^{-1}, $3.2 \pm 3.1 \times 10^{-8}$ for PZQ 200μg ml^{-1} and $3.5 \pm 2.1 \times 10^{-8} cm^2 sec^{-1}$ for PZQ 20μg ml^{-1}. These results indicate that MZD and PZQ caused a decrease in the fluidity of the surface membrane of schistosomula of *S. mansoni* and these effects could interfere with the survival mechanisms of the parasites.

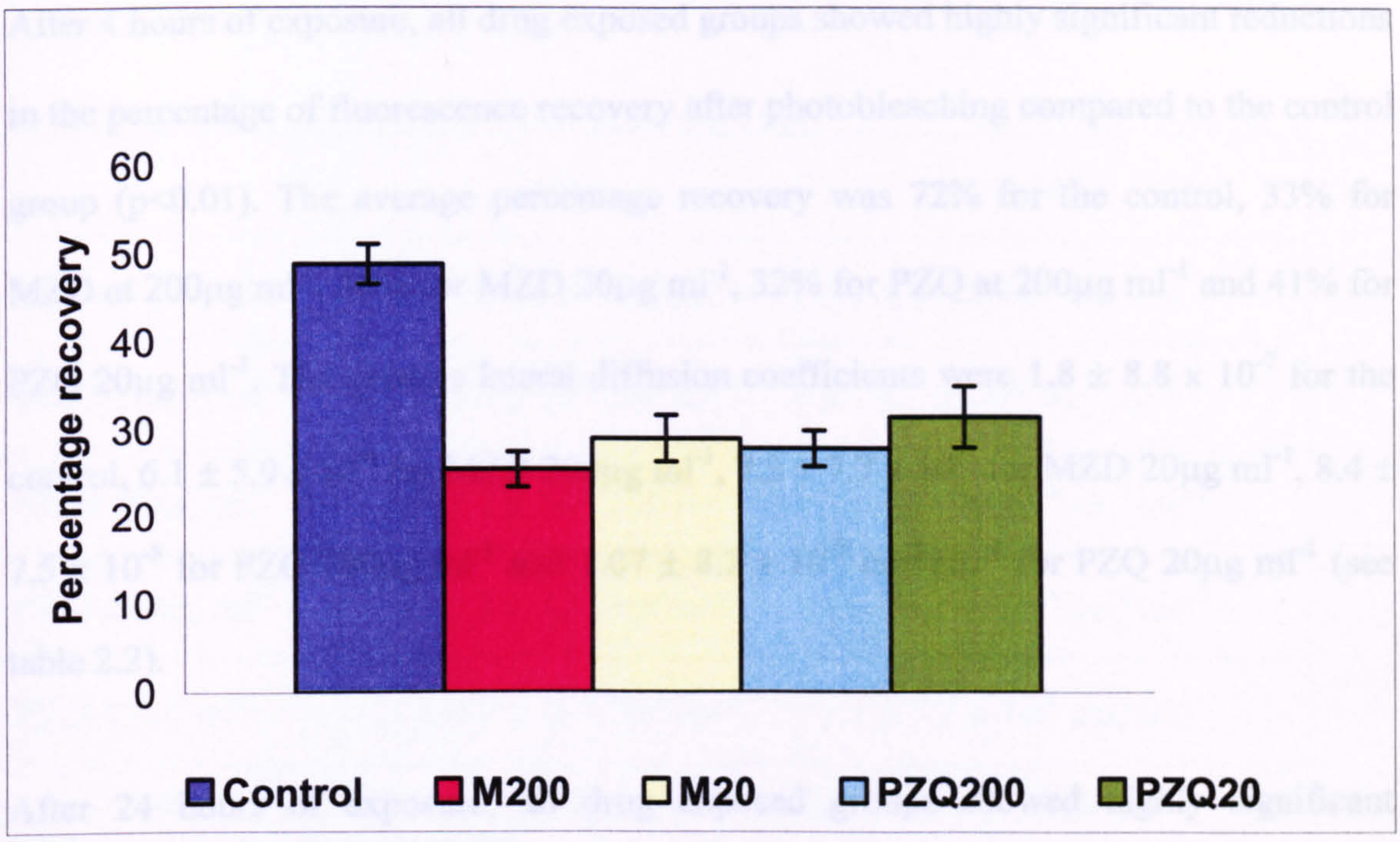

Figure 2.6 Effect of Mirazid® and praziquantel on the membrane fluidity of schistosomula of *S. mansoni*.

Four hours after exposure of the AF-18 labelled schistosomula to MZD or PZQ at 200µg ml^{-1} and 20µg ml^{-1}, their membranes were bleached with a laser beam and the percentage recovery was assessed. The columns represent the percentage recovery of fluorescence after laser photobleaching ± 95% confidence intervals. Control schistosomula incubated with 1% DMSO only (Control), MZD 200µg ml^{-1} (M200), MZD 20µg ml^{-1} (M20), PZQ 200µg ml^{-1} (PZQ200), PZQ 20µg ml^{-1} (PZQ20). All groups consisted of 100 schistosomula and these results represent the mean of 3 experiments.

2.3.5 Effects of Mirazid® and praziquantel on the membrane fluidity of adult *S. mansoni* male worms as determined by FRAP

The membranes of adult male worms were labelled with a fluorescent lipid analogue AF-18. Then they were treated with MZD or PZQ at 200µg ml^{-1} and 20µg ml^{-1}. Four and twenty four hours after exposure, the membrane was bleached with a 1.2µm diameter laser beam and the percentage fluorescence recovery after photobleaching was calculated using MPV-COMBI software program. Control worms were incubated in medium supplemented with the 1% DMSO (drug solvent).

After 4 hours of exposure, all drug exposed groups showed highly significant reductions in the percentage of fluorescence recovery after photobleaching compared to the control group ($p<0.01$). The average percentage recovery was 72% for the control, 33% for MZD at 200μg ml^{-1}, 38% for MZD 20μg ml^{-1}, 32% for PZQ at 200μg ml^{-1} and 41% for PZQ 20μg ml^{-1}. The average lateral diffusion coefficients were $1.8 \pm 8.8 \times 10^{-7}$ for the control, $6.1 \pm 5.9 \times 10^{-8}$ for MZD 200μg ml^{-1}, $7.3 \pm 7.7 \times 10^{-8}$ for MZD 20μg ml^{-1}, $8.4 \pm 7.5 \times 10^{-8}$ for PZQ 200μg ml^{-1} and $1.07 \pm 8.2 \times 10^{-7}$ cm^2 sec^{-1} for PZQ 20μg ml^{-1} (see table 2.2).

After 24 hours of exposure, all drug exposed groups showed highly significant reductions in the percentage of fluorescence recovery after photobleaching compared to the control group ($p<0.01$). The average percentage recovery was 56.5% for the control, 28% for MZD 200μg ml^{-1}, 34% for MZD 20μg ml^{-1}, 22% for PZQ 200μg ml^{-1} and 26.5% for PZQ 20μg ml^{-1} (see figure 2.7). There were highly significant differences between PZQ 200μg ml^{-1} and MZD 200μg ml^{-1} ($P<0.01$) and between PZQ 20μg ml^{-1} and MZD 20μg ml^{-1} ($P< 0.01$). The average lateral diffusion coefficients were $1.74 \pm 8.6 \times 10^{-7}$ for the control, $5.9 \pm 7 \times 10^{-8}$ for MZD 200μg ml^{-1}, $9.4 \pm 7.7 \times 10^{-8}$ for MZD 20μg ml^{-1}, $3.4 \pm 2.1 \times 10^{-8}$ PZQ 200μg ml^{-1} and $7.1 \pm 7.2 \times 10^{-8} cm^2$ sec^{-1} for PZQ 20μg ml^{-1} (see table 2.2). These results indicate that both MZD and PZQ caused reductions in the membrane fluidity of adult male *S. mansoni* and that after 24h of exposure PZQ had a greater effect than MZD.

	4h	24h
Control parasites	$1.8 \pm 8.8 \times 10^{-7}$ cm^2 sec^{-1}	$1.7 \pm 8.6 \times 10^{-7}$ cm^2 sec^{-1}
MZD 200µg ml^{-1}	$6.1 \pm 5.9 \times 10^{-8}$ cm^2 sec^{-1}	$5.9 \pm 7 \times 10^{-8}$ cm^2 sec^{-1}
MZD 20µg ml^{-1}	$7.3 \pm 7.7 \times 10^{-8}$ cm^2 sec^{-1}	$9.4 \pm 7.7 \times 10^{-8}$ cm^2 sec^{-1}
PZQ 200µg ml^{-1}	$8.4 \pm 7.5 \times 10^{-8}$ cm^2 sec^{-1}	$3.4 \pm 2.1 \times 10^{-8}$ cm^2 sec^{-1}
PZQ 20µg ml^{-1}	$1.1 \pm 8.2 \times 10^{-7}$ cm^2 sec^{-1}	$7.1 \pm 7.2 \times 10^{-8}$ cm^2 sec^{-1}

Table 2.2 Effect of Mirazid® and praziquantel on the lateral diffusion co-efficient of the fluorescent membrane of adult *S. mansoni* worms.

Four and twenty four hours after exposure of the AF-18 labelled schistosomula to MZD or PZQ at 200µg ml^{-1} and 20µg ml^{-1}, their membranes were bleached with a laser beam and the lateral diffusion co-efficient was assessed. This table represents the mean lateral diffusion co-efficient for each group ± 95% confidence intervals.

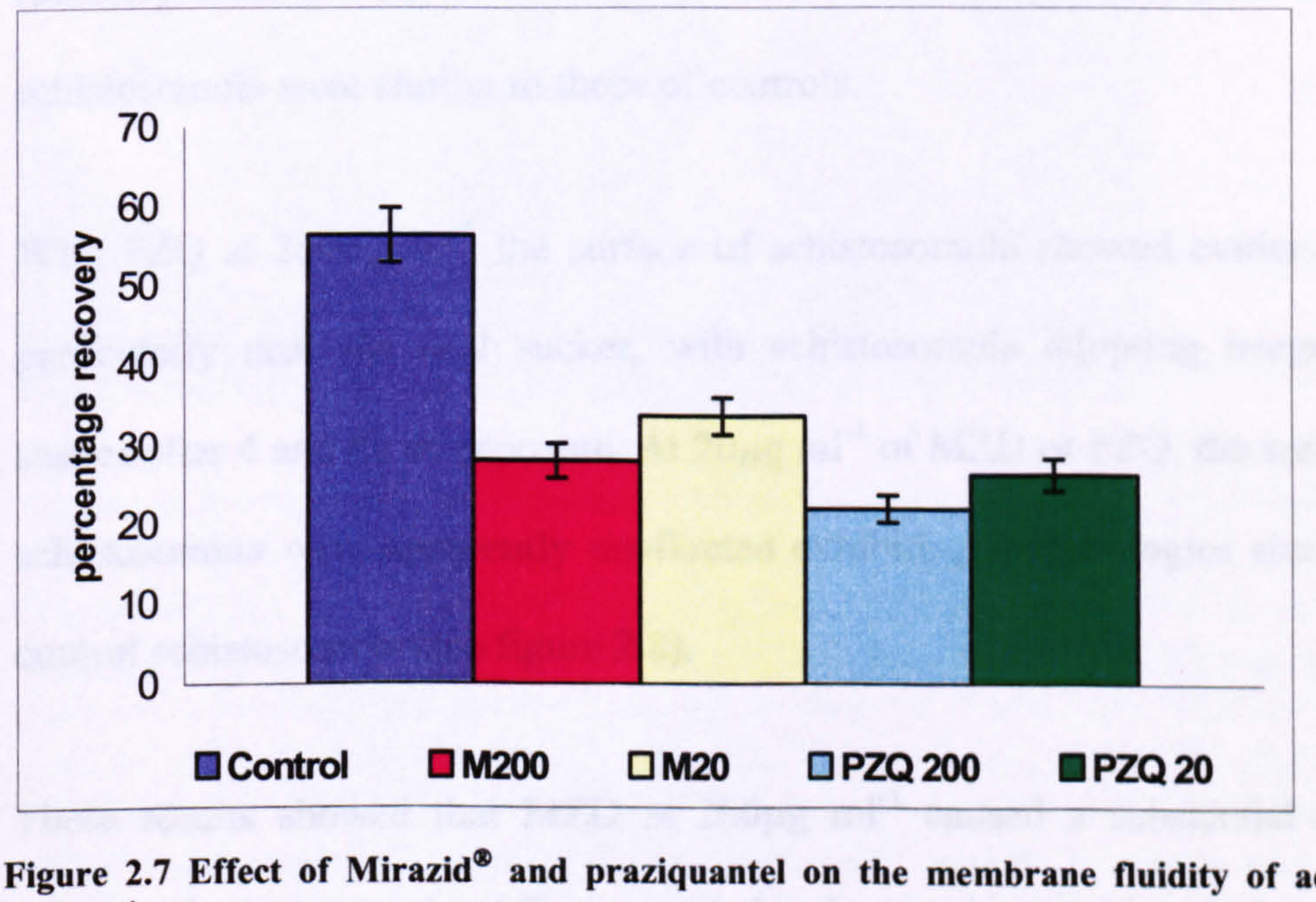

Figure 2.7 Effect of Mirazid® and praziquantel on the membrane fluidity of adult males of *S. mansoni*.

Adult worms were labelled with AF-18 and then exposed to either MZD or PZQ at concentrations of 200µg ml^{-1} and 20µg ml^{-1}. After 24h of exposure, the membrane of the parasite was bleached with a laser beam and the percentages recovery were calculated. The columns represent the percentage recovery of fluorescence after laser photobleaching ± 95% confidence intervals. Control worms incubated with 1% DMSO only (Control), MZD 200µg ml^{-1} (M200), MZD 20µg ml^{-1} (M20), PZQ 200µg ml^{-1} (PZQ200) and PZQ 20µg ml^{-1} (PZQ20). All groups consisted of 50 adult male worms and these results represent the mean of 3 experiments.

2.3.6 Scanning electron microscopy (SEM) of schistosomula of *S. mansoni* after exposure to Mirazid® or praziquantel

Schistosomula of *S. mansoni* were incubated with MZD or PZQ at 200μg ml^{-1} and 20μg ml^{-1}. Control schistosomula were incubated in medium supplemented with 1% DMSO. After 4 and 8 hours of exposure, schistosomula were fixed and processed for SEM (see section 2.2.6).

Control schistosomula retained their normal structure and showed no obvious changes. Four hours after exposure, MZD at 200μg ml^{-1} caused tegumental damage and protrusion of hair-like extensions from the outer surfaces of schistosomula in a high proportion but not all of the parasites. Eight hours after exposure, the damage was severe with sloughing of the tegument of the schistosomula leaving exposed areas (possibly basal membrane or muscle) behind. With MZD at 20μg ml^{-1}, the surfaces of schistosomula were similar to those of controls.

With PZQ at 200μg ml^{-1} the surface of schistosomula showed evidence of blebbing, particularly near the oral sucker, with schistosomula adopting irregular contracted shapes after 4 and 8h of exposure. At 20μg ml^{-1} of MZD or PZQ, the surfaces of treated schistosomula were apparently unaffected exhibiting morphologies similar to those of control schistosomula (see figure 2.8).

These results showed that MZD at 200μg ml^{-1} caused a substantial damage to the surface of schistosomula of *S. mansoni*, this damage increased with time supporting the results of the previous experiments that MZD is more effective than PZQ against the schistosomula stage of *S. mansoni*.

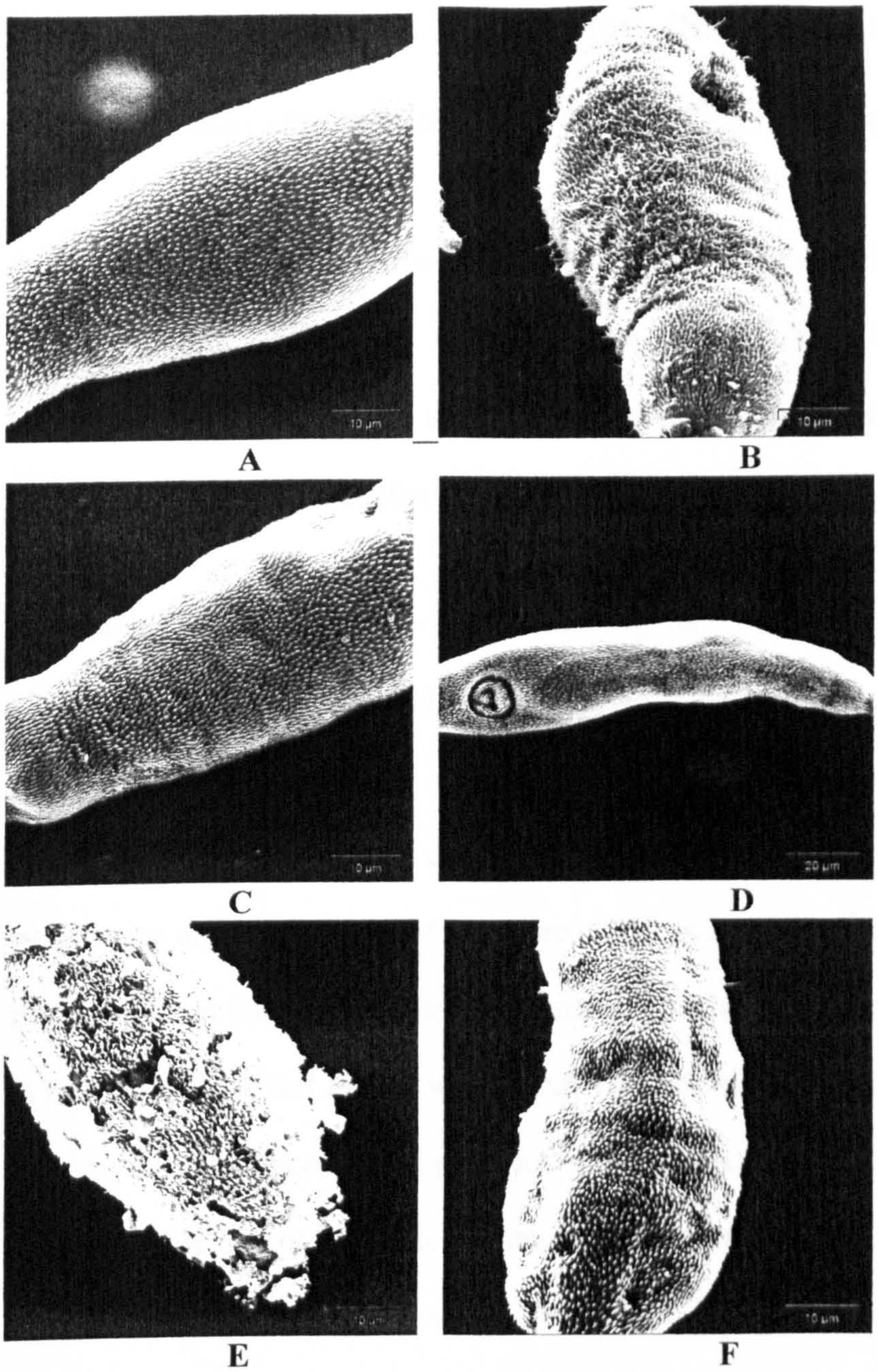

Figure 2.8 Scanning electron microscopy of schistosomula of *S. mansoni* after exposure to Mirazid® or praziquantel.

Schistosomula of *S. mansoni* were exposed to MZD and PZQ at concentrations of 200µg ml^{-1} and 20µg ml^{-1}. After 4 and 8 hours of exposure, schistosomula were fixed, processed for SEM and examined using an electron microscope (Phillips SEM 500). (A) Control schistosomula incubated with 1% DMSO for 4h, (B) MZD 200µg ml^{-1} 4h after exposure, (C) PZQ 200µg ml^{-1} 4h after exposure, (D) control schistosomula incubated with the drug solvent for 8h, (E) MZD 200µg ml^{-1} 8h after exposure and (F) PZQ 200µg ml^{-1} 8h after exposure. The scale bars measure 10µm for (A), (B), (C), (E) and (F) and 20µm for (D).

2.3.7 Scanning electron microscopy (SEM) of adult worms of *S. mansoni* after exposure to Mirazid® or praziquantel

Adult worms of *S. mansoni* were incubated with MZD at 200μg ml^{-1} or 20μg ml^{-1} or PZQ at 200, 20 and 5μg ml^{-1}. Control worms were incubated in medium supplemented with 1% DMSO. After 4, 24 and 72 hours of incubation, adult worms were fixed and processed for SEM (see section 2.2.6).

Four hours after exposure to MZD at 200μg ml^{-1} male worms demonstrated surface blebbing (small diameter) and a partial loss of the spines from the tubercles. At this concentration, female worms showed considerable swelling of the tegument such that the spines appeared to be deeply embedded within it. Damage appeared to be progressive and by 24 hours of exposure, MZD at 200μg ml^{-1} caused loss of the surface ridges between the tubercles, loss of some tubercular spines and more surface blebbing in both adult male and female worms. After 72 hours, MZD at 200μg ml^{-1} caused severe damage to the surfaces of the parasites with complete sloughing of the surfaces across much of the length of the adult worms. After 4 hours, MZD at 20μg ml^{-1} had no evident effects on the surface of adult worms, however it caused surface blebbing of the surface of the gynaecophoric canals of male worms. After 24 hours of exposure, MZD at 20μg ml^{-1} caused some loss of the surface ridges between the tubercles and loss of some tubercular spines of male worms and induced surface blebbing of female worms. At this concentration, the damage was more evident after 72 hours after exposure (see figure 2.9-12).

The most striking effect of PZQ was surface blebbing shown by both male and female worms. On female worms blebbing was mainly confined to the anterior region of the parasite, behind the ventral sucker whereas the effect on male worms was widespread. Blebbing was evident within 4 hours of exposure even to PZQ at 5μg ml^{-1} and increased

progressively with time. After 72 hours, blebbing was evident across all surfaces of the male worms but there was little evidence of any sloughing.

Given concerns about the potential development of drug resistance among parasites (and other microbial pathogens), combination therapies are often employed to reduce the likelihood of its emergence. This consideration led us to examine the effect on worms of simultaneous exposure to both PZQ and MZD. Surprisingly, the surface damage observed when using both drugs was less than that observed when either drug was used in isolation.

In conclusion, both MZD and PZQ cause dose and time-dependent damage to the surfaces of adult worms of *S. mansoni* though higher doses of MZD than PZQ are required to cause damage to the worms.

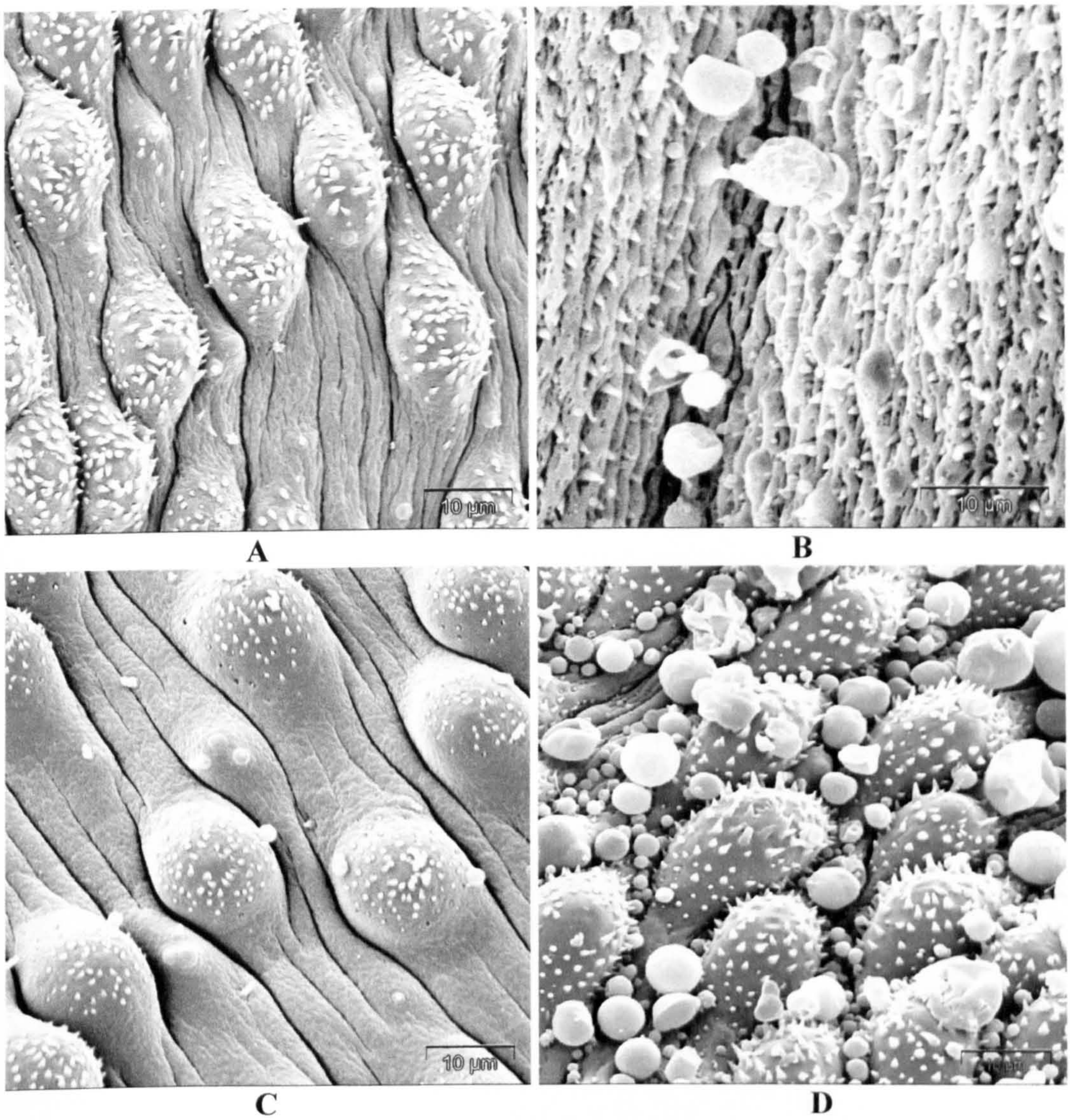

Figure 2.9 Scanning electron microscopy of adult male worms of *S. mansoni* after 4 hours of exposure to Mirazid® or praziquantel.

Adult male worms of *S. mansoni* were exposed to different concentrations of MZD or PZQ. Four hours after exposure, worms were fixed, processed and for SEM and examined using an electron microscope (Phillips SEM 500). (A) Control adult male incubated with 1% DMSO, (B) MZD 20µg ml^{-1}, (C) MZD 200µg ml^{-1} and (D) PZQ 20µg ml^{-1}. Scale bar 10µm.

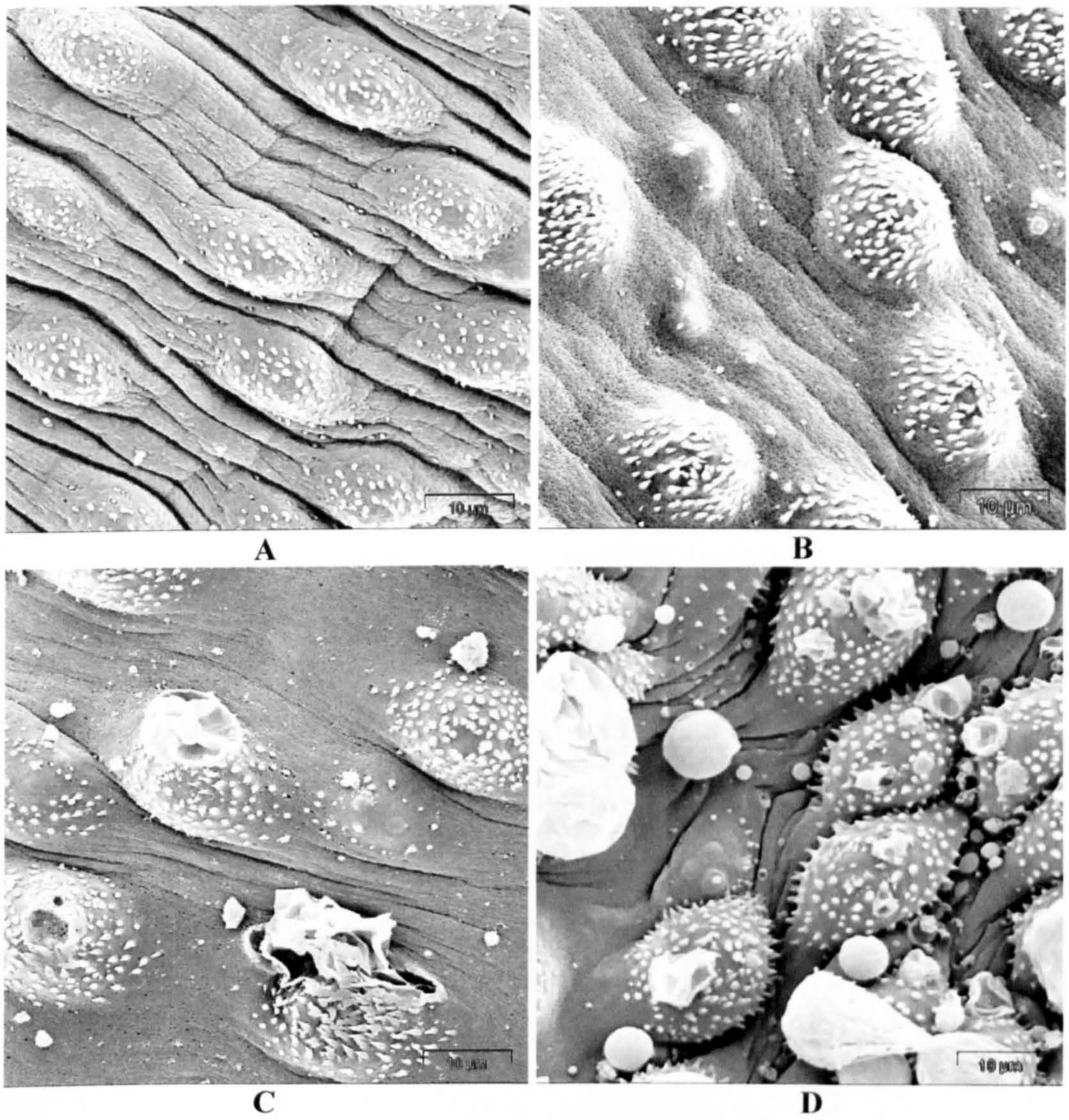

Figure 2.10 Scanning electron microscopy of adult male worms of *S. mansoni* after 24 hours of exposure to Mirazid® or praziquantel.

Adult male worms of *S. mansoni* were exposed to different concentration of MZD or PZQ. After 24 hours of exposure, worms were fixed, processed for SEM and examined using an electron microscope (Phillips SEM 500). (A) Control adult male incubated with 1% DMSO, (B) MZD 20μg ml^{-1} (C) MZD 200μg ml^{-1} and (D) PZQ 5μg ml^{-1}. Scale bar 10μm.

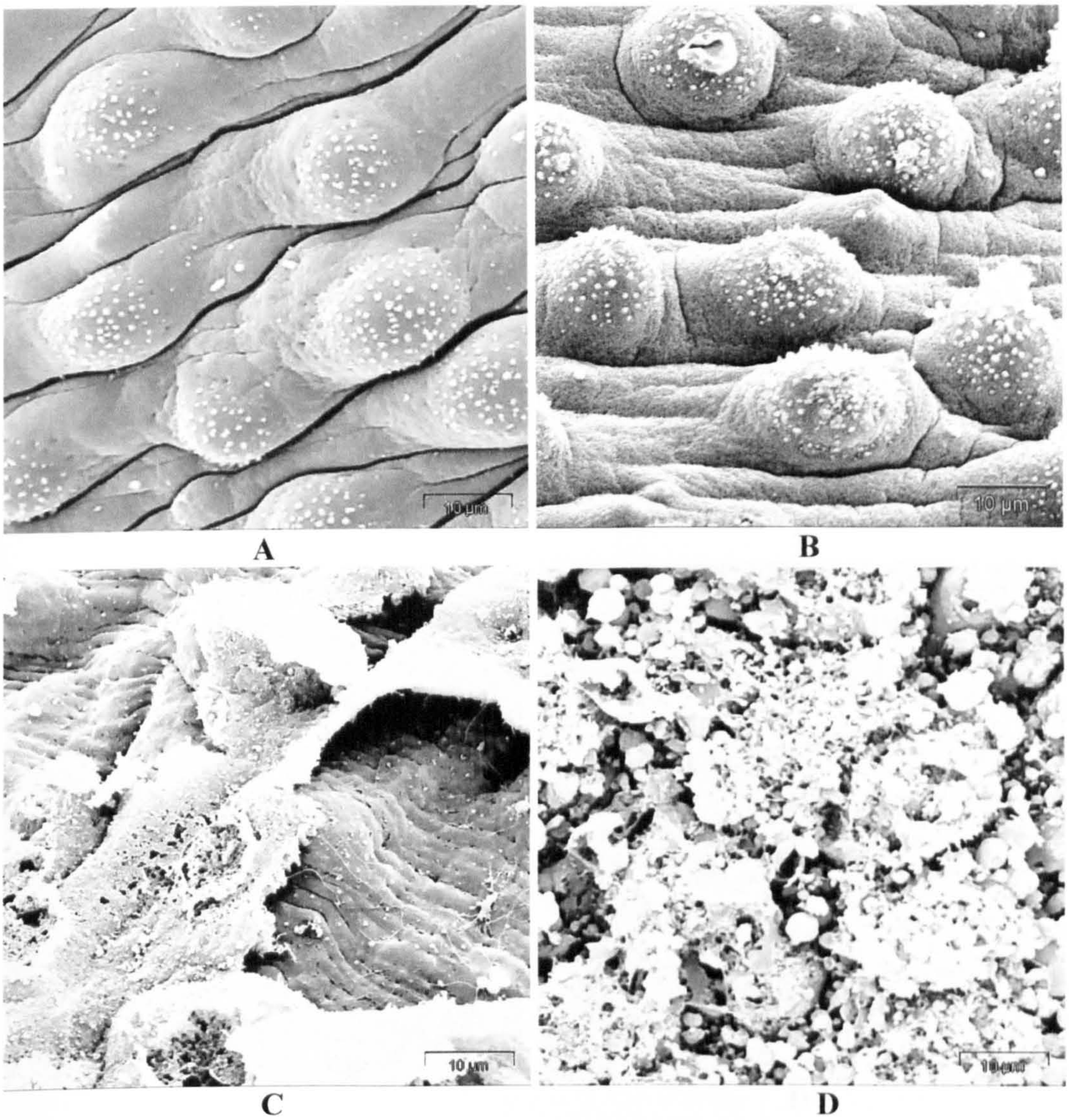

Figure 2.11 Scanning electron microscopy of adult male worms of *S. mansoni* after 72 hours of exposure to Mirazid® or praziquantel.

Adult male worms of *S. mansoni* were exposed to different concentration of MZD or PZQ. After 72 hours of exposure, worms were fixed, processed for SEM and examined using an electron microscope (Phillips SEM 500). (A) Control adult male incubated with 1% DMSO, (B) MZD 20µg ml^{-1}, (C) MZD 200µg ml^{-1} and (D) PZQ 5µg ml^{-1}. Scale bar 10µm.

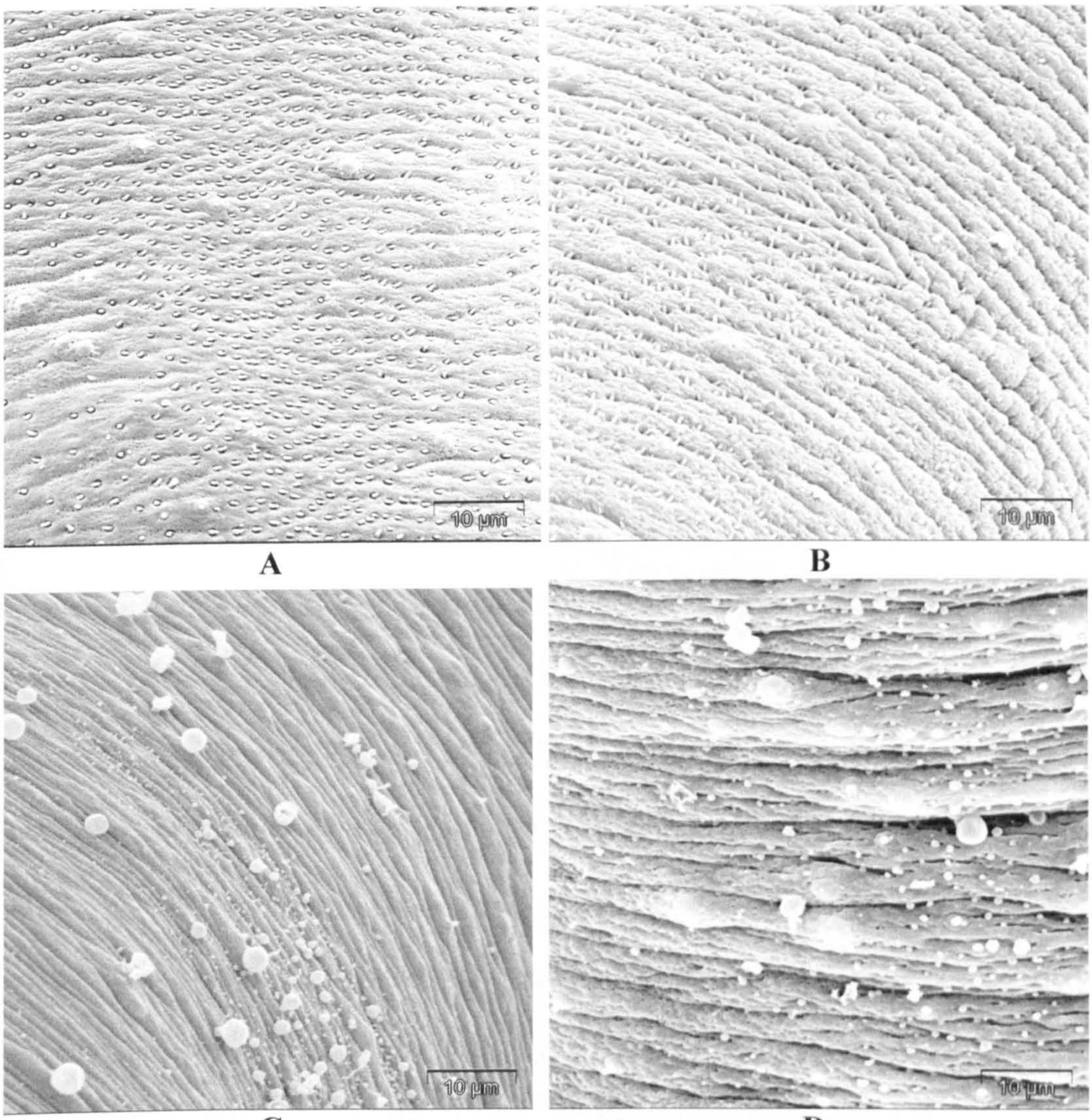

Figure 2.12 Scanning electron microscopy of adult female worms of S. mansoni after exposure to Mirazid® or praziquantel.

Adult female worms were exposed to different concentrations of MZD or PZQ. After 4 and 24 hours of exposure, worms were fixed, processed for SEM and examined using an electron microscope (Phillips SEM 500). (A) Control female exposed to 1% DMSO for 4h, (B) MZD 200µg ml^{-1} 4h after exposure, (C) PZQ 5µg ml^{-1} 4h after exposure and (D) MZD 20µg ml^{-1} 24h after exposure. Scale bar 10µm.

2.3.8 Transmission electron microscopy of adult worms of *S. mansoni* after exposure to Mirazid® or praziquantel

Having investigated the surface effects of MZD and PZQ through SEM, a more detailed investigation of damage was launched in a series of experiments designed to permit examination of worm ultrastructure using transmission electron microscopy (TEM). Adult worms of *S. mansoni* were incubated with different concentrations of MZD and

PZQ (200μg ml^{-1} and 20μg ml^{-1}). Control worms were incubated in medium supplemented with 1% DMSO. After 4 and 24h, adult worms were fixed and processed for TEM. Control worms retained their normal structure and showed no signs of damage throughout the experiment.

Four hours after exposure to MZD at 200μg ml^{-1}, both adult male and female worms showed sub-tegumental vacuolisation. The vacuoles were of variable sizes and originated from beneath the basal lamina of the tegument and extended into the tegumental cytoplasm. The tegumental cytoplasm showed increased numbers of discoid bodies. There was no or little evidence of damage to the parasites' musculature and parenchyma. Parasites exposed to MZD at 20μg ml^{-1} showed little damage and their structure was similar to that of control parasites (see figure 2.13). Twenty-four hours after exposure, worms exposed to MZD at 200μg ml^{-1} showed an increase in the number of the sub-tegumental vacuoles and an increase in the number of discoid bodies and membranous vesicles in the tegument cytoplasm. Worms exposed to MZD at 20μg ml^{-1} showed a small increase in the number of discoid bodies and membranous vesicles in the tegument cytoplasm. There was no or very little evidence of damage to the parasites' musculature and parenchyma at either drug concentration (see figure 2.14).

On the other hand, PZQ caused sub-tegumental vacuolisation and substantial damage and loss of integrity of the musculature and parenchyma of both adult male and female worms. The effects were evident after 4h of exposure to both drug concentrations and greatest after 24h of exposure to PZQ at 200μg ml^{-1}. The male worms showed more extensive damage than the adult females at all the concentrations used.

These observations showed that there is an apparent overlap in the type of damage caused by the drugs. Both drugs cause sub-tegumental vacuolisation of both male and female worms of *S. mansoni*. However, though the damage caused by MZD was mainly

confined to the tegument of the parasites in the form of multiple sub-tegumental vacuoles and small increase in the number of the discoid and membranous bodies. In contrast, the damage caused by PZQ affects the musculature and parenchyma as well as the tegument.

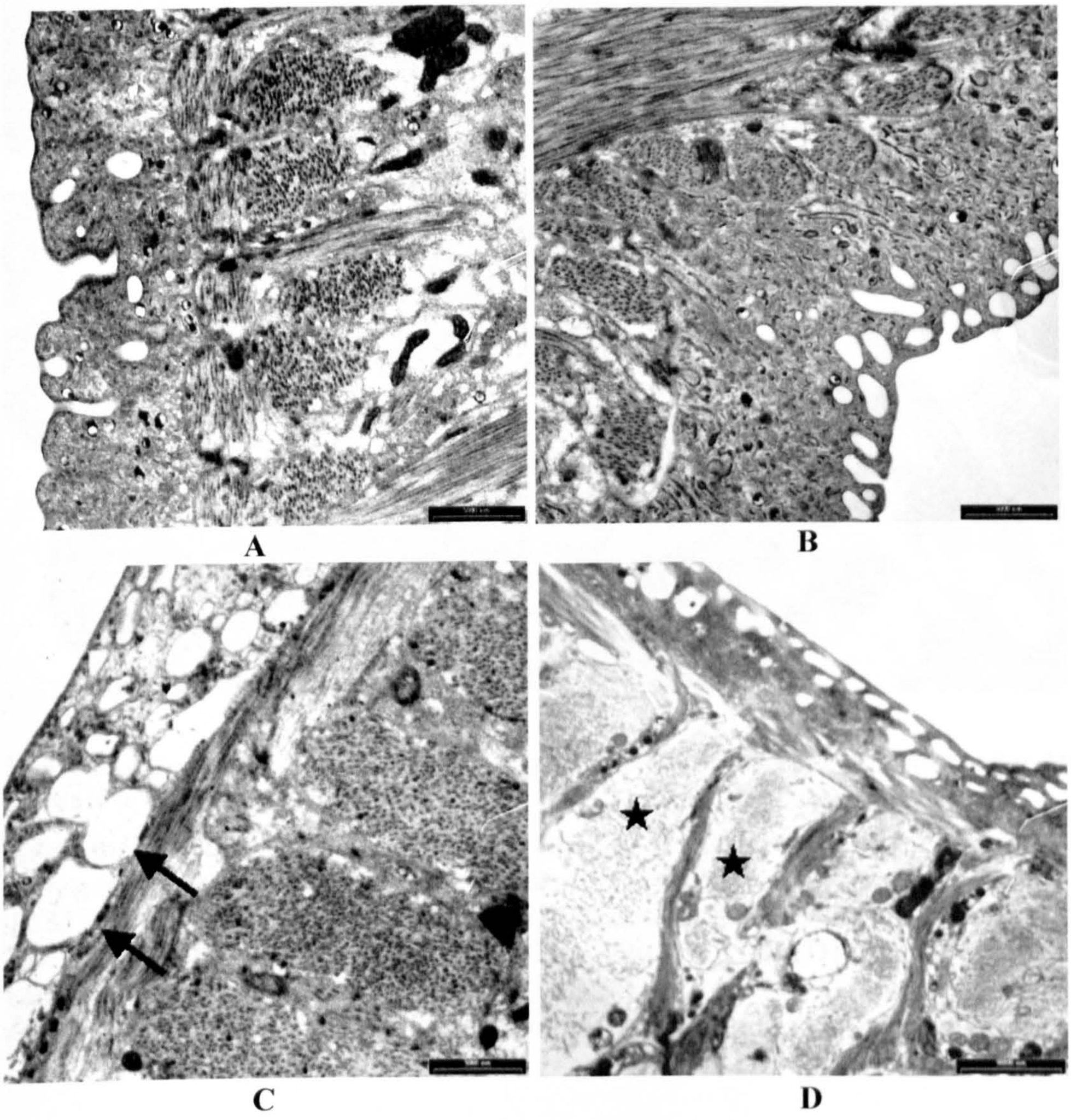

Figure 2.13 Transmission electron microscopy of adult male worms of *S. mansoni* after 4 hours of exposure to Mirazid® or praziquantel.

Adult male worms of *S. mansoni* were exposed to MZD or PZQ at concentrations of 200μg ml^{-1} and 20μg ml^{-1}. Four hours after exposure, worms were fixed, processed for TEM and examined using a TEM (Zeiss 902). (A) Control male worms exposed to 1% DMSO only, (B) MZD 20μg ml^{-1}, (C) MZD 200μg ml^{-1} and (D) PZQ 200μg ml^{-1}. Scale bars measure 5μm for (A), (B) and (C) and 10μm for (D). The arrows point to sub-tegumental vacuolisation. The stars point to muscle and parenchymal damage.

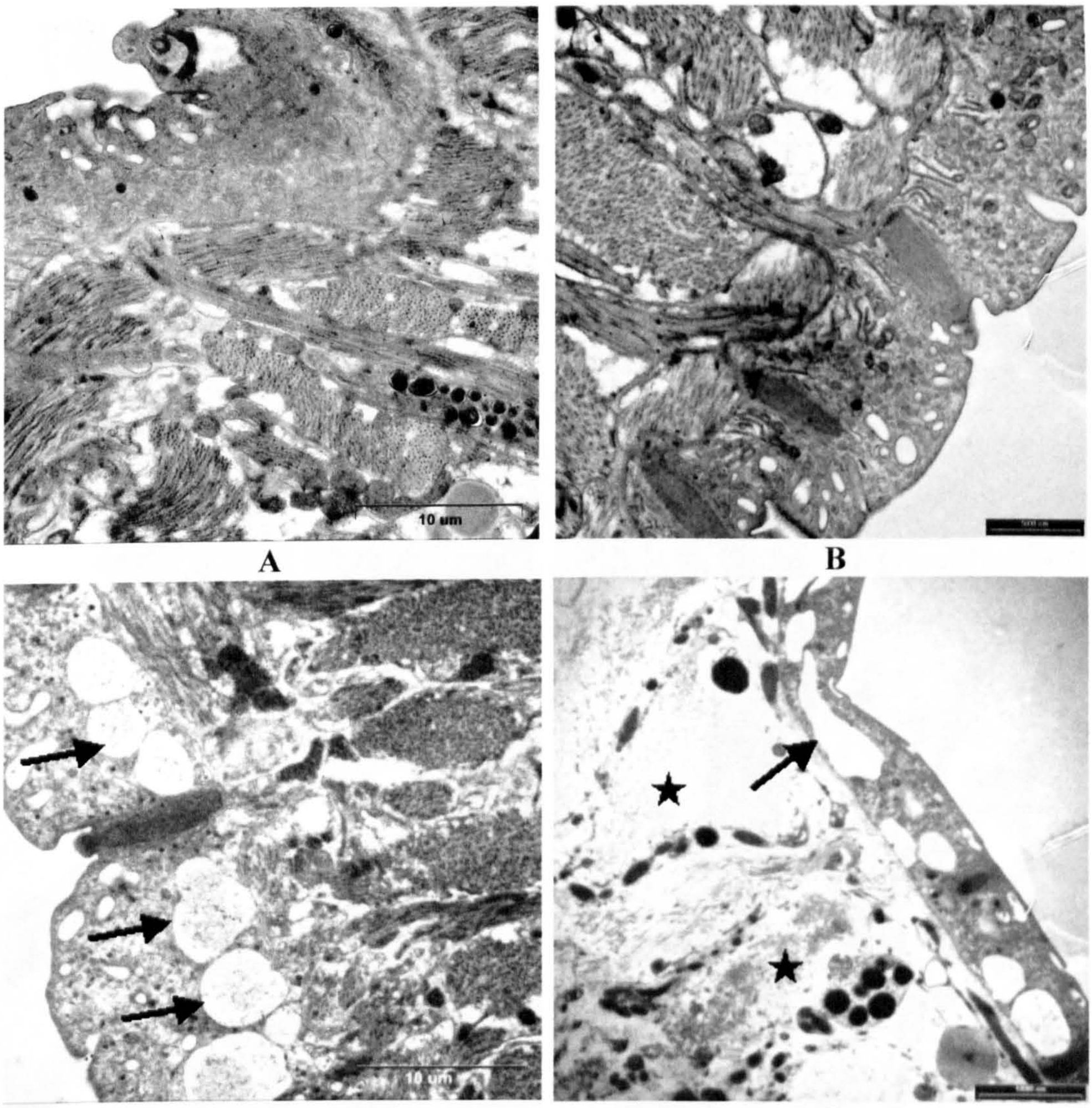

Figure 2.14 Transmission electron microscopy of adult male worms of *S. mansoni* after 24h after exposure to Mirazid® or praziquantel.

Adult male worms of *S. mansoni* were exposed to MZD or PZQ at concentrations of 200μg ml^{-1} and 20μg ml^{-1}. After 24 hours of exposure, worms were fixed, processed for TEM and examined using a TEM (Zeiss 902). (A) Control male worms exposed to 1% DMSO only, (B) MZD 20μg ml^{-1}, (C) MZD 200μg ml^{-1} and (D) PZQ 200μg ml^{-1}. Scale bars measure 10μm for (A), (C) and (D) and 5μm for (B). The arrows point to sub-tegumental vacuolisation. The stars point to muscle and parenchymal damage.

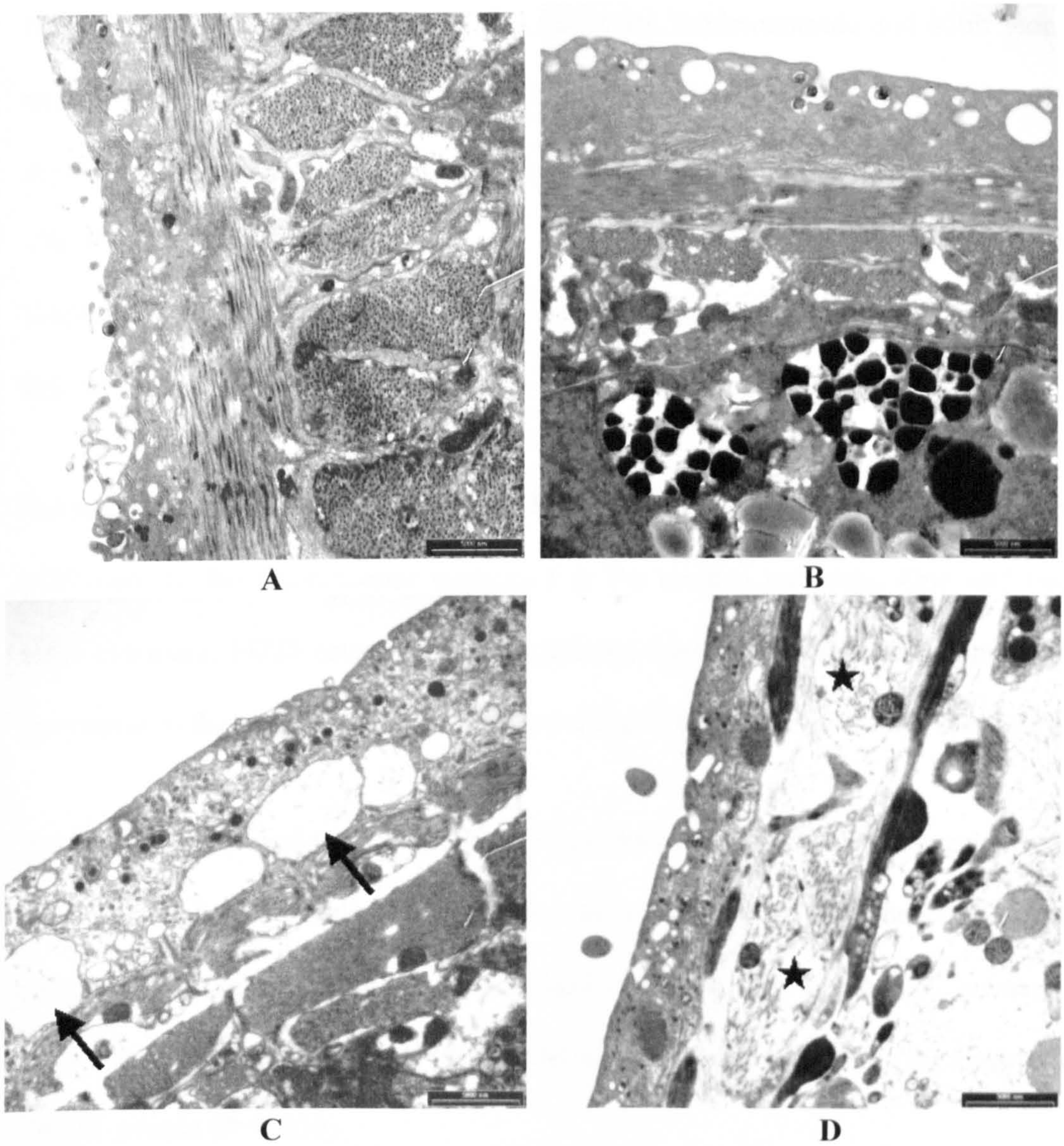

Figure 2.15 Transmission electron microscopy of adult female worms after 24 hours of exposure to Mirazid® or praziquantel.

Adult female worms of *S. mansoni* were exposed to either MZD or PZQ at a concentration of 200μg ml^{-1}. After 24 hours of exposure, worms were fixed, processed for TEM and examined using a TEM (Zeiss 902). (A) Control female worms exposed to 1% DMSO only, (B) MZD 200μg ml^{-1}, (C) MZD 200μg ml^{-1} and (D) PZQ 200μg ml^{-1}. Scale bar 5μm. The arrows point to sub-tegumental vacuolisation. The stars point to muscle and parenchymal damage.

2.3.9 Effect of Mirazid® and praziquantel on intra-parasite calcium

While the precise modes of action of MZD and PZQ have yet to be determined, it has been suggested that the effects of PZQ are in part a result of changes in calcium flux (Cioli *et al.*, 1995). To investigate the effects of PZQ and MZD on calcium balance within the parasites, we exploited fluo-3 AM which is a specific fluorescent dye that

increases its fluorescence when it binds calcium. Schistosomula and adult worms of *S. mansoni* were labelled with fluo-3 AM and then treated with either MZD or PZQ at a concentration of 200μg ml^{-1}. Control parasites were incubated with media containing 1% DMSO. Thirty minutes, 1, 2 and 4h after exposure, the amount of fluorescence (indicating the level of intracellular calcium) was measured using the fluorescent microscope.

Schistosomula exposed to PZQ or MZD for 30 minutes showed no significant difference in the fluorescence compared to the control parasites. One and two hours after exposure, MZD caused highly significant increases in the fluorescence ($P<0.01$) compared to the control group while PZQ caused no significant effect (see figure 2.16).

Adult worms exposed to PZQ for 30 minutes showed a highly significant increase in the fluorescence in both male and female worms compared to both the control and MZD groups ($P<0.01$). MZD on the other hand caused a non significant increase in the fluorescence of male worms ($P=0.27$) and a significant increase in the fluorescence of female worms ($P=0.036$).

One, 2 and 4 hours after exposure, both MZD and PZQ caused highly significant increases in the fluorescence in both male and female worms compared to the control group ($P<0.01$). However, the increases of fluorescence observed in worms treated with PZQ were always highly significant compared to MZD in both male and female worms ($P<0.01$) (see figure 2.17).

When adult worms were treated with either MZD or PZQ first, washed and then labelled with the fluorescent dye, the results were significantly different. Adult worms exposed to MZD or PZQ for 1 and 4 hours showed highly significant increases in the fluorescence of both male and female worms compared to the control group ($P<0.01$).

However, in this case worms exposed to MZD showed a highly significant increase in the fluorescence of both male and female worms compared to worms exposed to PZQ ($P<0.01$) (see figure 2.18).

These results indicate that both PZQ and MZD cause an increase of the intra-parasite calcium levels. Some aspects of similarities of the effect of both drugs on the tegument of schistosomes taken together with these results might indicate that both drugs could exhibit their effects at least partially through changes in calcium fluxes.

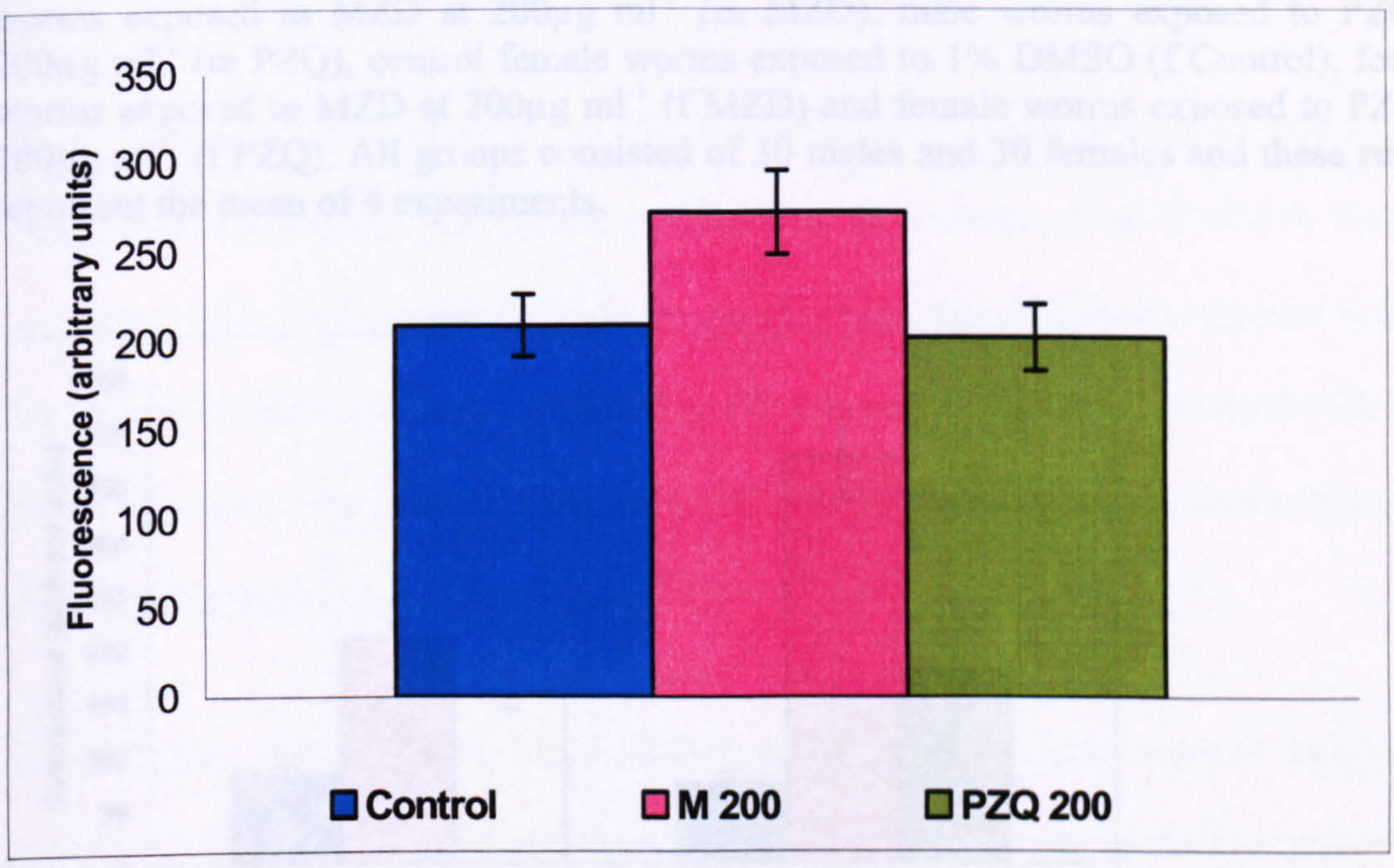

Figure 2.16 Effect of 1 hour of exposure to Mirazid® or praziquantel on the intra-parasite calcium of schistosomula of *S. mansoni*.

Schistosomula were labelled with fluo-3 AM and then treated with either MZD or PZQ at 200µg ml^{-1} for 1h before their fluorescence was quantified using a fluorescent microscope. The columns represent the quantities of fluorescence ± 95% confidence intervals. Control schistosomula exposed to 1% DMSO only (Control), MZD 200µg ml^{-1} (M 200) and PZQ 200µg ml^{-1} (PZQ 200). All groups consisted of 100 schistosomula and these results represent the mean of 3 experiments.

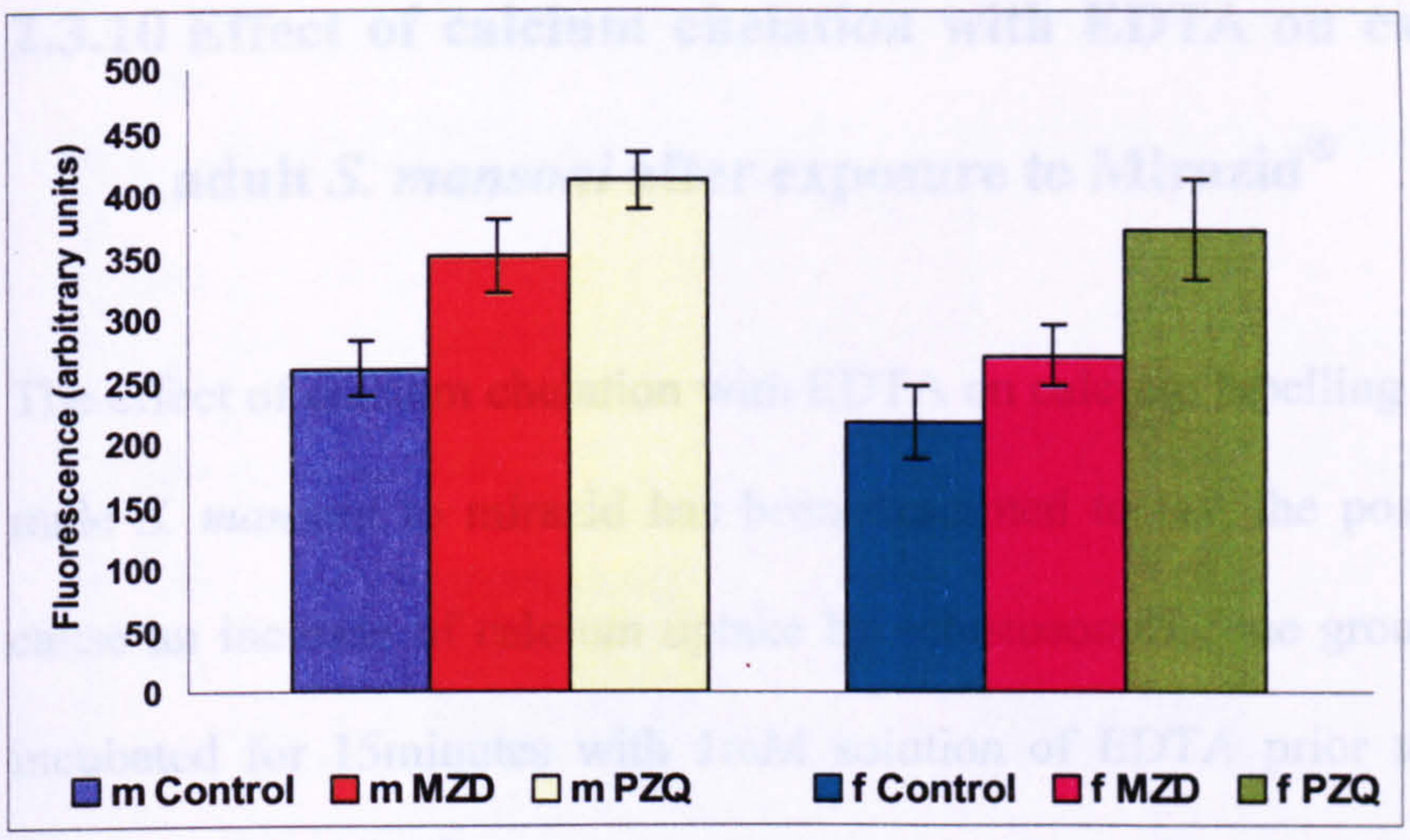

Figure 2.17 Effect of 2 hours of exposure to Mirazid® or praziquantel on the intra-parasite calcium of adult worms of *S. mansoni*.

Adult male and female worms were labelled with fluo-3 AM and then treated with either MZD or PZQ at 200µg ml^{-1} for 2h.The fluorescence was quantified using a fluorescent microscope. The columns represent the quantities of fluorescence ± 95% confidence intervals. Control male worms exposed to 1% DMSO (m Control), male worms exposed to MZD at 200µg ml^{-1} (m MZD), male worms exposed to PZQ at 200µg ml^{-1} (m PZQ), control female worms exposed to 1% DMSO (f Control), female worms exposed to MZD at 200µg ml^{-1} (f MZD) and female worms exposed to PZQ at 200µg ml^{-1} (f PZQ). All groups consisted of 30 males and 30 females and these results represent the mean of 4 experiments.

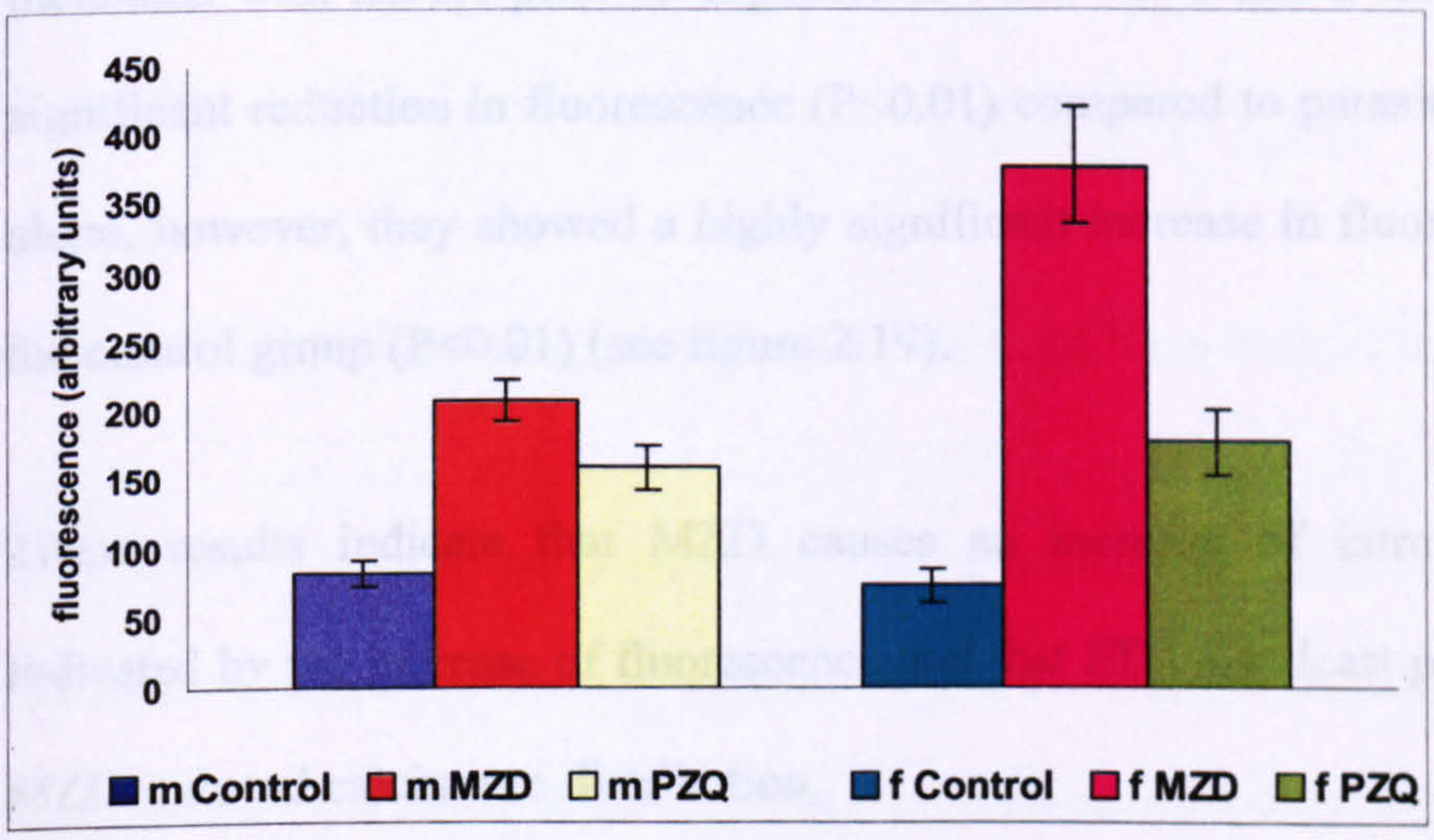

Figure 2.18 Effect of 1 hour of exposure of adult worms of *S. mansoni* to Mirazid® or praziquantel prior to labelling with fluo-3 AM.

Adult male and female worms were treated with either MZD or PZQ at 200µg ml^{-1} for 1h, washed and labelled with fluo-3 AM. The fluorescence was quantified by fluorescent microscope. The columns represent the quantities of fluorescence ± 95% confidence intervals. Control male worms exposed to 1% DMSO only (m Control), male worms exposed to MZD at 200µg ml^{-1} (m MZD), male worms exposed to PZQ at 200µg ml^{-1} (m PZQ), control female worms exposed to 1% DMSO only (f Control), female worms exposed to MZD at 200µg ml^{-1} (f MZD) and female worms exposed to PZQ at 200µg ml^{-1} (f PZQ). All groups consisted of 30 males and 20 females and these results represent the mean of 3 experiments.

2.3.10 Effect of calcium chelation with EDTA on calcium labelling of adult *S. mansoni* after exposure to Mirazid®

The effect of calcium chelation with EDTA on calcium labelling after exposure of adult male *S. mansoni* to mirazid has been examined to test the possibility that MZD can cause an increase of calcium uptake by schistosomes. One group of adult worms was incubated for 15minutes with 1mM solution of EDTA prior to their treatment with MZD at 200μg ml^{-1} for 1 and 2h. The second group was treated with MZD without EDTA and the third, the control group, was incubated in medium supplemented with 1% DMSO. Parasites were washed and labelled with fluo-3 AM. Fluorescence quantification was carried out as before (see section 2.2.8).

After 1 and 2 hours of exposure to MZD at 200μg ml^{-1}, adult worms showed a highly significant increase of fluorescence compared to the control group ($P<0.01$). Parasites incubated with EDTA prior to exposure to MZD for 1 and 2 hours, showed a highly significant reduction in fluorescence ($P<0.01$) compared to parasites treated with MZD alone, however, they showed a highly significant increase in fluorescence compared to the control group ($P<0.01$) (see figure 2.19).

These results indicate that MZD causes an increase of intra-parasite calcium as indicated by the increase of fluorescence and that EDTA at least partially interfere with MZD-induced calcium re-distribution.

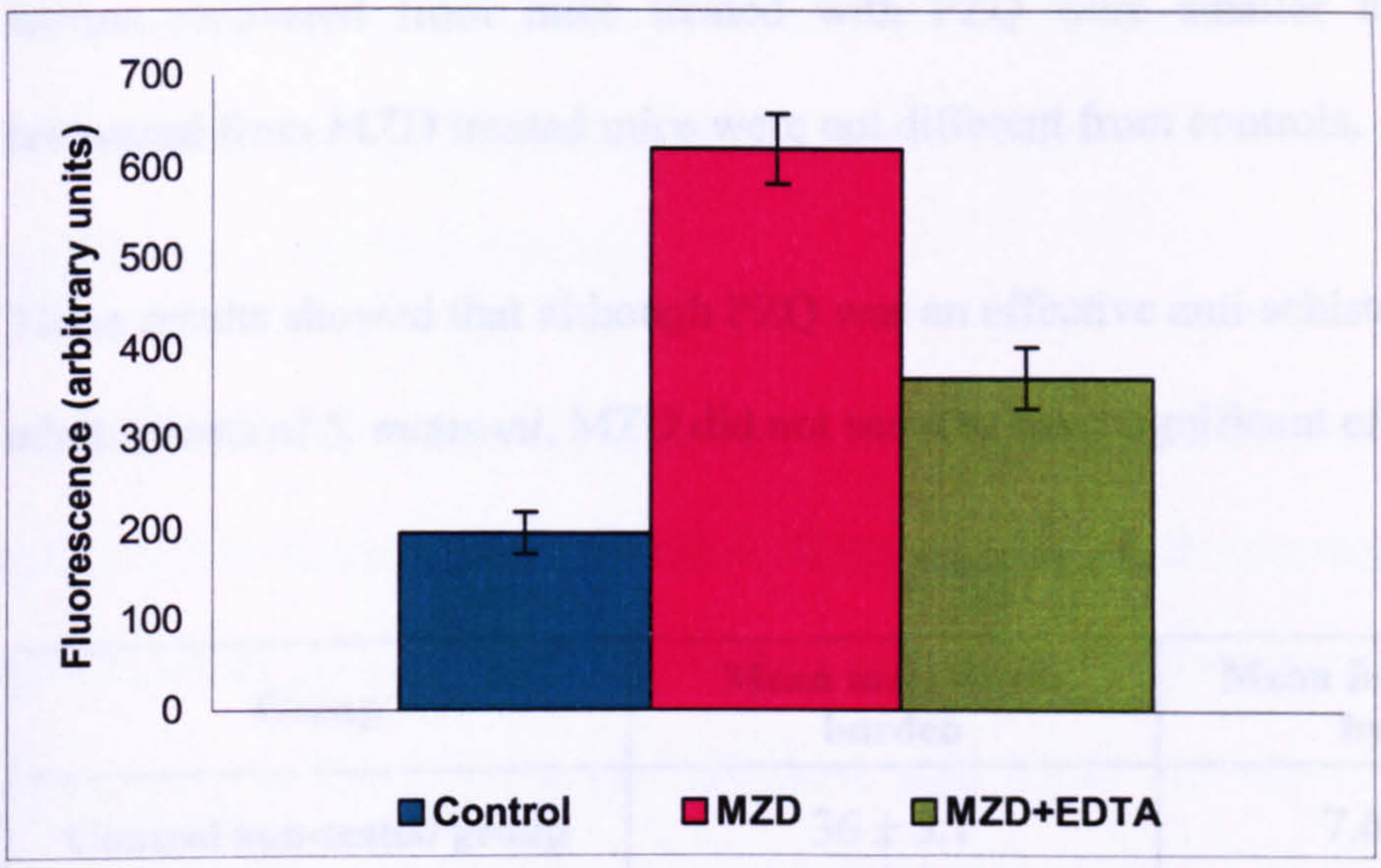

Figure 2.19 Effect of calcium chelation with EDTA on calcium labelling after exposure of adult worms of *S. mansoni* to Mirazid®

Worms were exposed to MZD at 200µg ml^{-1} for 1h with or without prior incubation with 1mM solution of EDTA. They were washed, labelled with fluo-3 AM and fluorescence was quantified using fluorescent microscope. The columns represent the quantities of fluorescent (in arbitrary units) ± 95% confidence intervals. Control worms incubated with the solvent only (Control), adult worms exposed to MZD 200µg ml^{-1} without EDTA (MZD) and adult worms treated with 1mM of EDTA prior to exposure to MZD 200µg ml^{-1} (MZD+EDTA). All groups consisted of 35 males and these results represent the mean of 3 experiments.

Results of the *in vivo* experiments

2.3.11 Effect of praziquantel and Mirazid® on worm burden

Mice infected with *S. mansoni* were treated with either MZD or PZQ 6 weeks after infection. One group, control infected, was left without treatment. Two weeks after treatment (8-weeks after infection), mice were killed and perfused (see section 2.2.2) for worm recovery.

The number of worms recovered from mice treated with MZD at 3 doses each of 400mg kg^{-1} was not significantly different from that of the non-treated mice (see table 2.3). On the other hand, mice treated with PZQ at a single dose of 400mgkg^{-1} showed a highly significant reduction in the numbers of both male and female worms ($P<0.01$). The

worms recovered from mice treated with PZQ were smaller in size while those recovered from MZD treated mice were not different from controls.

These results showed that although PZQ was an effective anti-schistosomal drug against adult worms of *S. mansoni*, MZD did not seem to have significant effects *in vivo*.

Group	Mean male worm burden	Mean female worm burden
Control non-teated group	36 ± 3.1	7.6 ± 0.9
Mirazid® treated group	35.8 ± 3.6	5.8 ± 0.3
Praziquantel treated group	16.5 ± 4.8	1.4 ± 1

Table 2.3 Number of worms recovered after perfusion of mice.

Control mice and mice treated with either MZD or PZQ were perfused 2 weeks after treatment and the worms recovered were counted. The table presents the mean number of male and female worms for each group ± standard error. All groups consisted of 10 mice.

2.3.12 Effect of treatment of infected mice with either Mirazid® or praziquantel on the egg burden in liver tissue

After perfusion of mice a piece of each liver was weighed and digested with KOH. The digested tissue was centrifuged and the eggs were re-suspended in PBS. Egg counting was carried out using a light microscope.

The number of eggs per gram of liver tissue of the MZD-treated mice showed a slight reduction (16% reduction) and this reduction was not significantly different from that of the control non-treated mice ($P=0.39$). In contrast the number of eggs per gram of liver tissue of the PZQ-treated mice showed a highly significant decrease compared to that in the infected non-treated mice ($P<0.01$) (see figure 2.20).

Most of the eggs in the livers of PZQ treated mice appeared to be granular and dark in colour while that of both MZD treated mice or infected non-treated mice were bright and showed normal morphology.

These results showed that PZQ caused a decrease in the number of eggs deposited per gram of liver tissues of infected mice. This reduction can be as a result of anti-fecundity effects of the drug or secondary to the decrease in the number of female worms after treatment. On the other hand, treatment with MZD did not cause any significant reduction in the number of eggs per gram of liver.

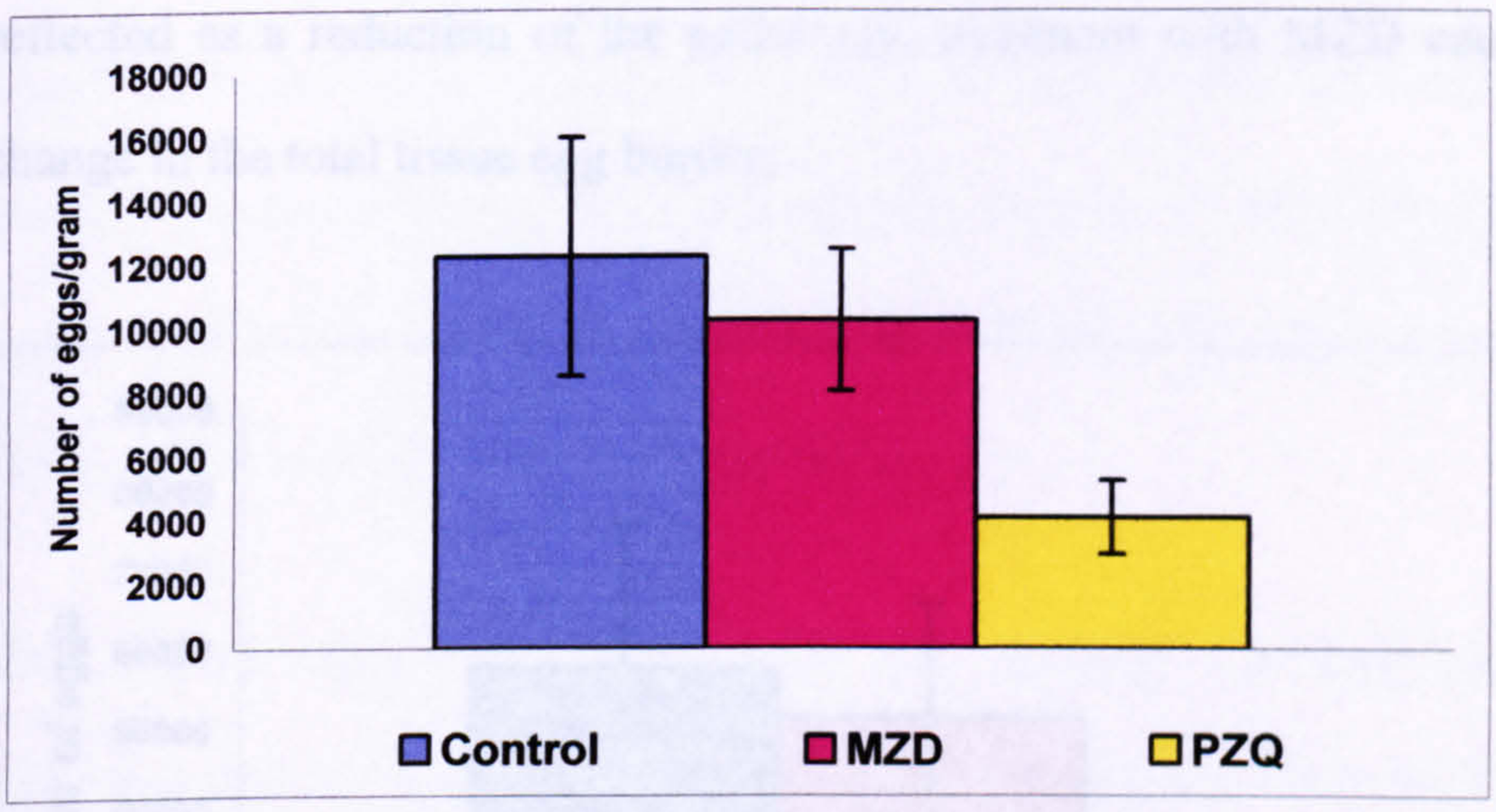

Figure 2.20 Egg burden per gram of liver tissue of mice infected with *S. mansoni* and treated with either Mirazid® or praziquantel

After killing of mice, a piece of each liver was weighed and digested in KOH. The eggs were counted using a dissecting microscope. The columns represent the number of eggs per gram of liver tissue ± 95% confidence interval. Control non-treated mice (Control), Mirazid® treated mice (MZD) and praziquantel treated mice (PZQ). All groups consisted of 10 mice.

2.3.13 Effect of treatment of infected mice with either Mirazid® or praziquantel on the total tissue egg load

After killing the infected mice, a piece of the liver and the whole gut of each mouse were digested with KOH. They were then processed exactly as mentioned previously.

The number of eggs in the whole liver was added to the number of eggs in the gut to represent the total tissue egg load.

Mice treated with PZQ showed a highly significant decrease in the total tissue egg load compared to the infected non-treated mice and to MZD-treated group ($P<0.01$). In contrast, the total tissue egg load in mice treated with MZD was not significantly different from that of the infected non-treated mice ($P=0.61$) (see figure 2.21).

These results showed that while treatment of *S. mansoni* infected mice with PZQ caused a reduction in the number of eggs deposited in the tissues of infected mice which will be reflected as a reduction of the pathology, treatment with MZD caused no significant change in the total tissue egg burden.

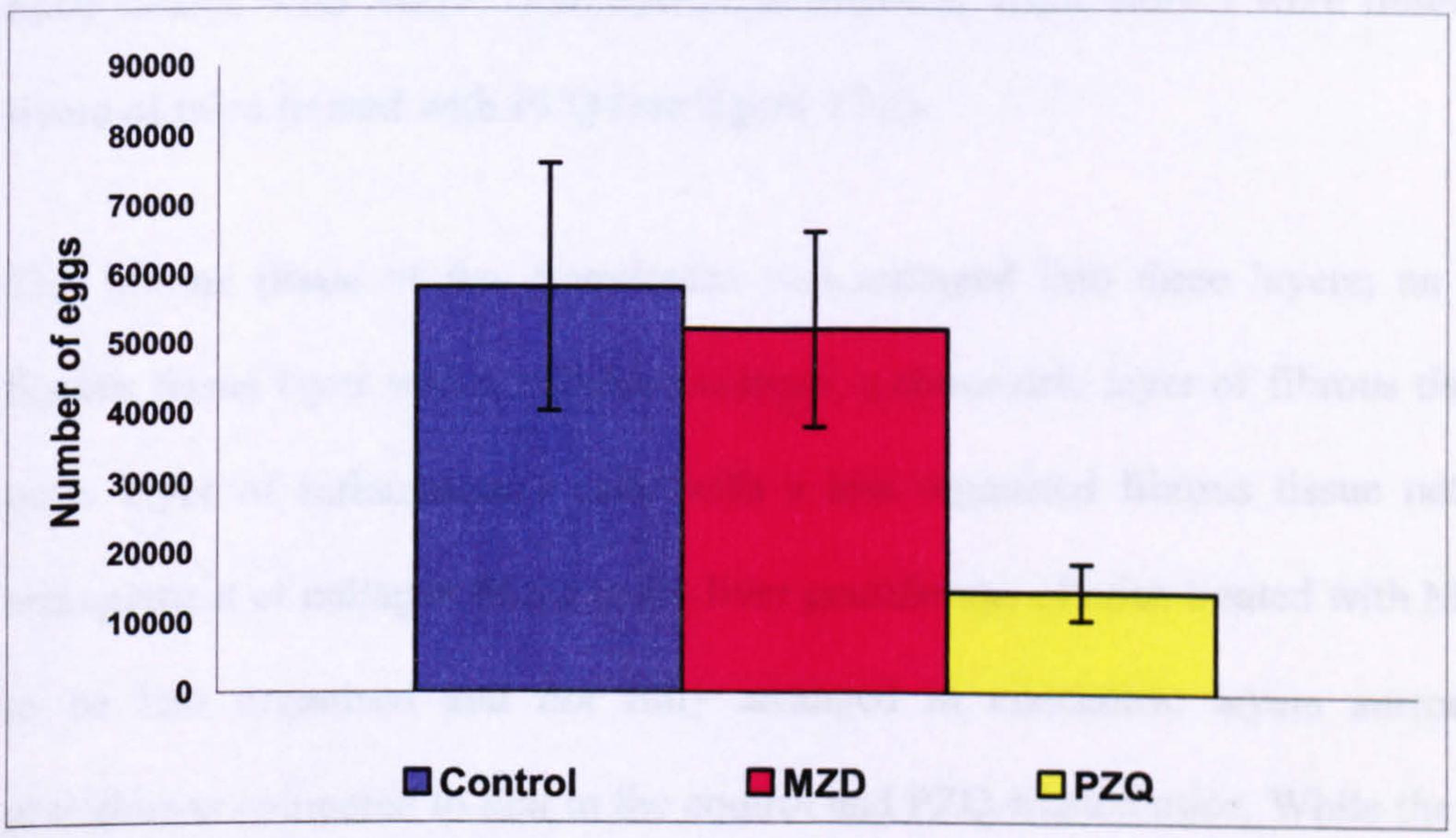

Figure 2.21 Total tissue egg burden of mice infected with *S. mansoni* after treatment with either Mirazid® or praziquantel.

After killing of the mice, the gut and a piece of liver were digested in KOH and their total egg count was calculated. The columns represent the mean of total number of eggs in mice tissues ± 95% confidence interval. Control non-treated mice (Control), Mirazid® treated mice (MZD) and praziquantel treated mice (PZQ). All groups consisted of 10 mice.

2.3.14 Effect of treatment of infected mice with either Mirazid® or praziquantel on the diameter, content and structure of liver granulomas

Mice were killed 2 weeks after treatment. A piece of the liver of each mouse were fixed, processed for histology and stained with H & E. The diameter of granulomas was measured using a light microscope with graduated eye-pieces. The mean of the diameters of granulomas of each mouse were used for the statistical analyses.

There was no significant difference in the diameter of liver granulomas of treated or non-treated mice. The average diameter of liver granulomas were 362 ± 23μm for control non-treated mice, 381 ± 15μm for mice treated with PZQ and 355.5 ± 28μm for mice treated with MZD. Granulomas surrounding adult worms were observed in the livers of mice treated with PZQ (see figure 2.22).

The fibrous tissue of the granulomas was arranged into three layers; an interlacing fibrous tissue layer with a cellular infiltrate, a concentric layer of fibrous tissue and an outer layer of inflammatory cells with a less organised fibrous tissue network. The arrangement of collagen fibres in the liver granulomas of mice treated with MZD tended to be less organised and not fully arranged in concentric layers surrounding the granulomas compared to that in the control and PZQ-treated mice. While the number of eosinophils in liver granulomas of mice MZD treated mice (78 ± 9/HPF) was slightly less than the control mice (84 ± 11/HPF) or mice treated with PZQ (91 ± 14/HPF), this difference was not significant.

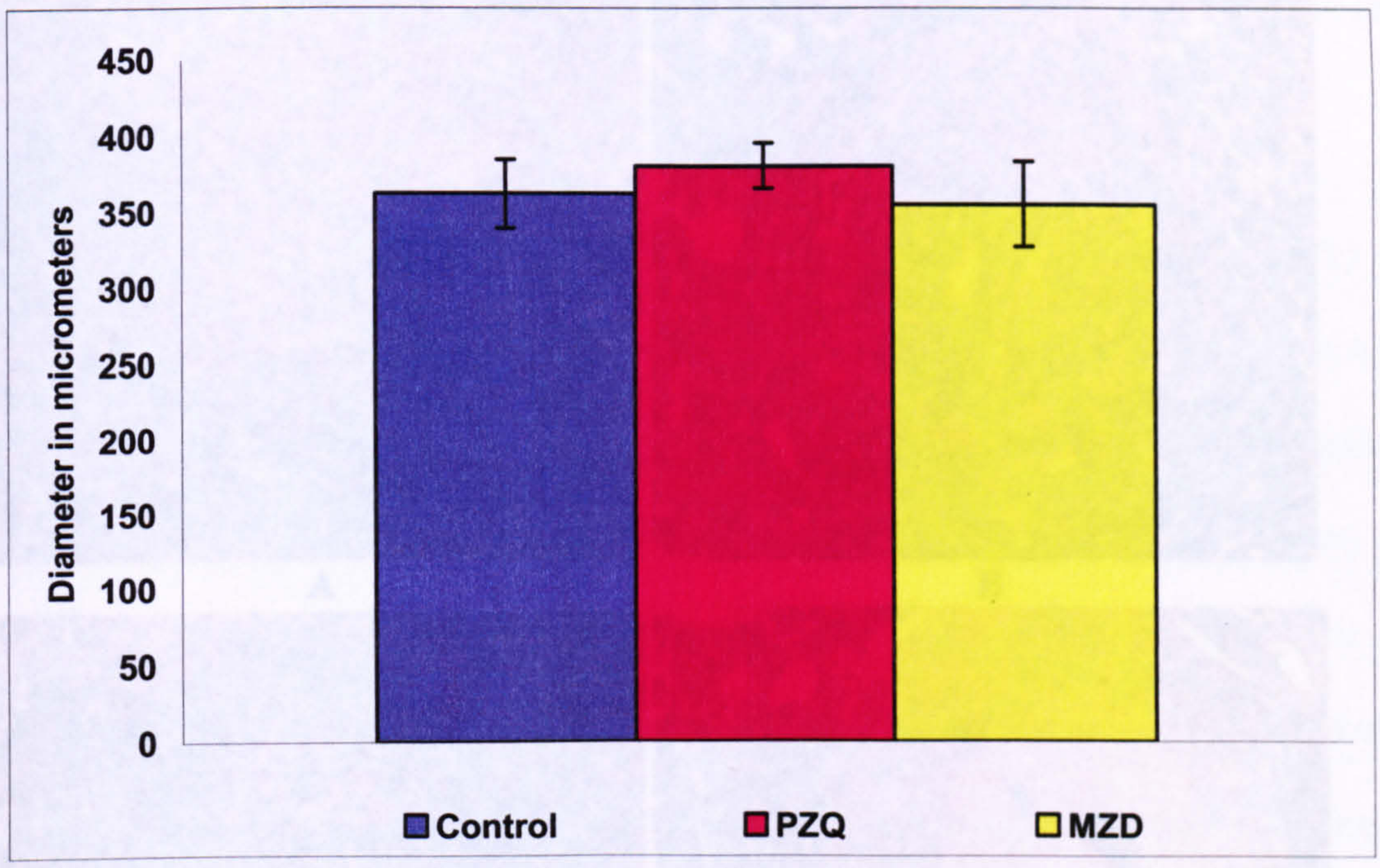

Figure 2.22 Effect of treatment of mice infected with *S. mansoni* with either Mirazid® or praziquantel on the diameter of liver granulomas.

After killing of mice, a piece of liver was processed for histology and stained with H&E. The diameter of the granulomas was measured using a light microscope. The columns represent the average of the diameter liver granulomas of each group ± 95% confidence intervals. Infected non-treated mice (Control), PZQ treated group (PZQ) and MZD treated group (MZD). All groups consisted of 10 mice.

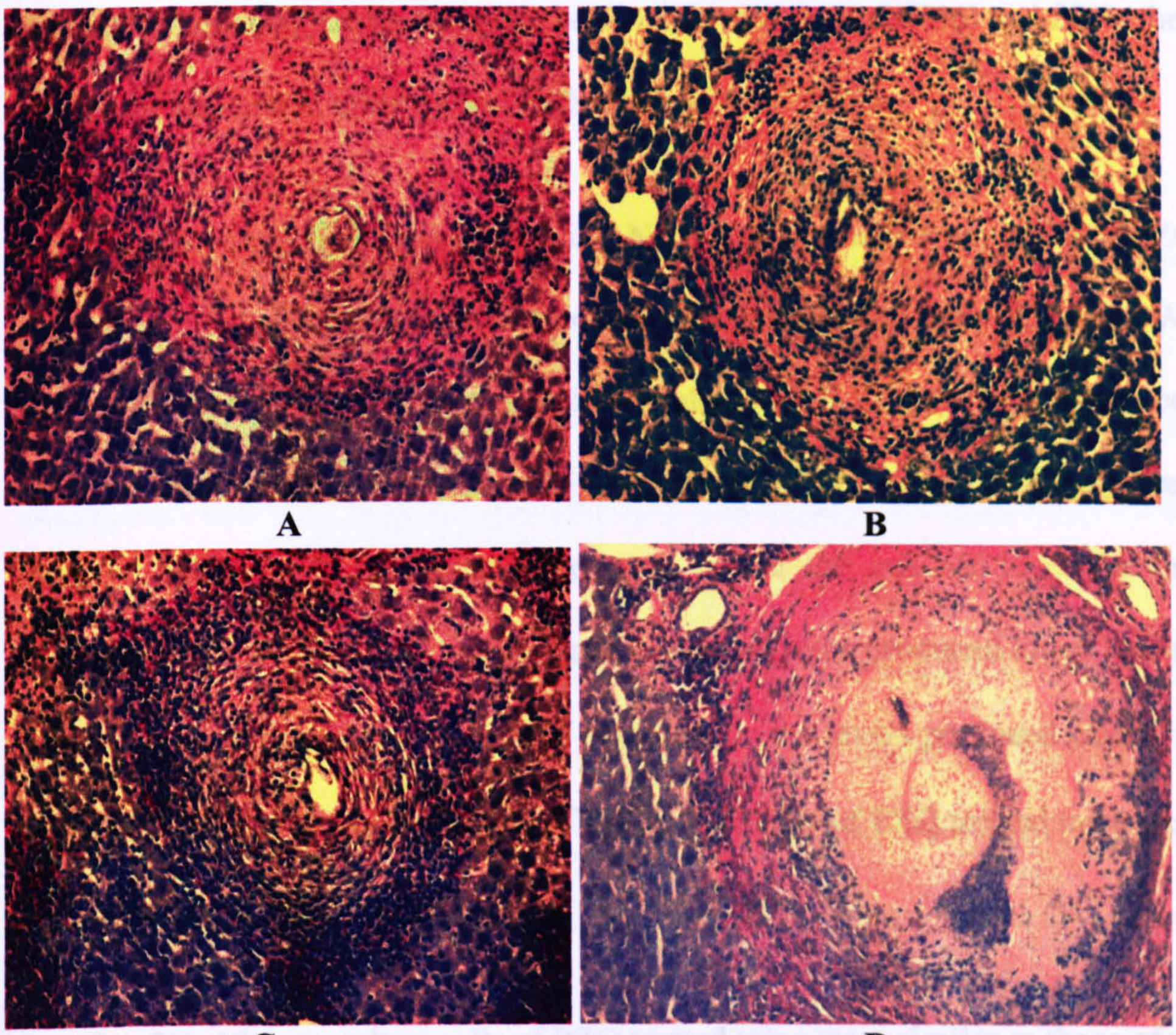

Figure 2.23 Sections of *S. mansoni* egg granuloma in the livers of infected treated or un-treated mice (Haematoxylin and Picro Sirius red stain).

Liver sections were processed and stained Picro Sirius red to localise collagen fibres. Egg granuloma in liver section from infected un-treated mouse (A), egg granuloma in liver section from PZQ treated mouse (B), egg granuloma in liver section from MZD treated mouse (C) and worm granuloma in liver section from PZQ treated mouse (D).

2.3.15 Effect of treatment of infected mice with either Mirazid® or praziquantel on lymphoblastogenesis and cytokine production

After killing of the mice, lymphocytes from mesenteric LNs and spleen from each group were stimulated *in vitro* with ConA, SWAP and SEA. Control cells were cultured without stimulation. After 5 days of culturing, the supernatants were collected for cytokine assays for IL-4, IL-10, IL-13 and IFN-γ. A blastogenesis assay was carried out using radioactive thymidine and the rate of cell multiplication was assessed by measuring the cellular radioactivity. All cells were stimulated to multiply with ConA indicating their viability. Although lymphocytes from LNs and spleen of infected

treated and infected un-treated mice were reactive to both SWAP and SEA, the level of reactivity was less in mice treated with either PZQ or MZD.

Levels of IL-4 and IL-13 were lower in LNs and spleen cells from MZD or PZQ treated mice than infected un-treated mice in response to stimulation with SEA and SWAP. The difference was greatest in spleen cells stimulated with SEA. While spleen cells from infected un-treated mice produced high levels of IFN-γ, levels of IFN-γ from spleen cells of MZD or PZQ treated mice were below the cut off level for the assay. Furthermore, LNs from MZD treated and un-treated mice produced lower levels of IFN-γ in response to stimulation with SEA compared to the levels produced after stimulation with SWAP. In both cases the level of IFN-γ from LNs of PZQ treated mice were below the cut off level for the assay.

Levels of IL-10 from spleen cells of PZQ treated mice were similar to those of untreated animals in response to stimulation with either SWAP or SEA. However, LNs from PZQ treated mice produced higher levels of IL-10 than un-treated animals after stimulation with either SWAP or SEA. Spleen cells and LNs from MZD treated mice produced lower levels of IL-10 in response to stimulation with either SWAP or SEA compared to the un-treated animals.

	IL-10				IL-4				IL-13				IFN-gamma			
	LNs		Spleen		LNs		Spleen		LNs		Spleen		LNs		Spleen	
	SEA	SWAP	SEA	SWAP	SEA	SWAP	SEA	SWAP	SEA	SWAP	SEA	SWAP	SEA	SWAP	SEA	SWAP
Control			13	30												
Infected	2948	1782	3004	612	2000	959	2000	72	8546	7654	9461	1551	76	541	426	411
MZD	2106	783	924	210	2000	685	758	97	8200	6429	2322	222		433		
PZQ	3167	1963	2693	606	2000	355	1031	286	6957	3938	4278	416				

Table 2.4 Levels of different cytokines produced by spleen and LNs cells from infected mice.

Cells from spleen and LNs were culture *in vitro* and stimulated with different antigens. Five days after culture, levels of different cytokines were measured using ELISA. The levels were measured in $pgml^{-1}$. Un-infected mice (Control), infected un-treated mice (Infected), MZD treated mice (MZD), PZQ treated mice (PZQ).

2.4 Discussion

Despite all efforts that have been made to control schistosomiasis this disease remains a major health problem in the tropics and subtropics. Chemotherapy is considered to be the keystone of any control programme against schistosomiasis, with praziquantel being the drug of choice because it is effective against all schistosome species, it is easy to administer, it is safe with few and transient side effects and most importantly is now cheap (Hagan *et al.*, 2004). However, the appearance of schistosome strains with lower susceptibility to praziquantel has highlighted the need for other anti-schistosomal drugs (Kusel and Hagan, 1999; Doenhoff *et al.*, 2002). Furthermore, praziquantel has modest effects against the immature stages of schistosomes (Andrews, 1981; Sabah *et al.*, 1986). In the last few years, Mirazid® has been licensed and released for the treatment of human schistosomiasis in Egypt. Since then, there has been much debate about its anti-schistosomal effects. In this study, the anti-schistosomal activities of Mirazid®, compared to those of praziquantel, were investigated both *in vitro* and *in vivo* against early schistosomula and adult stages of *S. mansoni*.

The effects of MZD and praziquantel on the viability of schistosomula of *S. mansoni* were assessed using the toluidine blue exclusion test. This test is appropriate for assessment of viability of schistosomula since results are not different from those obtained using the morphology and mobility parameters. It has the advantage of being quick and easy to perform (Gold, 1997). In this study, the viability of schistosomula exposed to MZD at concentrations of 200, 100 and 50μg ml^{-1} was significantly reduced after 4 and 8 hours after exposure. PZQ caused less, but significant, impairment in the viability of schistosomula at concentrations of 200μg ml^{-1} and 100μg ml^{-1}. The effects of both drugs were time and dose dependent. However, the most striking feature of schistosomula exposed to PZQ was the blebbing around or within the oral sucker of the

parasites that was evident, under light microscope, within 1h of exposure to PZQ at concentrations of 200, 100 and 50μg ml^{-1}. The results of the toluidine blue exclusion test were confirmed with the results of the Hoechst test. The number of worms stained with Hoechst dye, indicating damage, was significantly increased after exposure to the high doses of either MZD or PZQ. Based on the results of the toluidine blue exclusion test, two concentrations were used in the rest of experiments on schistosomula. The 200μg ml^{-1} was used as a high and effective concentration and the 20μg ml^{-1} was used as a low concentration that did not show any significant effects in the toluidine blue test but may have other effects not detectable with this method. SEM revealed that in schistosomula exposed to MZD there was surface damage within 4h of *in vitro* exposure to the high concentrations of the drug. The changes were progressive with time. After 8h of exposure, parts of the surface of schistosomula were sloughed leaving bare areas. PZQ on the other hand caused limited surface blebs and irregular contractions of the parasites after 4 and 8h of exposure. These results are in agreement with the results of (Xiao *et al.*, 1985). They reported that the immature stages were less susceptible to PZQ *in vitro* and that in early schistosomula stages about 2 to 4h of exposure is required to cause limited small blebs around the oral sucker and on the surface of the parasites. Similarly, *in vivo* studies showed that artemether (Utzinger *et al.*, 2001a) and Ro15-5458 (Guirguis, 2003) were more effective against the immature stages of schistosomes compared to PZQ. However, Andrews reported that all stages of *S. mansoni* are equally affected after *in vitro* exposure to PZQ (Andrews, 1981). However, higher concentrations of PZQ were used in this study than reported in the previous studies because the current study was designed to investigate the efficacy of MZD and to compare it to that of PZQ.

The earliest observed *in vitro* effect of MZD on adult worms of *S. mansoni* was enhanced worm motility with an increase in their length and subsequent separation of

worm pairs. The increased worm activity observed in this study accords well with the observations of Hassan and colleagues. They reported that MZD induced contractions of the somatic muscles of adult *S. mansoni* worms (Hassan *et al.*, 2003). Increased worm activity has been reported after *in vitro* exposure to other drugs, for instance, hycanthone (Senft and Hillman, 1973), oxamniquine (Chavasse *et al.*, 1978) and low doses of PZQ (Chavasse *et al.*, 1979; Xiao *et al.*, 1985). The increase in motility after exposure to hycanthone and oxamniquine was attributed to the blockade of acetylcholine receptors caused by the drugs, with subsequent suppression of the inhibitory effects of acetylcholine (Hillman and Senft, 1975). A similar effect may be produced by MZD. In support of this idea is the observation that a higher concentration of carbachol (a cholinergic compound) was needed to stop worm motility during the FRAP experiments in the worms treated with MZD.

Worm separation was evident as early as 15 minutes after exposure to the high doses of MZD (200μg ml^{-1} and 100μg ml^{-1}). The separation progressed with time to the extent that all worm pairs were separated after 6h of exposure to MZD at a concentration of 200μg ml^{-1}. The exact mechanism of worm separation is not known. A possible explanation is that it could be a result of the increased and irregular worm motility. However, separation of worm pairs continued to progress with time even though worm motility had returned to normal after 3-4h of exposure. Another possible cause of worm separation is the preferential tegumental damage observed in scanning electron microscopy. Surface blebs were observed only on the surface of gynaecophoric canals of male worms after 4h of *in vitro* exposure to MZD at a concentration of 20μg ml^{-1}. This damage could interfere with the interaction between male and female worms and promote their separation. These results accord with the *in vivo* results observed by Badria and colleagues (2001) who reported that MZD induces separation of *S. mansoni* worm pairs (Badria *et al.*, 2001).

After lengthy *in vitro* exposure of adult worms of *S. mansoni* to MZD at high doses, there was a marked damage to the surface of the parasites manifested by the loss of tubercular spines, loss of the surface ridges between the tubercles and surface blebbing. Both male and female worms appeared to be equally affected. The damage became extensive in the form of sloughing of parts of the surface of the parasites after 3 days of exposure. These observations agree with the observation of Hassan and colleagues. They showed that MZD causes tegumental damage of the parasite in the form of tegumental disruption, oedema and loss of the spines covering the tubercles (Hassan *et al.*, 2003).

The earliest observed effects after *in vitro* exposure of adult worms to PZQ were contractions and coiling of worms that became evident within seconds of exposure. After 30 minutes of exposure, worms uncoiled and remained shorter and thicker than the controls. These results are in agreement with the observations described by (Pax *et al.*, 1978), (Xiao *et al.*, 1985) and (Pica-Mattoccia and Cioli, 2004). All PZQ concentrations used in this study caused marked surface damage in the form of extensive surface blebbing. The damage was widely spread in male worms compared to a localised patchy damage in female worms. These results accord with previous findings that adult *S. mansoni* worms exposed to PZQ showed surface damage and vesicle formation which is more extensive in male worms (Becker *et al.*, 1980; Mehlhorn *et al.*, 1981; Staudt *et al.*, 1992). Similar surface damage was observed in adult *S. mansoni* worms recovered from mice treated with PZQ. The worms showed surface damage that was more extensive in male worms than that in female worms (Shaw and Erasmus, 1983; Shaw and Erasmus, 1987; Shaw, 1990). However, Gonnert and Andrews reported that both male and female worms of *S. mansoni* are equally susceptible to the effects of PZQ (Gonnert and Andrews, 1977). Unexpectedly, in the present study, after 72h of exposure of adult *S. mansoni* worms to both MZD (at a concentration of 200µg ml^{-1})

and PZQ (at a concentration of 5μg ml^{-1}) at the same time, the damage was less than when the parasites were exposed to either drug individually. However, the effect of PZQ prevailed. This may indicate that the drugs can interfere with each other either chemically or physiologically.

When considering membrane fluidity as an indirect indication of membrane functions, both MZD and PZQ caused significant reduction of the mobile fraction of the lipid molecules and of the lateral diffusion coefficient of both male and female worms and schistosomula of *S. mansoni*. These results are in agreement with the results of Lima and colleagues. They have shown that while both (PZQ-) and (PZQ+) caused reduction in the velocity of the lipid molecules, only (PZQ-) caused a reduction in the mobile lipid fraction. They suggested that PZQ inserts itself in the membrane and thus acts as a barrier for membrane fluidity and this insertion change the organisation of the tegument and this may be one of the ways PZQ exerts its action. The altered lipid organisation could lead to conformational changes in calcium pumps allowing an enhanced entrance of calcium ions (Lima *et al.*, 1994). In other biological systems, membrane fluidity is important in regulating enzyme activity (Rimon *et al.*, 1978) as well as the rate of second messenger production (Atlas *et al.*, 1980). PZQ and MZD, by reducing the membrane fluidity of the parasites, may interfere with some mechanisms important for their survival.

After exposure to PZQ at concentrations of 200μg ml^{-1} and 20μg ml^{-1}, adult worms of *S. mansoni* showed ultra-structural damage in the form of sub-tegumental vacuolisation and disruption of the musculature and parenchyma of both male and female worms. The damage was more evident in male worms than in the female worms. These results corresponded with the previously reported ultra-structural damage of adult *S. mansoni* worms exposed to PZQ (Becker *et al.*, 1980; Mehlhorn *et al.*, 1981). Similar changes were reported in worms recovered from mice treated with PZQ (Mehlhorn *et al.*, 1981;

Shaw and Erasmus, 1983; Shaw and Erasmus, 1987). On the other hand, MZD at high concentrations (200μgml^{-1}) caused sub-tegumental vacuolisation of both male and female worms. The damage was evident after 4h of exposure and progressed with time. There was no evidence of parenchymal or muscular damage even after 24h of exposure of adult worms to the high concentration of MZD. This provides evidence that while PZQ may act directly on the tegument and on the muscle and parenchyma as a consequence of increased calcium influx, MZD may act only locally on the tegument.

However, these ultrastructural changes of the tegument of schistosomes are not an exclusive feature of PZQ or MZD as they have been reported with other anti-schistosomal agents including Astiban (Otubanjo, 1981), hycanthone (Hillman *et al.*, 1977), oxamniquine (Popiel and Erasmus, 1984; Fallon *et al.*, 1996) and artemisinin derivatives (Xiao *et al.*, 2000; Xiao *et al.*, 2001; Shuhua *et al.*, 2002).

Although the mode of action of PZQ is not yet exactly determined, there is good evidence that PZQ, at least in part, exerts its action by increasing calcium influx. The increase in intra-parasite calcium may in turn be responsible for the increased muscular contraction and the tegumental damage (Pax *et al.*, 1978; Day *et al.*, 1992). Similarly, increased calcium uptake has been reported in the liver fluke *Opisthorchis viverrini* in response to praziquantel treatment (Ruenwongsa *et al.*, 1983). In this context, voltage-gated calcium channels (formed of an alpha chain and a beta chain) have been described in schistosomes. The beta subunits of schistosome voltage-gated calcium channels have been shown to confer PZQ sensitivity to an otherwise PZQ-insensitive mammalian calcium channel, indicating that a possible target for PZQ action is the interaction between beta subunits and pore-forming alpha 1 subunits in schistosomes (Kohn *et al.*, 2001a; Kohn *et al.*, 2003).

In the present study, the amount of intra-parasite calcium was indicated by the quantity of fluorescence after labelling of calcium with fluo-3. It has been shown that the intensity of fluo-3-related fluorescence depends on the Ca^{2+} concentration and that this fluorescence can be used as a cytoplasmic or cytosolic Ca^{2+} indicator (Kao *et al.*, 1989). In *S. mansoni*, it was shown that compounds which increase intra-parasite calcium concentration increase fluo-3/Ca^{2+} fluorescence and compounds that chelate calcium decrease the fluorescence (Sato *et al.*, 2004). In the present study, both PZQ and MZD caused a significant increase in the intra-parasite calcium of both adult male and female worms of *S. mansoni*. The increase in calcium was greater and was evident earlier with exposure to PZQ than with exposure to MZD. This corresponds with the previous *in vitro* experiments indicating that PZQ is more effective than MZD against adult worms of *S. mansoni*. However when adult worms were exposed to either drug, washed and then labelled with fluo-3, the fluorescent labelling of the intra-parasite calcium was significantly higher in worms exposed to MZD than PZQ. This can be explained by the finding that the binding of PZQ to the tissues of schistosomes can be easily reversed after incubation in drug-free medium (Andrews, 1981) to the extent that 45% and 93% of the ^{14}C-PZQ radioactivity was lost from schistosomes after 5min and 30min, respectively, after transfer to a drug-free medium (Andrews *et al.*, 1983). Similarly, the effects of praziquantel have been shown to be reversed, at least in part, when the parasites were moved to a drug-free medium (Andrews, 1981; Xiao *et al.*, 1985; Pica-Mattoccia and Cioli, 2004). On the other hand, the binding of MZD to schistosome tissues could be stronger and last longer promoting long lasting effects on the parasite calcium distribution.

Exposure of schistosomula of *S. mansoni* to MZD caused an increase in the labelling of intra-parasite calcium. PZQ, on the other hand, caused no significant change in the labelling of intra-parasite calcium. This indicates that the effect of MZD on both adult

and schistosomula stages of *S. mansoni* could be through changes in the calcium distribution in the parasite while with PZQ different mechanisms could be responsible for its actions. In support of the idea that MZD may act by increasing intra-parasite calcium, when adult worms were incubated with 1mM EDTA and 5mM EDTA for 20min before exposure to MZD, there was a significant reduction of intra-parasite calcium compared to the worms exposed to MZD without prior exposure to EDTA ($P<0.01$).

When *S. mansoni*-infected mice were treated, 6 weeks after infection, with MZD (at a dose of 400mg kg^{-1}), there was no significant difference in the number of worms recovered after perfusion compared to the infected un-treated mice. The recovered worms were mostly paired and did not show any gross morphological changes. There was no significant difference in the number of eggs per gram of liver tissue or in the total number of eggs deposited in the tissues of infected mice compared to the infected un-treated animals. At the dose used in the present study, MZD was safe and there were no deaths or signs of toxicity in the treated mice. These results provide no evidence that MZD had *in vivo* anti-schistosomal effects against adult worms of *S. mansoni*.

These results agree with the results of Botros and colleagues. They tried several doses of MZD and myrrh (180 to 10000mg kg^{-1}) against different strains of *S. mansoni* (Egyptian, Puerto Rican and Brazilian) in different hosts (mice and hamsters). The greatest observed reductions in the number of worms recovered were observed in mice infected with the Puerto Rican strain (36% reduction), mice infected with the Egyptian strain and treated with myrrh (26% reduction) and mice infected with the Egyptian strain and treated with MZD (19% reduction). There was no reduction in the number of recovered worms in hamsters infected with the Puerto Rican strain or in mice infected with the Brazilian strain and treated with myrrh. There was no significant reduction in

the number of eggs in the livers and guts of mice infected with the Egyptian strain after treatment with either MZD or myrrh (Botros *et al.*, 2004).

However, the results of the present study are in contrast with those of Badria and colleagues. They reported a 76% reduction of the number of worms recovered from mice infected with the Egyptian strain of *S. mansoni* after treatment with either myrrh or MZD. They also reported an increase in the percentage of mature stages of eggs deposited in the tissues of infected mice compared to immature stages as a result of cessation of new egg production (Badria *et al.*, 2001). One possible explanation for this distinction is the difference in the schistosome strain or the strain of mice used in both experiments. While Badria and colleagues used the Egyptian strain of *S. mansoni* to infect male albino mice, in the present study, the Puerto Rican strain was employed to infect female TO mice. However, Botros and colleagues reported a little effect on any of the strains of *S. mansoni* they used.

One other possibility is the composition of MZD. MZD is an extract of myrrh that can be obtained from different species of the plant *Commiphora*. It has been shown that myrrh is a very complex mixture of compounds and that myrrh derived from one species of *Commiphora* is different from that obtained from another species (El Ashry *et al.*, 2003). Myrrh from *C. molmol* contains 23-40% resin (myrrhin), 2-8% volatile oils (myrrhol) and 40-60% bitter principle (Al-Awadi *et al.*, 1991). The complex composition of myrrh may be difficult to ensure consistency of contents from batch to batch. However, the batch of MZD used in this study was different from that used by Botros and colleagues who, themselves, used several batches of MZD in their multicentre studies but they all confirmed the inefficacy of MZD against *S. mansoni*. Another possible explanation for difference between the results of the present study and those reported by Badria and colleagues is the methodology for counting adult worms. Whereas in the studies reported herein and those of Botros and colleagues used the

standard perfusion method and counting of recovered worms, Badria and colleagues, on the other hand, used a different method for counting the worms in portal and mesenteric veins. They used a 3x lens to count worms *in situ* (Botros *et al.*, 2004), and this is likely to be less accurate.

PZQ on the other hand, at a single dose of 400mg kg^{-1}, caused a significant reduction in the number of recovered worms. The recovered worms were smaller in size compared to worms recovered from other groups. There was a significant reduction in the number of eggs deposited in the liver and guts of infected and treated animals. These results are in agreement with the well documented effects of PZQ accumulated through enormous number of studies carried out on different schistosome species and using different experimental animals (Pellegrino *et al.*, 1977; Tanaka *et al.*, 1989; Ghandour *et al.*, 1990; Khalil *et al.*, 1995; Botros *et al.*, 2004).

The apparent discrepancy between the *in vitro* and *in vivo* results of the present study may be attributed to the pharmacokinetics of MZD. The pharmacokinetics of the drug is not known. It is possible that MZD is not readily absorbed from the gut of mice, it is rapidly metabolised into inactive compounds, or it is rapidly cleared from the circulation after being absorbed. In either case, the active compound of MZD will not be available in the circulation and will not have access to the parasite or it will be available only for a short duration. Another explanation is that the mode of action of MZD is through direct contact with the parasite and a prolonged contact with high doses of MZD is required which can be easily obtained and controlled *in vitro*. However, it might be very difficult to reach the same dose for prolonged duration to obtain the same results that were obtained *in vitro* as it was shown that increasing the dose of MZD was lethal to mice (Botros *et al.*, 2004).

In support of this idea, when MZD was given with foetal calf serum intra-peritoneally at a dose of 300mg kg^{-1} for two doses to mice infected with *S. mansoni* it caused a non significant reduction in the number of recoverd worms, a significant decrease in the egg burden per gram of liver tissue ($P<0.043$) and a non significant reduction in the diameters of liver granulomas and in the number of eosinophils in the granulomas (data not shown). However, this sort of prolonged slow release of the drug which promoted prolonged contact with the liver of mice and with the parasites caused liver changes which were manifested as gross swelling, dark colouration and roundness of the edges of the liver. On the microscopic level there was increased deposition of collagen fibres causing thickness of the liver capsule.

Furthermore, SEM and TEM revealed that while PZQ affects the surface as well as the musculature and parenchyma of the schistosome parasites, MZD only affects the tegument, without any obvious deeper effects. This pattern of lesions caused by *in vitro* exposure to MZD may support the idea that MZD acts locally through direct contact with the tegument of the parasite. In this context, it was reported that some detergents such as Triton X-100 caused disruption of the tegument of *S. mansoni* that never extend deeper to the basement membrane. There was a disturbed distribution of some ions including calcium which was accumulated into the parasite tissues to levels double those of control parasites (Depenbusch *et al.*, 1983). The later effects on calcium distribution are similar to those reported in parasites after *in vitro* exposure to MZD in the current study. The last possible explanation of this discrepancy is that in the *in vivo* study the effect of MZD was assessed against the adult stage, and not the immature stages, of *S. mansoni*. It was shown from the *in vitro* study that adult worms are less affected by MZD than the immature larval stage. In this context, other drugs particularly artemether was shown to have significant effects against immature developing

schistosome but modest effects against adult worms both *in vivo* and *in vitro* (Xiao and Catto, 1989; Lescano *et al.*, 2004).

One of the most striking reported advantages of treatment of human schistosomiasis with MZD is the improvement of symptoms shortly after treatment (Sheir *et al.*, 2001; Abo-Madyan *et al.*, 2004). The main pathological features of schistosome infections are attributed to development of inflammatory granulomas around the deposited eggs in different tissues of the hosts and the resulting fibroses (Boros, 1989; Auriault *et al.*, 1996; Fallon, 2000). The possibility that MZD can act by modulating the immunopathology of schistosomiasis and hence improving the symptoms of the disease was also investigated in this study. The mean diameter of liver granulomas and the structure of granulomas were investigated as parameters of the immunopathology. There was no significant difference in the diameter of liver granulomas of infected mice of different groups. The mean size of granulomas in the liver of mice treated with PZQ was slightly higher than that in the non-treated and in the MZD-treated mice. Similar observations were reported by Mehlhorn and colleagues. They reported that the mean diameters of liver granulomas of mice infected with *S. mansoni* and treated with PZQ were 350μm one hour after treatment, increased to 380μm 2 weeks after treatment and decreased to 140μm 12 weeks after treatment. In infected non-treated mice the mean diameter ranged between 350-360μm during the same period (Mehlhorn *et al.*, 1982). The reason for the slight increase in the diameter of liver granulomas of mice treated with PZQ may be explained by the possibility that most of the granulomas found in this group of mice were fully developed (mature) with scanty early developing (immature) granulomas as the drug caused decreased formation and deposition of new eggs. In the mice of the other groups, there were mature and immature granulomas resulting from the continued deposition of new eggs in the livers.

There was no significant difference in the number of eosinophils of liver granulomas from mice of different groups. However, the fibrous tissues of some granulomas of the livers from mice treated with MZD tended to be less than that found in the PZQ-treated and in the un-treated mice and the arrangement of collagen fibres tended to be less organised. In these mice, the decrease of granuloma diameter, eosinophils and collagen fibres was associated with decreased levels of IL-4, IL-13, and IL-10 in supernatants from spleen cells and LNs in response to stimulation with either SEA or SWAP. These results indicate that MZD may have a suppressor effects on the immune responses to schistosome antigens especially the Th2 associated immune responses. Similar anti-inflammatory effects were reported for myrrh (Kimura *et al.*, 2001). It is not clear in this study whether this change in the fibrous tissue in the liver granulomas is beneficial or harmful for the infected and treated animals. It has been shown that the formation of granulomas around the eggs helps to segregate them and thus protect the liver cells from exposure to their toxins (Doenhoff *et al.*, 1986). On the other hand, over-development of the granulomas and fibrosis leads to the main pathological consequences of the disease (Strickland and Ramirez, 2000). However, it is worth mentioning that in a previous experiment, infected mice treated with intraperitoneal MZD showed a similar pattern of granuloma structure and there was a high mortality rate in this group of mice (4 out of 7 mice were dead).

The exact mechanism of granuloma formation and fibrosis is not fully elucidated. Several cytokines have been shown to play a role in this immunopathological process. For instance, IL-4 has been shown to increase granuloma formation (Yamashita and Boros, 1992; Jankovic *et al.*, 1999a) and to increase the survival of infected hosts by preventing Th1 skewed immune responses (Brunet *et al.*, 1997; Fallon *et al.*, 2000a; La Flamme *et al.*, 2001; Patton *et al.*, 2001). IL-13 has been shown to increase granuloma formation and more importantly to be the main cytokine involved in fibrogenic

processes (Chiaramonte *et al.*, 1999; Fallon *et al.*, 2000a; Wynn, 2003; Mentink-Kane *et al.*, 2004; Kaviratne *et al.*, 2004). On the other hand, IL-10 has been shown to play an important role in the modulation of granulomas, reducing fibrosis and prevention of over expression of either Th1 or Th2 immune responses (Flores-Villanueva *et al.*, 1996; Boros and Whitfield, 1998; Zouain *et al.*, 2000; Sadler *et al.*, 2003; Hesse *et al.*, 2004; Booth *et al.*, 2004). Although IFN-γ may play a role in the early development of granulomas (Pearce *et al.*, 1991; Lukacs and Boros, 1992), it has been shown that IFN-γ plays a role in the reduction of granuloma size and fibrosis (Henri *et al.*, 2002; Booth *et al.*, 2004).

In PZQ treated mice, there was a decreased production of IL-4, IL-13 and IFN-γ from spleen cells and LNs in response to stimulation with either SWAP or SEA. The greatest reduction was that of IFN-γ. The levels of IL-10 in the supernatant from lymphocytes from mesenteric lymph nodes tend to be higher in PZQ-treated mice in response to stimulation with SEA and SWAP. When the levels of IL-10 produced by LNs after stimulation with SEA or SWAP were compared to the levels obtained in the absence of stimulation, there showed 12 and 7 fold increase, respectively. On the other hand, LNs from mice treated with MZD showed 4 and 1.5 fold increase and LNs from untreated mice showed 2.5 and 1.5 fold increase after stimulation with SEA or SWAP respectively. Furthermore, LNs from mice treated with PZQ produced 11 and 6 fold increase of IL-13 levels after stimulation with either SEA or SWAP compared to the levels produced in the absence of stimulation. LNs from mice treated with MZD or untreated mice showed only 1.5-2 fold increase in the level of IL-13 after stimulation with either SEA or SWAP. These results may indicate that PZQ treatment favours the stimulation of Th2 associated immune responses and this response is balanced by virtue of increased IL-10 levels. This pattern may explain the slight increase in the diameter and eosinophils of liver granulomas in mice treated with PZQ.

The preferential Th2 associated immune responses after treatment of schistosomiasis with PZQ were reported in the literature. In human schistosomiasis mansoni, the mean levels of tegument-specific IL-5 and IL-10 increased about 10-15 fold, and mean levels of IL-13 increased about 5 fold. Levels of SEA, SWA, and tegument-induced interferon- gamma were not significantly changed by treatment, and, with the exception of IL-10, which increased slightly, responses to SEA also remained largely unchanged (Joseph *et al.*, 2004). On the other hand, Fitzsimmons and colleagues investigated the early changes in immune reactions after praziquantel therapy in *S. mansoni* infected individuals. One day after PZQ treatment, a marked transient increase in plasma IL-5 levels was observed in 75% of the subjects. Blood cultures from the 16 subjects with the greatest increase in plasma IL-5 level displayed reduced IL-5, IL-13, and IL-10 responses to SWAP. They reported that there was a heterogeneity in early immune reactions to treatment, identifying subgroups who have different patterns of reaction and who may have different capacities to mount different immune responses (Fitzsimmons *et al.*, 2004). In human schistosomiasis haematobium, repeated treatment of infected school children led to high specific levels of interleukin (IL)-5 and low interferon-γ production but did not protect against reinfection (van den Biggelaar *et al.*, 2002).

In conclusion the work presented in this chapter has demonstrated that

1. Both MZD and PZQ showed *in vitro* anti-schistosome activities against *S. mansoni*.

2. MZD was more effective against schistosomula than PZQ *in vitro* but its efficacy was less against adult worms.

3. Lengthy exposures to high concentrations were necessary for the effects of MZD to be evident.

4. Although there were similarities in the effects of MZD and PZQ after *in vitro* exposure, the extent of the damage was distinct for each drug.

5. Both MZD and PZQ caused an increase in the intra-parasite calcium levels.

6. MZD had no anti-schistosome activity in infected mice. On the other hand PZQ caused a significant reduction in worm burden and liver and gut egg loads.

7. There were no significant differences in the diameter or the structure of liver granulomas in treated mice compared to untreated mice.

Chapter Three

Effects of *in vitro* exposure to Mirazid® and praziquantel on the soluble proteome of adult *Schistosoma mansoni*

3.1 Introduction

3.1.1 Two-dimensional gel electrophoresis and mass spectrometry

The term 'proteome' describes the total protein complement expressed by a genome (Wilkins *et al.*, 1996). The separation and characterisation of the protein content of complex mixtures has been made possible with the invention of the two-dimensional gel electrophoresis (O'Farrell, 1975). As is clear from the name, the two-dimensional gel electrophoresis (2-D electrophoresis) comprises two dimensions that separate proteins according to two different properties.

In the first dimension, isoelectric focusing (IEF), proteins are separated according to their isoelectric points (pI) which is determined by the relative contents of acidic (negatively charged) and basic (positively charged) residues of each protein. The isoelectric point (pI) is the specific pH at which the net charge of the protein is zero (Berg *et al.*, 2002b). The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. For instance, a protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. On the other hand, a protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the 'focusing' effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences (Berkelman and Stenstedt, 1998). The introduction of the immobilised pH gradient for isoelectric focusing has made the 2-D electrophoresis separations highly reproducible and has allowed greater amounts of protein to be loaded

(Bjellqvist *et al.*, 1982). After proteins have been separated by IEF, they are subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a second dimension.

In the second dimension, proteins are separated largely on the basis of their molecular masses. The mixture of proteins is first dissolved in a solution of sodium dodecyl sulfate (SDS), an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. Anions of SDS more importantly bind to peptide chains at a fixed ratio of one SDS anion for every two amino acid residues. The complex mixture of SDS and the denatured protein has a large net negative charge that masks the initial charge of the protein. When such a mixture is subjected to electrophoresis, small proteins move rapidly through the gel whereas large proteins stay near the point of application of the mixture. The mobility of most polypeptide chains under these conditions is linearly proportional to the logarithm of their mass (Berg *et al.*, 2002b). As an initial step for protein identification and comparison, they are stained with a specific dye. In theory, the analysis of up to 15000 proteins should be possible in one gel, however, in practice 5000 detected protein spots indicates a very good separation (Berkelman and Stenstedt, 1998).

Several methods have been used for protein identification. The methods of choice are now based on mass spectrometry (MS). They are applicable to all proteins, combining the advantages of greater sensitivity and high throughput without the expensive running costs of the earlier technologies. MS is a means of accurately determining molecular mass. The molecules are ionized in the gas phase, accelerated by an electric field in a vacuum and enter a mass analyser, which allows the measurement of their mass to charge ratio (Ashton *et al.*, 2001). After gel mapping, spots are excised and subjected to proteolytic digestion e.g. by trypsin that cuts peptide bonds C-terminal to lysine or arginine residues to create a diagnostic peptide mixture (Wilson *et al.*, 2004).

Until recently, the very low volatility of proteins and probably other macomolecules, made MS of no value for the investigation of these molecules. This difficulty has been circumvented by the introduction of techniques for effectively dispersing proteins and other macromolecules into the gas phase. These methods are called matrix-assisted laser desorption ionisation (MALDI) and electrospray spectrometry. With the MALDI technique, the protein sample embedded in an appropriate matrix is ionised by the application of a laser beam. The ions generated are then accelerated, using an electrical field, through the flight tube toward the detector with the smallest molecules traveling fastest and arriving first. Thus the time of flight (ToF) in the electric field is a measure of mass/charge ratio (Berg *et al.*, 2002b). For protein spots that have been excised from 2-D gels, the usual approach is to generate a peptide mass fingerprint (PMF). In theory, this should be diagnostic of a particular protein and searching a database of full-length protein sequences for the organism under investigation will lead to identification if the sequence for that protein is present in the database (Wilson *et al.*, 2004).

In the electrospray mass spectrometry (ESMS), liquid containing the analyte is pumped at low microliter-per-minute flow rates through a hypodermic needle at high voltage, to electrostatically disperse, or electrospray, small, micrometer-sized droplets, that rapidly evaporate and which impart their charge onto the analyte molecules. The molecules are transferred into the mass spectrometer with high efficiency for analysis. A wide range of compounds can be analyzed by ESMS; the only requirement is that the molecule be sufficiently polar to allow attachment of a charge. This includes proteins, oligonucleotides, sugars, and polar lipids. For a given compound, the signal strength (peak height in the spectrum) increases linearly with the analyte concentration over a wide range until saturation occurs (Mann *et al.*, 2001).

3.1.2 Schistosome proteome

The characterisation of parasite proteomes is a major task in biological research. The powerful combination of two-dimensional electrophoresis and mass spectrometry can identify selected parasite proteins rapidly and cheaply, providing a direct route to the cDNA and hence recombinant protein, and requiring as little as 500fmol of the original protein (Ashton *et al.*, 2001). However, such a combination has been available, for schistosomes, only in the last few years partly because of the recent advances in the sequencing of the schistosome genome. For instance, before 1994, there were only 220 sequences in the database for all schistosome species. With the use of the expressed sequence tag (EST) strategy for schistosomes now there are over 139064 public *S. mansoni* ESTs, representing 92% of the *S. mansoni* gene complement (El-sayed *et al.*, 2004).

Using the combination of two-dimensional electrophoresis and peptide mass fingerprinting Curwen and colleagues (2004) compared the proteome profiles of the soluble antigen preparations from different stages in the life cycle of *S. mansoni*, namely, cercariae (SCAP), lung-stage schistosomula (SLAP), adult worms (SWAP) and eggs (SEA). The pattern of protein spots from preparations of different stages showed great similarity especially between adjacent life-cycle stages reflecting in part the slow transition that occurs in schistosome 'stages' within the mammalian host. The MALDI-ToF mass spectrometric analyses of the top 40 spots from each gel revealed thirty-two individual protein species, 70% of which were identified in all the life cycle stages. However, there were some proteins that were unique for each stage. Amongst the twenty-four proteins identified by blotting the gels with sera from hosts with patent infections or hosts vaccinated with radiation attenuated cercariae or parasite extracts, several of the WHO priority vaccine candidates, including glutathione-S-transferases,

fatty acid binding proteins and paramyosin, were strongly recognized in all the life cycle stages (Wilson *et al.*, 2004; Curwen *et al.*, 2004).

As an extension of the study of the effects of MZD and PZQ on *S. mansoni*, the effect of MZD and PZQ on the soluble proteome of adult worms of *S. mansoni* was studied.

3.2 Materials and methods

3.2.1 Reagents and Equipment

Unless otherwise stated, all the reagents and equipment used were purchased from Amersham Biosciences UK limited, Buckinghamshire, UK.

3.2.2 Exposure of adult *S. mansoni* to MZD or PZQ

Adult worms were recovered from mice infected with the Puerto Rican strain of *S. mansoni*. Adult worms (10 worm pairs/ml^{-1} of complete RPMI-1640) were exposed for 24h to either PZQ or MZD at a concentration of 200μg ml^{-1}. Control worms were exposed to 1% DMSO (the drug solvent). The worms of each group were then pooled and washed three times with 40mM Tris solution.

3.2.3 Sample preparation

Washed worms were homogenised using a glass tissue homogeniser immersed in ice. The whole worm homogenate was transferred into a universal tube and sonicated on ice using a Soniprep 150 sonicator for 4 cycles of 1 minute each with a 1 minute interval between pulses to prevent over heating of the antigens. The supernatant was centrifuged for 20 minutes at 70000g at 4°C. The supernatant was collected in a universal tube to which a protease inhibitor was added. The protein was then concentrated with acetone

precipitation method. Briefly, three volumes of ice-cold acetone was added to one volume of the sample and incubated at –20°C for 2h. The protein was pelleted by centrifugation for 20 minutes at 70000g at 4°C. The supernatant acetone was gently removed and the remaining acetone was air-dried. The pellet was resuspended in DIGE lysis buffer and stored at –70°C.

3.2.4 Preparation for the 2-D fluorescence difference gel electrophoresis (2-D DIGE)

Determination of the protein content of the samples

The protein assay was carried out using the 2-D Quant Kit. Aliquots of different quantities (0, 10, 20, 30, 40 and 50µg) of bovine serum albumin (BSA) standard solution were prepared. Two microlitres of each sample were placed in an ependorff tube (run in duplicate). A 500µl of the precipitant reagent was added to each tube (including the standard curve tubes). They were mixed briefly by vortexing and incubated for 2–3 min at room temperature. A 500µl co-precipitant reagent was added to each tube and mixed briefly by vortexing. The tubes were centrifuged at 10000g for 5 min. The supernatant was decanted gently and the process of precipitation and centrifugation was repeated. The tubes were immediately removed from the centrifuge and the supernatants were carefully decanted. A 100µl of copper solution and 400µl of distilled de-ionized water were added to each tube. The precipitated protein was dissolved by brief vortexing. A 1ml volume of working color reagent (100 parts of colour reagent A to 1 part of colour reagent B) was added to each tube and mixed thoroughly. The tubes were incubated at room temperature for 15–20min. The absorbance of each sample and standard was read using a spectrophotometer at a wavelength of 480nm using water as the reference. A standard curve was generated by

plotting the absorbance of the standards against the quantity of protein. Using this standard curve the protein concentration of the samples was determined.

Fluorescent labeling of the samples with the CyDye

With the use of the different CyDye™ fluoros dyes, it is possible to compare between more than one protein sample in the same gel. The protein concentration of the samples was adjusted to a 5mg ml^{-1} concentration. Aliquots of 50µg proteins in 10µl DIGE lysis buffer were prepared. The pH was adjusted to between pH8 and pH9. On ice, 40pmol of either CyDye 3 or CyDye 5 was added to each aliquot and mixed immediately. The aliquots were then incubated on ice for 30min in the dark. The reaction was stopped by addition of 1µl of 10mM lysine and incubation for 10min on ice in the dark. The labelled samples were stored at –70°C.

3.2.5 Isoelectric focusing of the labelled samples

Control samples (stained blue with CyDye5) were compared with either sample from MZD-treated or PZQ-treated worms. A 150µg of unlabelled proteins was added to each of the 50µg labelled proteins aliquot and mixed well. For comparison, 200µg of the sample from MZD-treated or PZQ-treated worms (stained red) was mixed with 200µg of the control sample (stained blue) resulting in a loading amount of 400µg for each immobilised pH gradient (IPG) strip. This amount of proteins was loaded in 450µl of sample rehydration buffer (see appendix). The rehydration buffer with the proteins was placed in the Ettan IPGphor strip holder to which a 24cm-IPG strip, with a pH gradient pH3-10, was gently positioned, covered with few drops of oily solution, to prevent evaporation of samples, and then covered with the strip holder cover. The isoelectric focusing was carried out using an Ettan IPGphor isoelectric focusing system. The isoelectric focusing conditions were as follows: phase 1 as a rehydration phase and was

run under low voltage of 30V for 10h, phase 2 at 300V for 1h, phase 3 at 600V for 1h, phase 4 at 1000V for 1h, phase 5 as a gradient phase was run at 8000V for 3h and 30min and phase 6 at 8000V for 8h.

3.2.6 Equilibration of the sample

After completion of the isoelectric focusing, the IPG strips were carefully removed and equilibrated over two steps using two different equilibration buffers. The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye. Each strip was placed in the tube containing the first equilibration buffer. The tubes were shaken for 15min. The IPG strips were then moved into other tubes containing the second equilibration buffer and shaken for 15min. The IPG strips were then subjected to the SDS-PAGE.

3.2.7 SDS-PAGE

After separation of the protein mixture of the samples on the basis of their pIs using isoelectric focusing, the proteins were then separated according to their mass/charge using the SDS-PAGE. Twenty-four centimetre PAGE gels with 12.5% acrylamide were prepared (see appendix). The IPG strip was dipped in SDS electrophoresis buffer and then carefully placed onto the top of the PAGE, avoiding air bubbles between the IPG strip and the gel. The IPG strip was sealed in place with agarose sealing solution (see appendix). The gel cassettes were placed in the Ettan DALT separation unit containing SDS electrophoresis buffer (see appendix). More buffer was added until the fill line was reached. The electrophoresis was carried out at a current of 5W/gel for 30min followed by 17W/gel for 4h and 30min.

3.2.8 Scanning of the gels and image analysis

Each gel was scanned at 100μm resolution on a Typhoon™ 9400 Variable Mode Imager using 580BP30 (Cy3) and 670BP30 (Cy5) emission filters. The gels were stained with SYPRO™ orange (Bio-Rad) (1/10000 solution in 7.5% acetic acid) to ensure uniform staining of protein spots. The gels were destained in 7.5% acetic acid and scanned using a 555BP20 filter. Images were analyzed using DeCyder differential analysis software, v5.0 in both the differential in gel analysis (DIA) and the biological variation analysis (BVA) modules. Individual images were created for the different Cy3 and Cy5 labeled gels and matched together using DeCyder software. Automatic detection was followed by minimal manual, and therefore subjective, intervention, but in some regions on each gel spots were split and/or redrawn. Parameters for minimum spot volume and contrast were adjusted and the so called 'spiky spots' were also removed. Two preparative gels were added to the experimental study and parallel pick lists that included all spots of interest (i.e. those differentially regulated following treatment with the candidate drug) were created. The pick list was matched to the SYPRO gel images and the spot map was exported for subsequent spot handling prior to mass spectrometry.

3.2.9 Automated spot handling

Selected proteins were subjected to fully automated spot handling in Ettan Spot Handling Workstation. A method that included spot picking, digestion, and spotting on Ettan MALDI target slides was selected in the software, and the whole procedure was run automatically overnight without any manual intervention either within or between steps. In this automated procedure, gel plugs were cut by a 2-mm picking head and washed twice in 50% methanol and 50mM ammonium bicarbonate and once in 75% acetonitrile before drying. For digestion, 10μl trypsin solution (0.2μg Trypsin,

Sequencing Grade) was added before incubation at 37°C for 2h. Extraction was performed in two steps by addition of 50% acetonitrile and 0.1% trifluoroacetic acid. The pooled extract was finally dried prior to the two-step spotting procedure, where the matrix solution of 5mg/ml solution of recrystallized α-cyano-4-hydroxycinnamic acid in extraction liquid was deposited on the target. In the final step before MALDI-ToF, a tenth of the dissolved sample was mixed with the matrix layer on the target, leaving the rest for the electrospray.

3.2.10 Identification of proteins

Mass spectrometry peaks were obtained from an Ettan MALDI-ToF biospectrometry workstation followed by protein identification by peptide-mass fingerprinting (PMF) (Voyager DE™ Pro, Applied Biosystems, Framingham, MA, USA). The MALDI target, obtained from spot handling workstation, was loaded into the mass spectrometer. Spectra were acquired in reflector mode using the Voyager 5.1 software; all instrument settings were optimized for sensitivity. Raw spectrum files were opened in data explorer software (Applied Biosytems) and calibrated on two internal peaks resulting from autolysis of trypsin (m/z = 842.510 and 2211.104). Peak lists were then prepared from each spectrum following application of the process for PMF macro (modified from an original macro by Melanie Lin, Applied Biosystems). This corrects the baseline, removes noise (S.D. = 0.7), de-isotopes the peak list so that only the monoisotopic peaks remain and outputs a list of the remaining peaks as a text file, which represents the peptide mass fingerprint (PMF) for the original spot. The National Centre for Biotechnology Information non-redundant protein (NCBInr) database, was searched with each PMF using Mascot Daemon (Matrixscience, London, UK) with the peptide mass tolerance set to ± 50ppm.

When the MALDI-ToF method was unable to identify proteins, electrospray tandem mass spectrometry (ESTMS) was used. The tryptic peptides, resulting from the spot handling workstation, were solubilized in 0.5 % formic acid and fractionated by nanoflow high performance liquid chromatography (HPLC) on a C18 reverse phase column, eluting with a continuous linear gradient to 40% acetonitrile over 20 minutes. Eluate was analysed by online electrospray tandem mass spectrometry using a Qstar Pulsar (Applied Biosystems). A 3 second survey scan preceded each MS/MS data collection cycle of 4 x 3 second product ion scans, giving a total duty cycle of 15 seconds. Data was submitted to an MS/MS ions search *via* the Mascot search engine (Matrix Science), searching both locally established databases for *S. mansoni* DNA sequence and the current non-redundant NCBI database.

3.3 Results

3.3.1 2-D electrophoresis gel

As a preliminary step, different samples were run at pH3-7 gradient in the isoelectric focusing and the immobilised strip was subjected to a 4-12% gradient mini gel. The majority of spots were similar in control parasites and the drug treated parasites. Few spots showed different intensities between different samples. The experiment was expanded by using a wider pH range in the isoelectric focusing and the immobilised strip was subjected to electrophoresis using 12.5% 24cm gels. After 2-D electrophoresis and image analysis, DeCyder software matched about 3000 spots in each gel. After exclusion of the spiky spots the number of spots were reduced to about 2700. The intensity of protein spots tended to be less in drug treated samples especially with MZD. This presumably is due to lower protein contents of samples from MZD treated worms. However, there were eight spots in MZD treated samples showing more than a 2-fold

difference in their expression compared to control worms. Nine spots were differentially expressed in PZQ treated worms by at least a 2-fold difference compared to the control parasites. The different spots were picked and processed for protein identification.

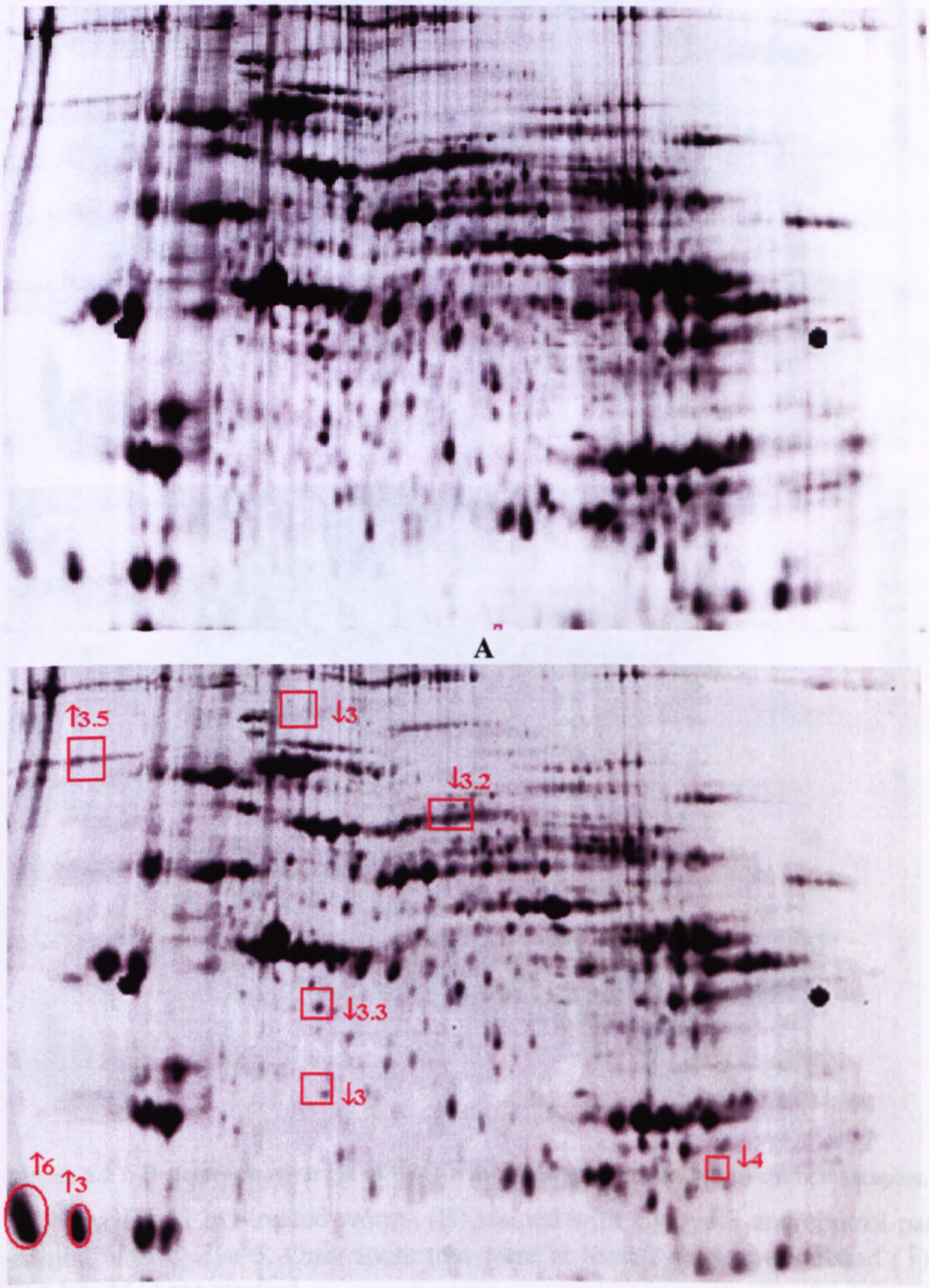

Figure 3.1 2-D electrophoresis gel of MZD-treated samples compared to control samples.

2-D PAGE of MZD-treated worms (B) stained with CyDye 3 and control parasites (A) stained with Cydye 5. Only spots that were at least 2-fold up-regulated (↑) or down-regulated (↓) are marked.

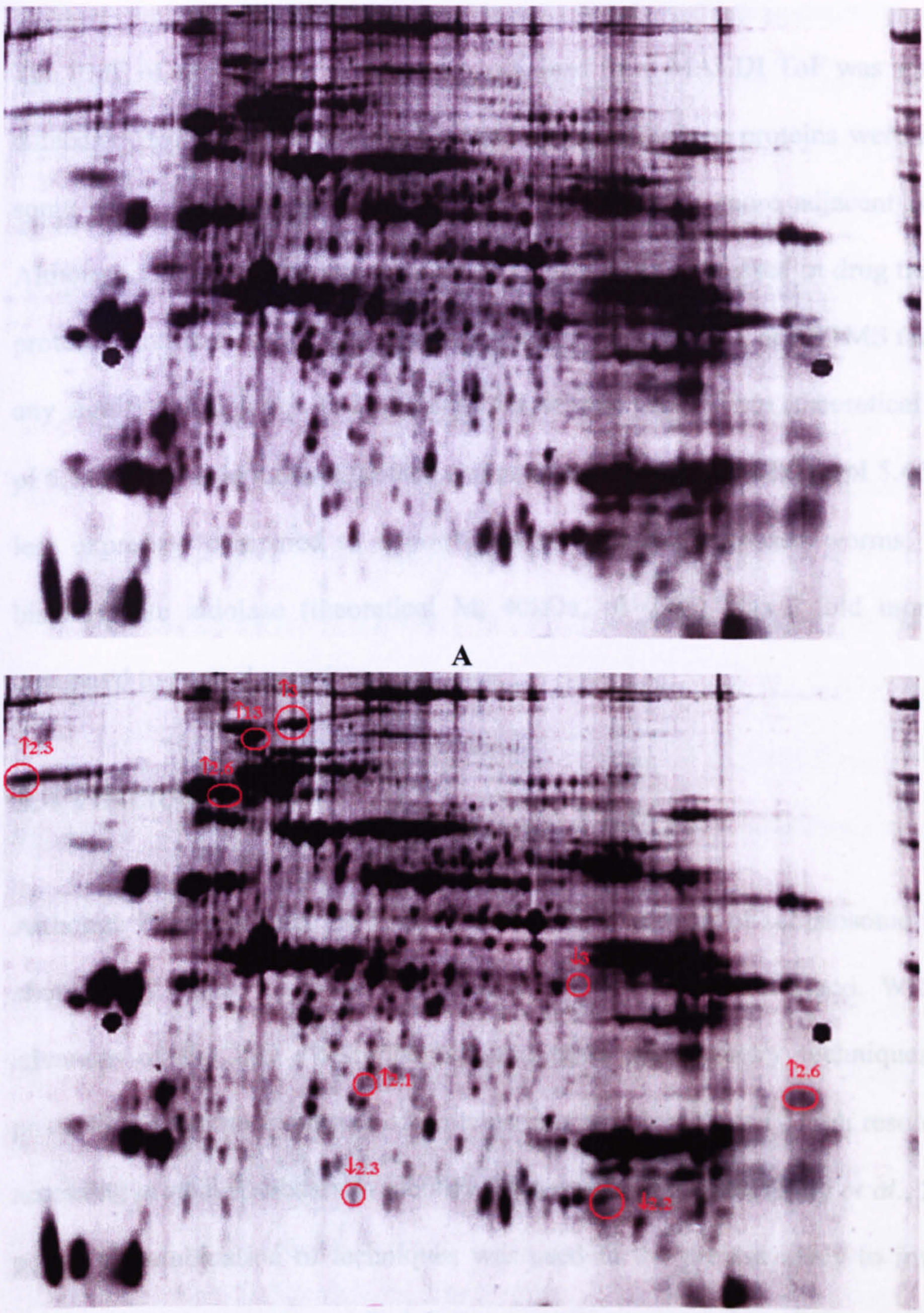

Figure 3.2 2-D electrophoresis gel of PZQ-treated samples compared to control samples.

2-D PAGE of PZQ-treated worms (B) stained with CyDye 3 and control parasites (A) stained with Cydye 5. Only spots that were at least 2-fold up-regulated (↑) or down-regulated (↓) are marked.

3.3.2 Protein identification using MALDI-ToF

The PMF of each picked protein spot, obtained from MALDI ToF was searched in the database. However, out of the 17 picked spots, only three proteins were identified. In some cases, the same protein was identified in two or more adjacent protein spots. Although some protein spots were up to 13-fold more expressed in drug treated worms, protein identification of these spots using either MALDI-ToF or ESTMS failed to reveal any significant matches. In MZD-exposed worms, paramyosin (theoretical M_r 100kDa, pI 5.31) was 4-fold more expressed and actin (theoretical M_r 42kDa, pI 5.46) was 3-fold less expressed compared to control parasites. In PZQ-exposed worms, fructose 1,6 bisphosphate aldolase (theoretical M_r 40kDa, pI 7.63) was 2-fold more expressed compared to control parasites.

3.4 Discussion

Although PZQ has been the drug of choice for treatment of schistosome infection for about thirty years, its mode of action is still not fully understood. With the major advances of the 2-D electrophoresis and mass spectrometry techniques, it is now possible to separate a mixture of proteins in a sample with very high resolution and by accessing protein databases to identify the proteins of interest (Berg *et al.*, 2002b). This powerful combination of techniques was used in the present study to investigate the effects of *in vitro* exposure of adult *S. mansoni* worms to either PZQ or MZD. Another powerful technique, DIGE was used for the comparison between samples from worms exposed to either drugs with samples from control worms in one gel as a reference (Berkelman and Stenstedt, 1998).

Protein separation using the 2-D PAGE revealed more than 3000 protein spots in both drug-treated and control parasites. The protein spots tended to be less intense in drug

treated parasites especially MZD-treated parasites. This is perhaps attributed to the amount of protein loaded in the samples. In order to avoid this possibility, only proteins that showed more than 2 fold differences were picked. Of the 17 spots, only 3 proteins were matched using the PMF obtained from MALDI ToF. Protein identification was retried using ESTMS but no additional proteins were identified. This may be explained by the possibility that these spots might have small amounts of proteins. It is estimated that about 500fmol of the original protein is essential to allow protein identification (Ashton *et al.*, 2001). The decreased number of identified proteins may be attributed to the incomplete status of the genome of *S. mansoni*. Against this, Curwen and colleagues compared the soluble proteomes of different life cycle stages of *S. mansoni* and they were able to identify many proteins (Curwen *et al.*, 2004). However, in their study they set the peptide mass tolerance to ± 2 dalton while in the present study it was set to ± 50 parts per million which, as expected, limits the range of proteins likely to be identified by the search tools. Though it would increase the degree of confidence that the matches are significant.

In the sample from worms exposed to MZD, two closely related spots were 4-fold more expressed compared to samples from control worms. The database search for the PMF from these spots revealed homology with *S. mansoni* paramyosin.

Paramyosin is a myofibrillar protein and is usually organised into the core structure of the thick filaments of the invertebrate muscle where it interacts with myosin molecules. The immunolocalisation studies of paramyosin were controversial. Although it was often localized in the muscle of the parasite, it was also localized in the tegument of adults and schistosomula and in the post-acetabular glands of the cercariae (Kojima, 2004). However, paramyosin may be multifunctional, playing a role not only as structural components but also serving several functions in the host-parasite interaction including interference with the complement cascade, impairing augmentation of the host

inflammatory response and binding of host immunoglobulins, thus making the parasite insusceptible to the immune system (Kalinna and McManus, 1997).

In this context, a surface protein of 94kDa designated schistosome complement inhibitory protein-1 (SCIP-1) exhibits antigenic and functional similarities with the human complement inhibitor protein CD59. Soluble SCIP-1, partially purified from schistosome tegument extracts using anti-CD59 antisera, was shown to bind to purified human C8 and C9 and inhibit lysis of sheep and rabbit RBCs by human complement (Parizade *et al.*, 1994). Subsequent sequence analysis of the purified SCIP-1 revealed it to be paramyosin. Furthermore, native and recombinant paramyosin was shown to bind to human C8 and C9 and to inhibit C9 polymerization on RBCs (Deng *et al.*, 2003). Like CD59, SCIP-1 (paramyosin) is viewed as 'mopping-up' any activated complement at the terminus of the pathway that may have escaped the multiple earlier inhibitors (Skelly, 2004).

If the increased expression of paramyosin observed in the present study occurs *in vivo*, in response to drug treatment, one would speculate that this could be a compensatory mechanism of the parasites in an effort to avoid both innate and acquired immune responses that exploit the complement system. Such immune responses might potentiate the damage caused by the drug.

Whatever the mechanism of increased expression of paramyosin in adult worms in response to *in vitro* exposure to MZD, this observation might support the hypothesis that some anti-schistosome drugs might have a temporary immunising effect promoting the stimulation of protective immune responses.

Paramyosin is a well-recognized vaccine candidate and is one of the vaccine candidate recommended by the WHO. Paramyosin, both the native and the recombinant molecules

from *S. mansoni*, administered intradermally with BCG at total doses of 4-40μg per mouse were found to confer significant resistance (26-33%) against challenge infection. In addition, paramyosin was shown to stimulate T lymphocytes from vaccinated mice to produce lymphokines especially IFN-γ that activate macrophages to kill schistosomula (Pearce *et al.*, 1988). In mice, subcutaneous immunization with *S. japonicum* paramyosin with BCG or alum produced 20–60% worm reduction in challenge infection (Kojima, 2004).

Interestingly, the reported preferential Th1 immune responses stimulation by immunization with paramyosin may fit with the observed preferential reduction of Th2-related cytokine production by LNs and spleen cells from mice treated with MZD (shown in chapter 2). This reduction in Th2 responses may be a consequence of the related boosting of the Th1 immune responses by the increased expression of paramyosin.

Another protein spot was expressed 3-fold less in samples from worms exposed to MZD compared to control worms. Identification of this spot revealed a protein that showed sequence homology to actin.

Actins constitute a highly conserved family of proteins found in all eukaryotes. These proteins are found predominantly in the cytoplasm of cells where monomers polymerise to form microfilaments. Microfilaments of actin participate in various cell functions such as muscle contraction, cell cytoskeleton and motility (Sheterline and Sparrow, 1994). In *S. mansoni*, indirect immunofluorescence studies localized actin predominantly in surface spines and tubercles of adult schistosomes (Abbas and Cain, 1987). Furthermore, using immunoelectron microscopy, the surface spines were shown to be composed of paracrystalline arrays of actin filaments. Actin was also present in

areas recovering from damage, implying an important role for this structural protein in tegumental repair (Matsumoto *et al.*, 1988).

The mechanism of decreased expression of actin in worms exposed to MZD in the present study is unknown. However, after treatment of *S. mansoni* with the anti-schistosome drug Ro 15-5458, the total RNA content of parasites recovered from mice 12, 72 and 96h after dosing was reduced by 14, 30 and 41%, respectively. Quantitative filter hybridization of blots of RNA extracted from treated and control parasites with specific probes indicated a decline in actin and superoxide dismutase mRNA as well as rRNA of treated parasites. The decline was observed 12h after dosing, 48h before parasites showed drug-induced changes in other vital biological processes (Eshete and Bennett, 1991). A similar mechanism may be involved after exposure to MZD.

In samples from worms exposed to PZQ, there was one protein spot that was 2-fold more highly expressed that control worms. Protein identification revealed a sequence homology with fructose 1,6 bisphosphate aldolase. Fructose 1,6 bisphosphate aldolase is a key enzyme in the glycolytic pathways and in energy metabolism. It catalyses the reversible aldol cleavage of the fructose 1,6 bisphosphate into two trioses, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Berg *et al.*, 2002a).

Schistosomes have a high rate of metabolism and energy consumption necessary for several vital functions including egg laying, muscular activity and transport of nutrients through the tegument. Although schistosomes live intravascularly with an unlimited supply of oxygen, most of their metabolic pathways including glycolysis are essentially anaerobic (Coles, 1984). Immunocytochemical studies localized fructose 1,6 bisphosphate aldolase in all tissues of adult schistosomes. In male worms it was mostly localised in the tubercles and in the subtegumental region (El Dabaa *et al.*, 1998).

The increased expression of fructose 1,6 bisphosphate aldolase in worms exposed to PZQ might indicate an increase in glycolysis and increased energy production. This could be attributed to the increased energy demands for repair mechanisms. However, this increased protein expression might result in priming of the immune response and thus favour the hypothesis of an immunising role for PZQ.

In conclusion, the present study indicated that *in vitro* exposure to either PZQ or MZD caused changes in the expression of some proteins in schistosomes. However, the results of the present study are preliminary and further studies are recommended, especially now that the genome sequence of *S. mansoni* is nearing completion. When the genome has been completed, it will facilitate the identification of more proteins using mass spectrometry. In any case it is possible to investigate the differential expression on the genomic level with the recent advanced techniques including microarrays. These studies will undoubtedly reveal invaluable information that will revolutionise our understanding of the actions of PZQ, MZD and other drugs.

Chapter Four

Antibody isotype recognition of antigens of *Schistosoma haematobium* by sera from infected individuals before treatment, after treatment and after reinfection

4.1 Introduction

4.1.1 Humoral immunity to schistosomiasis

All stages in the life cycle of the schistosome may be recognised by antibodies in the serum of infected humans (Butterworth *et al.*, 1985; Hagan *et al.*, 1987). Such antibodies can be detected in the serum of various experimental animals and humans within days of infection. It is clear that not all of these antibodies are relevant to protective immunity and there is a powerful evidence that some may prevent the expression of immunity (Hagan, 1987).

In humans with schistosomiasis a number of immuno-epidemiology studies have demonstrated that different antibodies are correlated with resistance to infection or to intensity of reinfection. For instance, adult worm and/or schistosomula specific IgE antibodies (Rihet *et al.*, 1991; Hagan *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993; Caldas *et al.*, 2000), IgA antibody (Ndhlovu *et al.*, 1996; Khalil *et al.*, 1999), IgG1 antibody (Satti *et al.*, 1996) and IgM antibodies against a 68kDa antigen (King *et al.*, 1989) have been associated with immunity to reinfection. On the other hand, a number of studies have reported that parasite specific IgM (Butterworth *et al.*, 1987; Ndhlovu *et al.*, 1996), IgG4 (Hagan *et al.*, 1991; Demeure *et al.*, 1993; Ndhlovu *et al.*, 1996) and IgG2 (Demeure *et al.*, 1993; Satti *et al.*, 1996) antibodies have been correlated with susceptibility to re-infection. The finding of such associations has stimulated attempts to determine which schistosome antigens are recognised by antibodies that may result in protection. The identification of the putative 'protective antigens' would be of significance in attempts to develop a vaccine that could be used to artificially stimulate protection through vaccination.

The pattern of recognition of schistosome antigens by antibodies has been described on a number of occasions. In some studies antigen recognition has been correlated with resistance to re-infection (Roberts *et al.*, 1987; Dessein *et al.*, 1988; King *et al.*, 1989; Grzych *et al.*, 1989; Dunne *et al.*, 1992; Webster *et al.*, 1996; Ndhlovu *et al.*, 1996). Roberts and colleagues (1987) used western blotting analyses to examine the different recognition patterns of *S. mansoni* surface antigens using sera from Kenyan children previously infected with *S. mansoni* and who were classified as resistant or susceptible to re-infection after treatment. The antibody response to three antigens of molecular mass 100, 50 and 27kDa were detected more frequently in the resistant than in the susceptible group. However, Roberts and colleagues concluded that the differences in total antibody levels to the tegumental membrane preparation were insufficient to account for the resistance or susceptible status of the children (Roberts *et al.*, 1987).

In a similar study, Dunne and colleagues (1992) reported on the different recognition patterns of *S. mansoni* antigens. Using western blotting analyses with serum from individuals classified as resistant or susceptible to reinfection with *S. mansoni*, they were able to demonstrate that the recognition of a 22kDa *S. mansoni* antigen by IgE antibodies was correlated with lower intensities of reinfection after treatment (Dunne *et al.*, 1992). Critically, the recognition of the rSm22 (rSm22.6) by human IgG3, Ig4 and IgA was found to be increased in the resistant group, but only the IgE response was significantly correlated with resistance to reinfection after treatment (Webster *et al.*, 1996; Webster *et al.*, 1998).

In another treatment and reinfection study, Dessein and colleagues (1988) showed that IgG antibodies from sera of individuals classified as resistant to reinfection with *S. mansoni* recognised larval surface antigens of 202, 165, 90 to 92, 85, 72 and 37kDa. The reactivity of IgG against the 37kDa antigen showed an association with potential resistance to reinfection (Dessein *et al.*, 1988). In *S. haematobium* infections both IgE

and IgG4 recognised different soluble schistosomula antigens but no difference was reported in the pattern of antigen recognition between the older 'putatively resistant' (≥ 20years) and the younger 'putatively susceptible' (< 20 years) individuals except for the 37kDa antigen. IgE antibody recognition of the 37kDa antigen was higher in the older (putatively resistant) group (Ndhlovu *et al.*, 1996).

King and colleagues (1989) studied the recognition pattern to *S. mansoni* surface antigen (SmW68) using sera from Egyptian individuals chronically infected with *S. mansoni*. They reported a negative correlation between the IgM response to SmW68 and the intensity of infection (King *et al.*, 1989). Similarly, IgA recognition of *S. mansoni* glutathione-S-transferase (Sm28) has been correlated with resistance to human schistosomiasis mansoni infection (Grzych *et al.*, 1989).

Interestingly, the antibody recognition pattern can be a marker for detection of early schistosomiasis infection. In a study of previously non-exposed individuals, the recognition of *S. mansoni* antigens of 32-35kDa with IgG1 and IgG3 antibody isotypes has been reported to be a potential marker for early schistosome infection (Evengard *et al.*, 1990). Similarly, Shaheen and colleagues (1996) reported that the IgG1 and IgG3 antibody recognition of 30-40kDa adult worm antigens of *S. mansoni* was prominent during the acute pre-patent phase of schistosomiasis. As the disease progressed to chronicity the IgG1 and IgG3 recognition shifted to the higher molecular weight antigens (≥ 80kDa) (Shaheen *et al.*, 1996).

4.1.2 Effects of chemotherapy on antibody immune responses against schistosomes

In schistosome infection there is usually a delay of the development of the protective immune response. The delay in the development of protective immunity may be

attributed to many factors. Some groups favour the idea that blocking antibodies (IgM, IgG2 and IgG4) which appear early in infection might prevent or delay the onset of protective response (Hagan *et al.*, 1991; Demeure *et al.*, 1993). Others have suggested that children are not able to mount a protective response simply because their immune responses are not fully developed (the immunocompetence hypothesis). Only when their immune responses mature are they able to mount a protective response. The modulation and evasion of the immune system by schistosomes can be itself an explanation for the delay in development of protective immune responses (Barsoum *et al.*, 1982). One final explanation is the lack of availability to the host's immune system of schistosome antigens. Only when parasites die are such antigens released to the immune system. During natural infection antigens are released in small amounts over a prolonged time period. Only when the host has had sufficient experience of these antigens, a process that may take years, is the immune response effectively armed to deal with new infection. However, none of these hypotheses can on their own completely explain the delayed onset of immunity to schistosomiasis and it is likely that several factors are involved (Hagan and Sharaf, 2003). Praziquantel, and probably other drugs, damage or kill worms perhaps exposing antigens that were previously concealed, or accelerate antigen release (Harnett and Kusel, 1986; Woolhouse and Hagan, 1999) that could stimulate protective immunity. In this context, treatment of schistosome infections at the skin schistosomula stage by Ro11-3128 resulted in immunity to reinfection. Sera from mice immunized by infection plus Ro11-3128 treatment on days 1 or 2, co-precipitated essentially the same pattern of ^{125}I-labelled surface antigens as did serum from mice vaccinated with 20 krad irradiated cercariae, *viz.* M_r 38, 32, 23and 15kDa (Bickle *et al.*, 1990).

In an early attempt to study the effect of praziquantel treatment on the responses of *S. haematobium* infected Gambians, Hagan and colleagues (1987) studied the differences

in antibody recognition of *S. haematobium* whole worm homogenate (WWH) and soluble egg antigens (SEA) before treatment, 3 months after treatment and after reinfection. They reported that antibodies against WWH increased after treatment and fell after reinfection while antibodies against SEA fell after treatment and increased after reinfection (Hagan *et al.*, 1987). However, they reached no conclusion as to whether or not particular antigen recognition patterns were associated with protection against infection.

In a study of the effects of praziquantel treatment on the immune responses in *S. mansoni* infection in occupationally hyper-exposed canal cleaners in the Sudan, Satti and colleagues (1996) reported that individuals resistant to re-infection showed no significant difference in the levels of IgG4 to WWH after treatment. In contrast there was a significant increase in the levels of IgG4 to WWH in the susceptible group. Levels of specific IgG4 to SEA showed a highly significant decrease after treatment in the resistant group. The same antibody subclass increased after treatment in the susceptible group. However, none of the groups studied after treatment showed a significant change in their specific IgE to WWH (Satti *et al.*, 1996).

Further support for the effect of chemotherapy on the immune response comes from the work of Mutapi and colleagues (1998). They reported that treatment of *S. haematobium*-infected Zimbabwean children, 5-16 years old, with praziquantel induced a switch from a predominantly IgA-specific antibody response to a predominantly IgG1 response, similar to that of adults naturally exposed to infection over many years and supposed to be resistant to re-infection. The switch of the immune response which occurs naturally after several years of exposure in this community occurred in the children within 12 weeks of treatment with praziquantel (Mutapi *et al.*, 1998). The same authors studied the effect of praziquantel and oxamniquine on the humoral immune responses directed against *S. mansoni* soluble egg antigens (SEA) in Zimbabwean children. The proportion

of treated children producing IgE and IgG3 increased six weeks post-treatment. There was also an increase in the mean levels of IgE, IgM and IgG3 in these children. At 18 weeks post-treatment, the proportion of treated children producing IgA, IgE, and IgG3 increased while the proportion producing IgG1 and IgG4 decreased. Mean levels of IgA, IgE, and IgG3 were higher than pre-treatment levels while levels of IgG1, IgG4 and IgM were lower. Statistical analyses showed that the magnitude of change in levels of IgE, IgM and IgG3 at 6 weeks post-treatment and of IgE, IgG3 and IgG4 at 18 weeks post-treatment were significantly greater in treated compared to untreated children. There were no significant differences in immune responses between children treated with praziquantel and those treated with oxamniquine (Mutapi *et al.*, 2003).

Furthermore, Grogan and colleagues (1997) studied specific IgG4 and IgE responses against *S. haematobium* adult worm antigens and SEA in *S. haematobium* infected individuals before and after chemotherapy. In this study there was a substantial drop in the IgG4 response to SEA after chemotherapy in both adults and children. However, IgG4 and IgE antibodies to adult worm antigen (AWA) and IgE antibodies to SEA increased in children but remained unchanged in adults (Grogan *et al.*, 1996). Similarly, in a study of the antibody responses against *S. mansoni* AWA and SEA in Brazilian individuals chronically infected with *S. mansoni* before and after treatment with oxamniquine, there was a decrease in the schistosome-specific IgG level observed 6 months after treatment. Although there was significant decrease of anti-SEA IgG1 and IgG3 levels, the decrease of anti-SEA IgG4 level was much more evident (Vendrame *et al.*, 2001).

These studies together indicate that treatment of schistosomiasis with praziquantel induces some changes of the immune response in the form of an increase of IgE levels, an antibody isotype which has been correlated with resistance, and decrease of IgG4 levels, an antibody isotype which has been correlated with susceptibility to reinfection.

To further investigate the possibility that schistosome antigen recognition is associated with susceptibility or resistance to reinfection, a unique serum bank containing samples obtained in the reinfection study reported by (Wilkins *et al.*, 1987) was provided together with access to the parasitological database. Using this material it was possible to compare the isotype specific recognition patterns of *S. haematobium* soluble adult worm antigens. Serum samples from a resistant group (showing no or few eggs in their urine after exposure to re-infection) and a susceptible group (showing high numbers of eggs in their urine after re-infection) before treatment, after treatment with praziquantel and after re-infection were exploited.

4.2 Materials and Methods

4.2.1 Serum samples

Background information on the serum samples

The serum samples were collected from two villages (Madina Samaco and Njarinjufa) in Upper River Division of The Gambia (Hagan *et al.*, 1987). The communities were mainly dependent on subsistence agriculture. The transmission of *S. haematobium* in these villages was seasonal. Pools forms in the rainy season between June and November. No transmission of schistosomiasis occurred from December to June/July. A cohort of 40% of the population of the villages was included in this study. In February and March 1983, urine samples were collected for egg count studies with intensities of infection being assessed. After blood samples were collected (pre-treatment sera), the participants were treated with a single dose of 40mg kg^{-1} of praziquantel. Three months after the treatment, further urine and blood samples were collected (post-treatment sera). During the transmission season, careful observation of water contact by trained observers allowed an index of cumulative exposure to infection throughout the

transmission season to be calculated for each individual. In April 1984, 5 months after the end of the transmission season, a third urine sample was collected and the intensity of re-infection was calculated. Blood samples were also collected at this time (reinfection sera).

This study showed that among groups of subjects with an apparently similar intensity of exposure to infection, reinfection tended to be much heavier in children under 10 years of age than in 10 to 14-year-olds, while only light infections were found in the few adults who became reinfected. This trend for re-infection to decrease with increasing age, after an allowance for variation in exposure, was highly significant ($P<0.001$) (Wilkins *et al.*, 1987). Levels of parasite specific IgE were positively correlated with resistance to reinfection while levels of IgG4 showed a positive correlation with susceptibility to reinfection (Hagan *et al.*, 1991).

Grouping of patients' sera

Individuals who became re-infected with *S. haematobium* after treatment with praziquantel were considered to be susceptible. On the other hand, individuals with no eggs detected in their urine after exposure to reinfection, were considered to be resistant. Sera from five control non-infected individuals living in a non-endemic area were included in this antigen recognition study.

Comparisons were carried out between resistant and susceptible groups at each time point and between the different time points within the same group.

4.2.2 Antigens

Freeze-dried adult *S. haematobium* worms were kindly provided by Professor Fred Lewis (Biomedical Research Institute, Rockville, Maryland, USA). They were re-

suspended in PBS (pH 7.4) and homogenised using a tissue homogeniser immersed in ice to prevent overheating. The whole worm homogenate was transferred into a universal tube and sonicated on ice using a sonicator (Soniprep 150) for 4 cycles of 1 minute each with a minute interval between pulses to prevent over heating of the antigens. The supernatant was centrifuged for 20 minutes at 70000g at 4°C. The supernatant was collected in a universal tube to which a protease inhibitor was added. The protein content of the soluble adult worm antigens preparation (SWAP) was then quantified.

4.2.3 Determination of protein concentration of SWAP

Five microlitres of PBS (as blank), 5µl of each of the standard's concentrations (1500, 1000, 500, 200, 100, 50 and 20µg ml^{-1}) and 5µl of the sample were pipetted in each well of a microwell plate. Standards and samples were run in triplicate. A 250µl of Coomassie-brilliant blue reagent was added to each well and their protein concentrations were measured, immediately after an initial shaking for 30 seconds at a wavelength of 595nm using a Dynex ELISA plate reader. The protein concentration of the homogenate was adjusted to 1mg ml^{-1} and it was then stored at –80°C.

4.2.4 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method used in this study was a modification of that described by Laemmli (1970). The conditions of electrophoresis and immunoblotting were adjusted after several preliminary experiments. All reagents used for SDS-PAGE were purchased from Invitrogen (Paisley, UK). *Schistosoma haematobium* SWAP was treated with sample buffer (NuPAGE® LDS Sample Buffer) 4:1 volume to volume, respectively, and then boiled for 5 minutes. 4-12% gradient precast gels (NuPAGE® Novex Bis-Tris Gels)

were used. Each gel is 1mm thick and has one well for the molecular weight markers (MWM) and a 7cm trough for the sample. Five hundred microlitres (containing 500μg) of the SWAP was loaded in the trough on each gel and run in parallel with 7μl of a prestained wide range molecular weight markers (SeeBlue® Pre-Stained Standard) in the single well. The running buffer was prepared by diluting the running buffer (NuPAGE® MES Running Buffer) 20 times with deionised distilled water and 500ml was put into the electrophoresis cell. The gel was run at 80mV for the stacking gel and then at 140mV until the bromophenol blue dye was approximately 0.5 cm from the bottom of the gel. The power was then switched off, the cast removed and the gel carefully extracted.

4.2.5 Staining of proteins

After running the proteins on SDS-PAGE, the proteins were stained to confirm that they were adequately resolved. Gels were stained with Coomassie blue stain for 2h with shaking at room temperature. The gels were removed from the stain and were destained with Coomassie destain for 4h with shaking at room temperature (until the background became clear). The gels were then removed from the destain solution and the molecular weights of the protein bands were measured using UV gel documentary system and using a software program (Labworks™ analysis software).

4.2.6 Western blotting

After protein resolution onto the SDS-PAGE gel, a sheet of nitrocellulose membrane was placed against the surface of the gel and a current was allowed to pass across the gel. This current caused the proteins to move from the gel onto the nitocellulose membrane. Once there, they are bound firmly by non-covalent forces (Hudson and Hay, 1989). Three Whatman filter papers, soaked in the cathode buffer (see appendix), were

placed onto the flat surface of the immunoblot apparatus. The gel was placed over the cathode papers. A piece of nitrocellulose membrane, 0.45um (Amersham) was cut slightly bigger than the size of the gel and was placed facing the gel, taking care to exclude air bubbles. One filter paper soaked in anode 2 (see appendix) was placed over the nitrocellulose membrane. Two filter papers soaked in anode 1 (see appendix) were then put over the anode 2 paper. The immunoblot plate was then covered and the current passed. The conditions of the protein transfer were adjusted after several experiments. A current of 200mA for 35minutes gave the best results with these samples. The nitrocellulose membrane was then removed and stained with Ponceau S (a red dye) to confirm that the proteins were properly transferred. The membrane was washed with distilled water to remove the dye and the membrane allowed to dry.

For proper alignment and comparison of the protein bands, a line was drawn near the bottom of the membrane, outside the protein migration area. Each membrane was cut into 18, numbered, longitudinal strips (enough for blotting the six antibody isotypes with three serum samples). The nitrocellulose strips were blocked with 0.3% PBS-Tween solution for 30minutes at room temperature, with shaking. The strips were removed from the blocking buffer and washed with 0.05% PBS-Tween wash buffer 3 times of 10 minutes each. In the mean time, serum samples were diluted 1:100 with 0.05% PBS-Tween solution. The diluted serum samples were put in universal tubes and 6 strips (one strip for each antibody isotype; IgG, IgM, IgG1, IgG2, IgG3 and IgG4) were incubated with the serum in each universal tube. The tubes containing the serum samples and the strips were then shaken over night at 4°C. The strips were removed from the sera and washed with washing buffer 3 times for 10 minutes each. One strip from each serum sample was put into a universal tube containing a 1:750 dilution of the secondary antibodies [anti-human IgG, IgM, IgG1, IgG2, IgG3 or IgG4 labelled with horse radish peroxidase (HRP) enzyme]. The strips were incubated with the secondary

antibodies over night at 4°C. The strips were removed from the secondary antibodies and washed with the washing buffer 3 times for 10 minutes each. The strips were incubated with the substrate solution (see appendix) until the bands became visible. The reaction was stopped by washing the strips with distilled water, 3 times, for 10 minutes each. The strips were then rearranged in their original sequence, the protein bands were visualised using a UV gel documentary system and the molecular weight of the bands was determined. The recognition was expressed as the percentage of serum samples of each group recognising a particular protein band.

The same procedure was employed for IgE with increasing the concentration of serum samples to up to 1:10 and of the anti-human IgE antibodies to 1:50. Despite repeated attempts, no recognition of parasite antigens by IgE was detected even after pooling the serum samples from each group.

4.2.7 Comparison between groups

After probing, identification of each antigen by each serum sample of those within the susceptible or resistant groups recognising a particular antigen was assessed. Recognition of each antigen was then expressed as a percentage of those within each group. For comparison between resistant and susceptible groups, the percentage recognition of the susceptible group of each band was subtracted from the value of the resistant group. The resultant value is the net difference between both groups. The same method was employed when assessing the impact of chemotherapy and reinfection on the antibody isotypes in each group.

4.3 Results

4.3.1 SDS-PAGE gel

A wide range of glycoproteins/proteins was separated on the SDS-PAGE gel. In total thirty distinct bands were recognised and their molecular masses ranged from 5 to 280kDa. An initial scan to identify which proteins were recognised by sera from patients included in this study showed that sera reacted to many of the separated proteins (see figure 4.1).

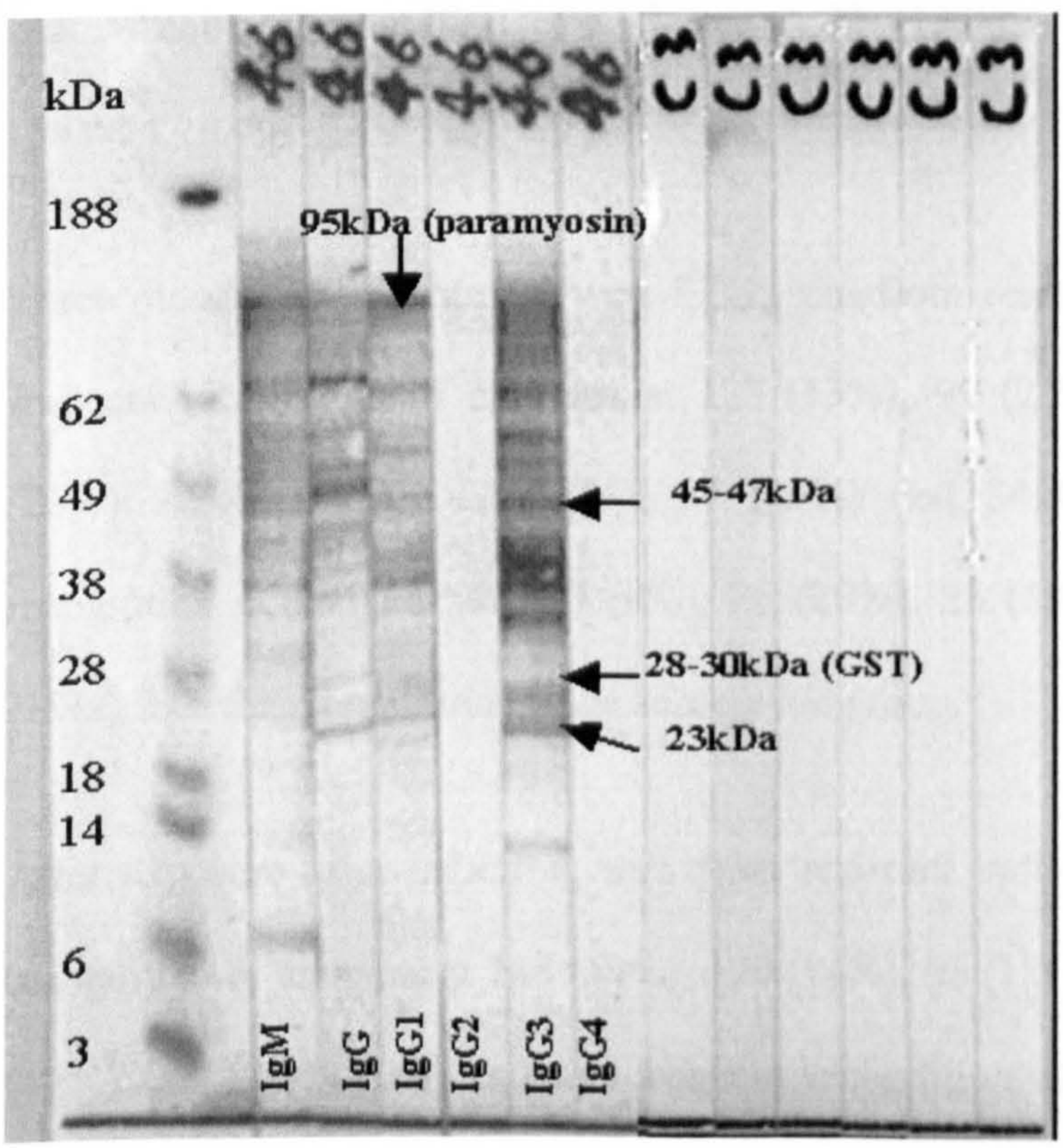

Figure 4.1 Western blotting of *S. haematobium* SWAP with different antibody isotypes.

Each serum sample was allocated six strips, one strip for each antibody isotype. The strips were then aligned and visualised using UV system. The left lane is the molecular weight marker. Serum from an infected individual (number 46) was blotted against a non-infected control serum (C3) for comparison.

4.3.2 Antigen recognition by IgM antibodies

Most of parasite antigens were recognised by at least some of the serum samples from the resistant and susceptible groups. Sera from control individuals did not recognise any parasite antigens.

Differences in antigen recognition between resistant and susceptible groups

Before treatment, sera from resistant individuals showed more frequent recognition of antigens at 170 (13%), 150 (27%), 133 (27%), 123 (27%), 95 (17%), 84 (27%), 78 (14%), 74 (18%), 65 (14%), 52-54 (29%), 45-47 (46%), 42 (12%) and 37kDa (27%) than the recognition by the susceptible group. Sera from susceptible individuals showed more frequent recognition of antigens at 23 (12%) and 16-18kDa (39%) than the resistant group.

Three months after treatment with PZQ, sera from resistant individuals showed more frequent recognition of antigens at 123 (13%), 95 (25%), 84 (38%), 78 (30%), 74 (26%), 45-47 (42%), 42 (11%), 37 (26%) and 34kDa (13%) and less frequent recognition of antigens at 150 (8%), 26 (20%), 23 (13%), 16-18 (26%) and 13kDa (18%) than the recognition by the susceptible group.

After exposure to re-infection, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 170 (14%), 95 (17%), 84 (30%), 65 (29%), 52-54 (47%) and 13kDa (6%) and less frequent recognition of antigens at 34 (10%), 28-30 (29%) and 23kDa (12%) than the recognition by the susceptible group.

Table 4.1 Percentage recognition of antigen bands in resistant and susceptible groups at different time points.

	IgM						Total IgG						IgG1					
	Pre-ttt		Post-ttt		Reinf		Pre-ttt		Post-ttt		Reinf		Pre-ttt		Post-ttt		Reinf	
kDa	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
186	0	8	0	8	14	0	7	0	0	17	0	0	7	0	0	8	14	0
170	21	8	0	0	14	0	7	0	0	8	0	0	7	0	0	0	14	0
150	36	8	0	8	0	0	7	8	7	17	7	0	0	8	14	8	0	17
133	36	8	14	8	0	0	14	17	21	33	7	8	7	8	21	33	0	17
123	36	8	21	8	7	8	21	25	21	42	7	17	7	8	21	33	7	25
95	100	83	100	75	100	83	93	83	86	100	79	100	100	83	93	83	100	75
84	86	58	71	33	71	42	64	67	64	75	50	83	64	58	57	50	64	58
78	64	50	71	42	64	58	57	42	71	75	57	83	43	33	43	58	57	50
74	93	75	93	67	100	92	86	67	86	92	79	100	79	42	71	92	64	75
65	14	0	21	17	29	0	50	8	36	25	29	17	21	0	21	8	7	8
52-54	71	42	36	42	82	35	71	42	50	58	43	33	54	33	33	50	7	8
45-47	88	42	84	42	79	75	89	50	79	65	64	84	71	48	21	67	21	42
42	79	67	86	75	71	75	86	67	86	100	71	75	71	83	36	92	43	75
37	36	8	43	17	21	25	36	8	29	33	29	25	21	8	14	25	0	0
34	7	8	21	8	7	17	14	33	14	25	7	17	0	25	0	25	0	17
28-30	47	50	64	64	21	50	55	25	20	33	37	42	14	51	59	50	57	58
26	14	17	21	42	14	17	7	42	21	42	7	33	7	33	14	50	7	33
23	21	33	29	42	21	33	50	42	43	58	36	25	36	50	43	67	43	50
20	7	0	7	0	7	0	21	17	0	17	14	0	7	0	7	17	7	0
16-18	21	60	29	55	14	19	43	50	36	81	29	33	36	33	43	58	28	42
13	21	25	7	25	14	8	43	33	36	42	36	33	14	17	21	42	14	17
11	0	0	0	8	0	0	0	8	0	8	0	8	0	0	0	8	0	0

The percentage recognition of different S. haematobium antigens by sera from resistant (R) and susceptible individuals (S) prior to treatment (Pre-ttt), three months after treatment with PZQ (Post-ttt) and after exposure to reinfection (Reinf). (Note that the table is continued over the next page).

	IgG2						IgG3						IgG4					
	Pre-ttt		Post-ttt		Reinf		Pre-ttt		Post-ttt		Reinf		Pre-ttt		Post-ttt		Reinf	
kDa	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
186	0	0	0	0	0	0	0	17	14	8	7	0	14	17	14	8	0	8
170	0	0	0	0	0	0	7	0	21	0	7	0	14	8	21	0	0	8
150	0	0	0	8	0	0	21	33	21	17	7	8	14	25	36	17	0	17
133	7	0	0	8	0	0	21	25	29	25	7	8	21	25	36	42	7	17
123	7	0	0	8	0	0	21	17	29	25	7	8	21	25	29	33	7	17
95	21	25	21	25	21	25	93	100	79	83	71	92	57	67	71	67	64	67
84	14	17	14	17	14	25	64	92	64	58	71	58	36	42	29	33	36	50
78	0	0	14	17	7	8	64	58	50	50	64	50	50	58	50	58	50	58
74	14	0	14	17	7	8	79	83	57	75	71	92	71	92	57	75	50	83
65	0	0	0	0	0	0	29	8	36	8	14	0	14	0	14	8	7	0
52-54	0	0	7	0	0	0	78	50	50	64	64	33	29	60	29	36	29	43
45-47	7	0	0	17	0	0	92	62	64	75	86	75	29	81	36	56	21	61
42	0	0	0	17	7	8	79	67	64	83	71	67	57	58	71	75	50	83
37	0	0	0	0	0	0	43	25	29	25	43	42	7	8	7	33	0	8
34	0	0	0	0	0	0	14	17	21	17	21	17	7	42	0	33	0	17
28-30	0	0	0	17	0	8	29	72	39	33	45	50	29	58	21	35	29	54
26	0	0	0	0	0	8	21	33	29	42	21	25	14	42	21	58	29	25
23	0	0	0	8	0	0	7	25	21	42	36	17	43	42	43	50	29	42
20	0	0	0	0	0	0	0	0	7	0	0	0	14	25	14	33	14	8
16-18	0	0	0	0	0	0	14	43	0	17	20	25	21	54	29	32	14	44
13	0	0	0	0	0	0	29	25	21	42	21	25	43	58	29	67	21	75
11	0	0	0	0	0	0	0	8	7	8	0	0	7	17	7	33	0	33

Impact of treatment and re-infection on antigen recognition

After treatment with PZQ, sera from resistant individuals showed increased frequency in the recognition of antigens at 34 (14%) and 28-30kDa (21%) and less frequent recognition of antigens at 170 (21%), 150 (36%), 133 (21%), 123 (14%), 84 (14%), 52-54 (21%) and 13kDa (14%) than the recognition patterns prior to treatment. Sera from susceptible individuals showed more frequent recognition of antigens at 65 (17%), 52-54 (17%), 45-47 (17%), 28-30 (16%) and 26kDa (25%) and less frequent recognition of antigens at 84kDa (25%) than the recognition patterns of the same individuals prior to treatment (see figure 4.3).

After exposure to re-infection, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 170 (14%) and 52-54 (41%) and less frequent recognition of antigens at 133 (14%), 123 (14%), 45-47 (21%), 42 (14%), 37 (21%), 34 (14%) and 28-30kDa (28%) than the recognition patterns of the same individuals three months after treatment. Sera from susceptible individuals showed more frequent recognition of antigens at 78 (17%), 74 (25%), 45-47 (18%), 34 (8%) and 34kDa (8%) and less frequent recognition of antigens at 65 (17%), 52-54 (33%), 26 (25%), 16-18 (24%) and 13kDa (17%) than the recognition patterns three months after treatment.

When comparing the recognition pattern after exposure to re-infection with the pre-treatment recognition, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 65 (14%) and 52-54kDA (21%) and less frequent recognition of antigens at 170 (7%), 150 (36%), 133 (36%), 123 (29%), 84 (14%), 45-47 (22%) and 37kDa (14%) than the recognition patterns of the same individuals prior to treatment. Sera from susceptible individuals showed more frequent recognition of antigens at 74 (17%), 45-47 (33%), 37 (17%) and 28-30kDa (17%) and less frequent recognition of

antigens at 186 (8%), 84 (17%), 52-54 (17%), 16-18 (24%) and 13kDa (17%) than the recognition prior to treatment (see figure 4.2).

These results showed that the effect of treatment with PZQ on the pattern of recognition of parasite antigens in both resistant and susceptible individuals (and groups) was heterogeneous. However, sera from resistant individuals showed less frequent recognition of antigens of high molecular weights (123 to 170kDa) after treatment with PZQ. After exposure to re-infection, while sera from susceptible group showed more frequent recognition of antigens at 45-47 (33%) and 37kDa (17%) compared to the recognition before treatment, sera from resistant individuals showed less frequent recognition of the same antigens (22% and 14%, respectively) as well as of antigens at molecular masses between 123 and 150kDa. On the other hand, while sera from resistant individuals showed more frequent recognition of antigens at 186 and 52-54kDa, sera from susceptible individuals showed less frequent recognition of the same antigens compared to the recognition before treatment.

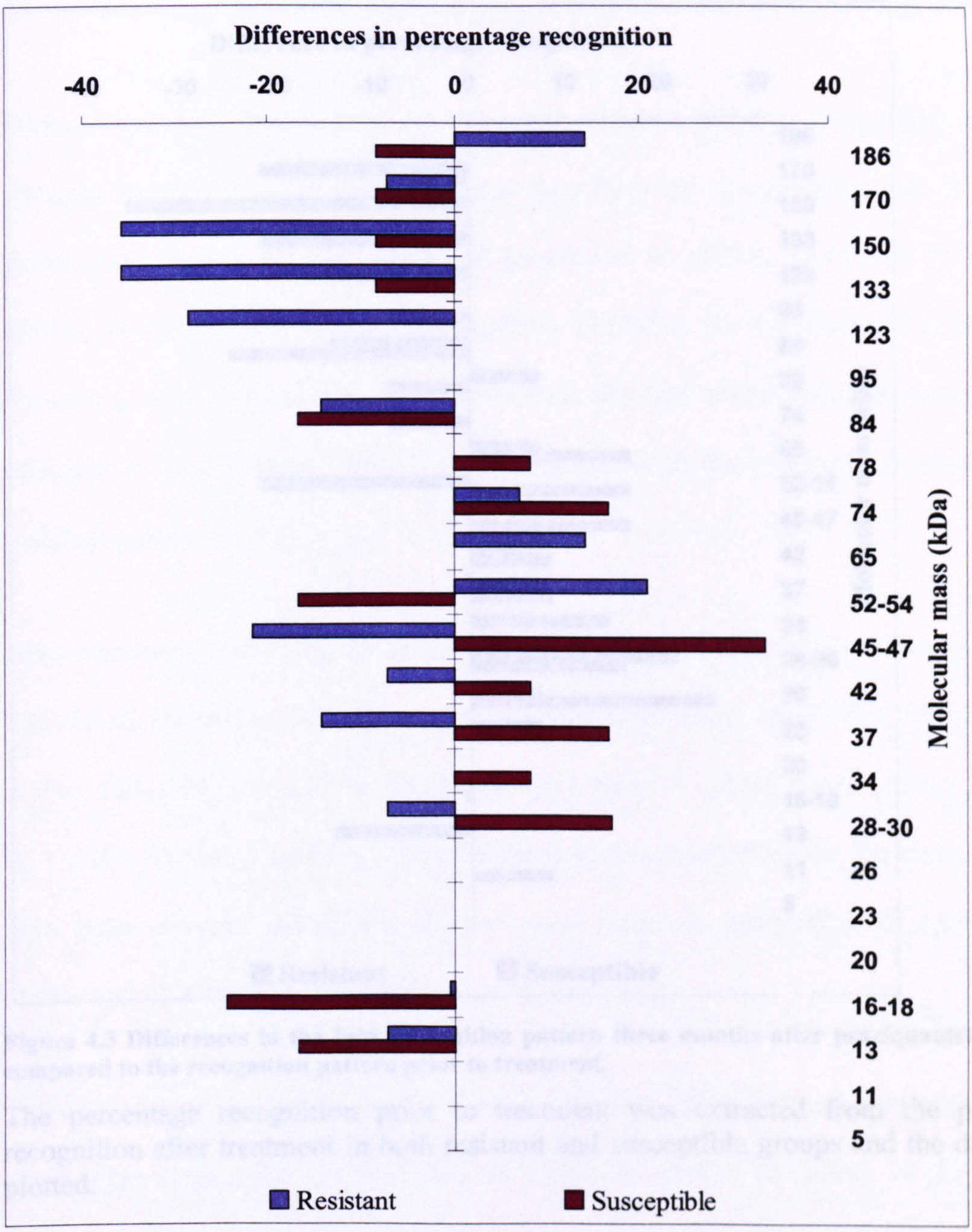

Figure 4.2 Differences in the IgM recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

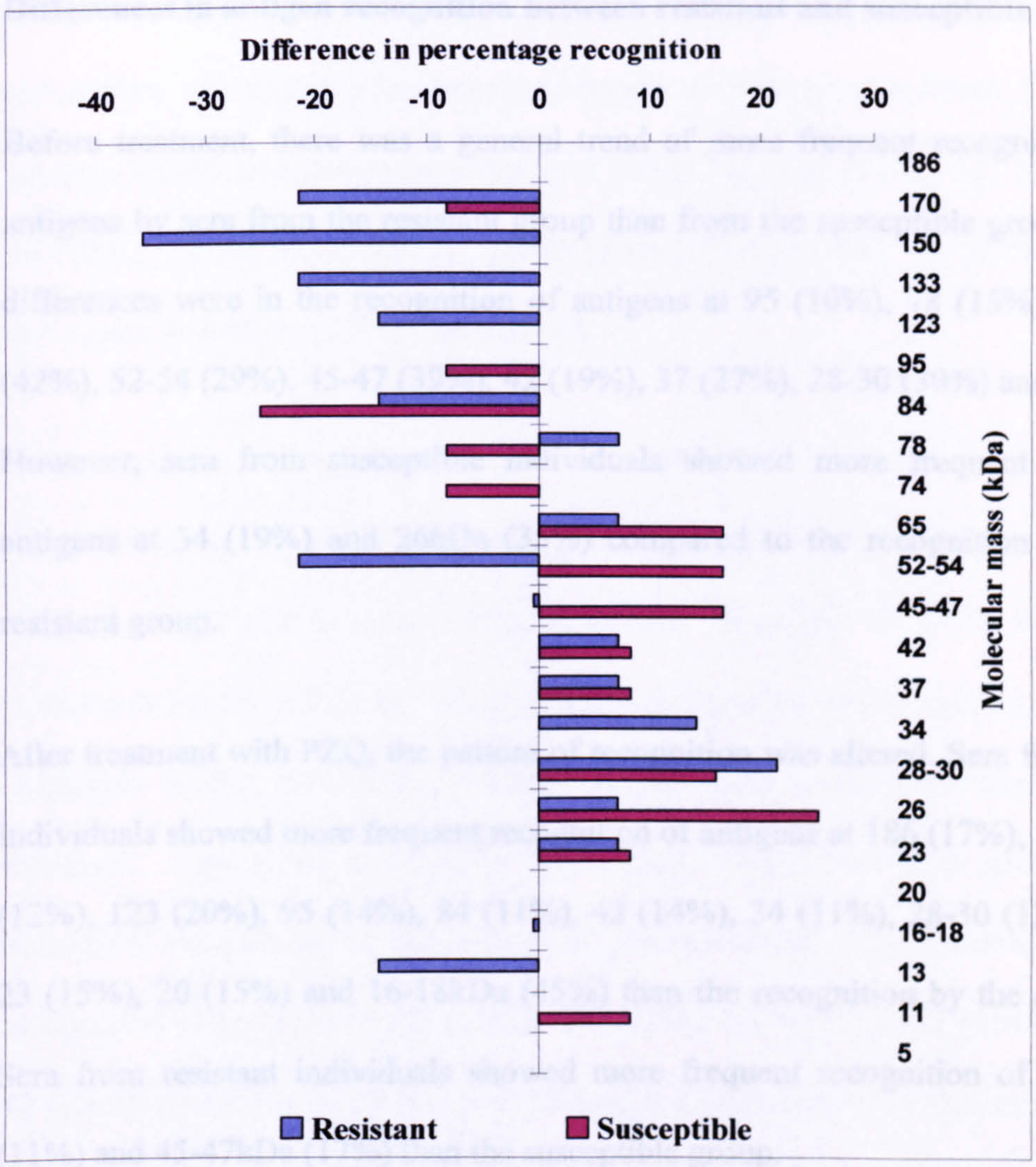

Figure 4.3 Differences in the IgM recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.3.3 Antigen recognition by total IgG antibodies

Most of parasite antigens were recognised by at least some of the serum samples from the resistant and susceptible groups. Sera from control individuals did not recognise any parasite antigens.

Differences in antigen recognition between resistant and susceptible groups

Before treatment, there was a general trend of more frequent recognition of parasite antigens by sera from the resistant group than from the susceptible group. The marked differences were in the recognition of antigens at 95 (10%), 78 (15%), 74 (19%), 65 (42%), 52-54 (29%), 45-47 (39%), 42 (19%), 37 (27%), 28-30 (30%) and 13kDa (10%). However, sera from susceptible individuals showed more frequent recognition of antigens at 34 (19%) and 26kDa (35%) compared to the recognition patterns of the resistant group.

After treatment with PZQ, the pattern of recognition was altered. Sera from susceptible individuals showed more frequent recognition of antigens at 186 (17%), 150 (10%), 133 (12%), 123 (20%), 95 (14%), 84 (11%), 42 (14%), 34 (11%), 28-30 (13%), 26 (20%), 23 (15%), 20 (15%) and 16-18kDa (45%) than the recognition by the resistant group. Sera from resistant individuals showed more frequent recognition of antigens at 65 (11%) and 45-47kDa (17%) than the susceptible group.

After exposure to re-infection, sera from susceptible individuals showed more frequent recognition of antigens at 123 (10%), 95 (21%), 84 (33%), 78 (26%), 74 (21%), 45-47 (20%), 34 (10%) and 26kDa (26%) than the recognition patterns of the resistant group. Sera from resistant individuals showed more frequent recognition of antigens at 65 (12%), 52-54 (10%) 23 (11%) and 20kDa (14%) than the susceptible group.

Impact of treatment and re-infection on antigen recognition

After treatment with PZQ, sera from resistant individuals showed more frequent recognition of antigens at 78 (14%) and 26kDa (14%) and less frequent recognition of antigens at 186 (7%), 170 (7%), 95 (7%), 65 (14%), 28-30 (44%), 23 (7%), 20 (21%), 16-18 (7%) and 13kDa (7%) than sera taken from the same individuals prior to

treatment. Sera from susceptible individuals showed an increased frequency in the recognition of antigens after treatment compared with pre-treatment. Differences were greatest for the recognition of antigens at 186 (17%), 133 (17%), 123 (17%), 95 (17%), 78 (33%), 74 (25%), 65 (17%), 52-54 (30%), 45-47 (17%), 42 (33%), 37 (25%), 23 (17%) and 16-18kDa (32%) (see figure 4.5).

After exposure to re-infection, resistant individuals showed a general pattern of decreased recognition of parasite antigens. They showed less frequent recognition of antigens at 133 (14%), 123 (14%), 84 (14%), 78 (14%), 74 (7%), 45-47 (24%), 42 (14%) and 26kDa (14%) and more frequent recognition of the antigen at 20kDa (14%) compared to the recognition patterns of the same individuals three months after treatment. Sera from susceptible individuals, also, showed a general pattern of decreased recognition of antigens than post-treatment. They showed less frequent recognition of antigens at 186 (17%), 150 (17%), 133 (25%), 123 (25%), 52-54 (17%), 42 (25%), 23 (33%), 20 (17%) and 16-18kDa (39%) and more frequent recognition of antigens at 84 (8%), 78 (8%), 74 (8%) and 45-47kDa (10%) than the recognition patterns of the same individuals three months after treatment.

When comparing the recognition pattern after exposure to re-infection with the pre-treatment recognition, sera from resistant individuals showed a decreased recognition of most antigens after exposure to re-infection. They showed less frequent recognition of antigens at 123 (14%), 95 (14%), 84 (14%), 74 (7%), 65 (21%), 45-47 (34%), 42 (14%), 37 (7%), 28-30 (42%) and 23kDa (14%) compared to the recognition patterns of the same individuals prior to treatment. Sera from re-infected susceptible individuals showed increased frequency in the recognition of antigens at 95 (17%), 84 (17%), 78 (42%), 74 (33%), 65 (8%), 52-54 (17%), 45-47 (25%) and 37kDa (17%) and less frequent recognition of antigens at 34 (17%), 23 (17%) and 20kDa (17%) compared to the recognition prior to treatment (see figure 4.4).

These results showed that after treatment with PZQ, while sera from resistant individuals showed a general pattern of decreased frequency of recognition, sera from susceptible individuals showed increased frequency in the recognition of most antigens after treatment compared to the recognition prior to treatment. Furthermore, while sera from re-infected susceptible individuals showed increased frequency in the recognition of antigens at molecular masses between 37 and 95kDa (especially the 78, 74 and 45-47kDa) sera from resistant individuals exposed to re-infection during the same period showed less frequent recognition of the same antigens compared to the recognition prior to treatment.

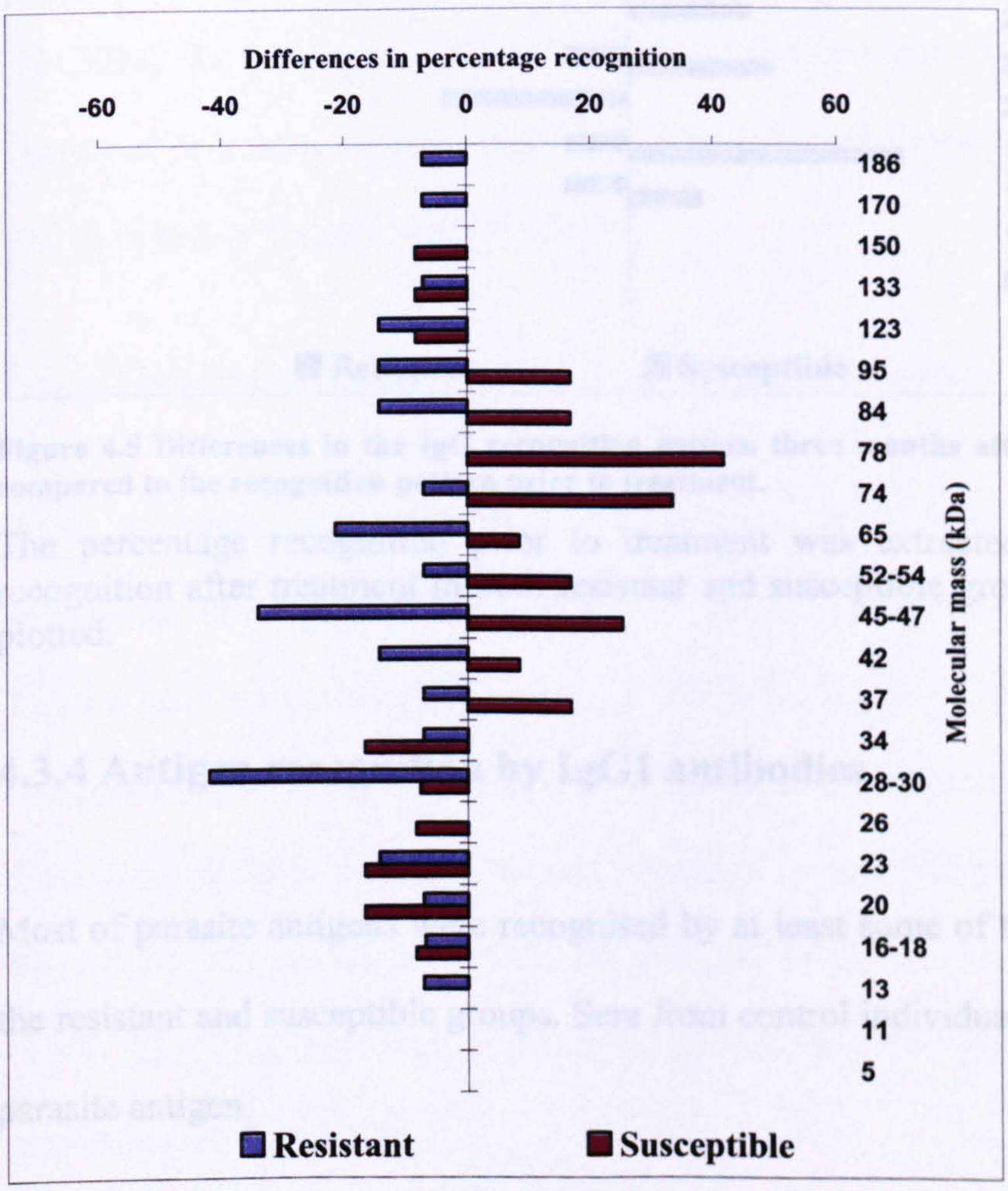

Figure 4.4 Differences in the IgG recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

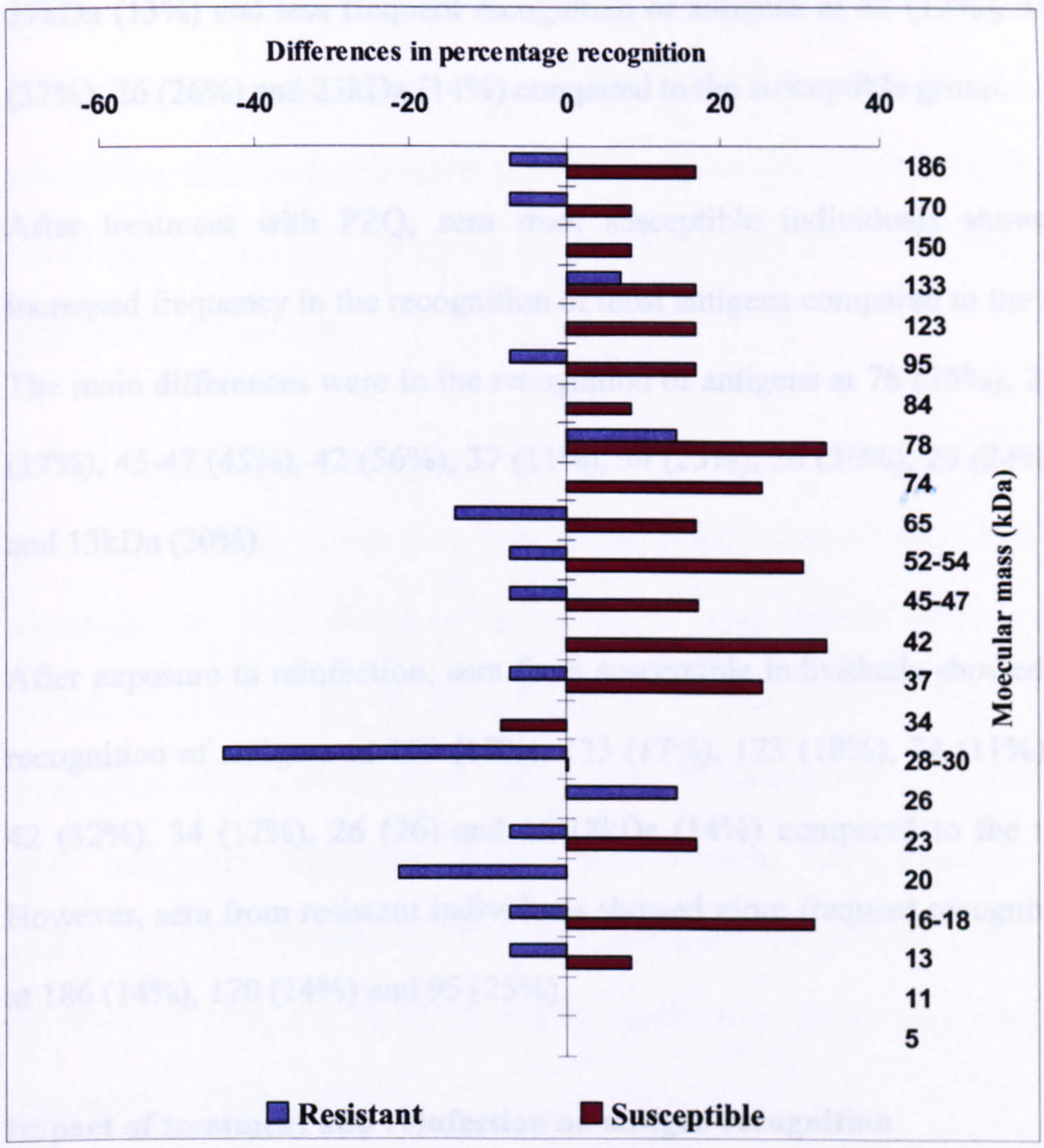

Figure 4.5 Differences in the IgG recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.3.4 Antigen recognition by IgG1 antibodies

Most of parasite antigens were recognised by at least some of the serum samples from the resistant and susceptible groups. Sera from control individuals did not recognise any parasite antigen.

Differences in antigen recognition between resistant and susceptible groups

Before treatment, sera from resistant individuals showed more frequent recognition of antigens at 95 (17%), 78 (10%), 74 (37%), 65 (21%), 52-54 (21%), 45-47 (23%) and

37kDa (13%) and less frequent recognition of antigens at 42 (12%), 34 (25%), 28-30 (37%), 26 (26%) and 23kDa (14%) compared to the susceptible group.

After treatment with PZQ, sera from susceptible individuals showed a generally increased frequency in the recognition of most antigens compared to the resistant group. The main differences were in the recognition of antigens at 78 (15%), 74 (20%), 52-54 (17%), 45-47 (45%), 42 (56%), 37 (11%), 34 (25%), 26 (36%), 23 (24%), 16-18 (15%) and 13kDa (20%).

After exposure to reinfection, sera from susceptible individuals showed more frequent recognition of antigens at 150 (17%), 133 (17%), 123 (18%), 74 (11%), 45-47 (20%), 42 (32%), 34 (17%), 26 (26) and 16-18kDa (14%) compared to the resistant group. However, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 170 (14%) and 95 (25%).

Impact of treatment and reinfection on antigen recognition

After treatment with PZQ, sera from resistant individuals showed more frequent recognition of antigens at 150 (14%), 133 (14%), 123 (14%) and 28-30kDa (21%) and less frequent recognition of antigens at 52-54 (21%), 45-47 (42%) and 42kDa (36%) compared to the recognition patterns of the same individuals prior to treatment. Sera from susceptible individuals showed increased recognition particularly of antigens at 133 (25%), 123 (25%), 78 (25%), 74(50%), 52-54 (17%), 45-47 (25%), 37 (17%), 26 (17%), 23 (17%), 20 (17%), 16-18 (25%) and 13kDa (25%) after treatment than prior to treatment (see figure 4.7).

After exposure to re-infection, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 170 (14%), 78 (14%) and 42kDa (7%) and less frequent recognition of antigens at 150 (14%), 133 (21%), 123 (14%), 65 (14%), 37

(14%), 28-30 (36%) and 16-18kDa (14%) compared to the recognition patterns of the same individuals after treatment. Sera from susceptible individuals showed decreased recognition after treatment especially of antigens at 133 (17%), 74 (17%), 52-54 (17%), 45-47 (25%), 42 (17%), 37 (25%), 28-30 (25%), 26 (17%), 23 (17%), 20 (17%), 16-18 (16%) and 13kDa (25%).

When comparing the recognition pattern after exposure to reinfection with the pre-treatment recognition, sera from resistant individuals showed more frequent recognition of antigens at 78kDa (14%) and less frequent recognition of antigens at 74 (14%), 65 (14%), 52-54 (22%), 45-47 (24%), 42 (29%), 37 (21%) and 28-30kDa (14%) compared to samples taken from the same individuals prior to treatment. On the other hand, sera from susceptible individuals showed more frequent recognition of antigens at 123 (17%), 78 (17%) and 74kDa (33%) and less frequent recognition of antigens at 42 (8%), 37 (8%), 34 (8%) and 28-30kDa (45%) compared to the recognition patterns prior to treatment (see figure 4.6).

These results showed that while the effect of treatment with PZQ on the recognition pattern of the resistant individuals was heterogeneous, sera from susceptible individuals showed a general pattern of increased recognition of most antigens especially antigens of molecular masses between 34 and 74kDa as a direct result of treatment with praziquantel. After exposure to re-infection, sera from susceptible individuals showed more frequent recognition of antigens at 74 (33%), 65 (8%) and 45-47kDa (8%) compared to the recognition patterns prior to treatment. Sera from resistant individuals showed less frequent recognition of the same antigens (14, 14 and 24%, respectively) than the recognition prior to treatment.

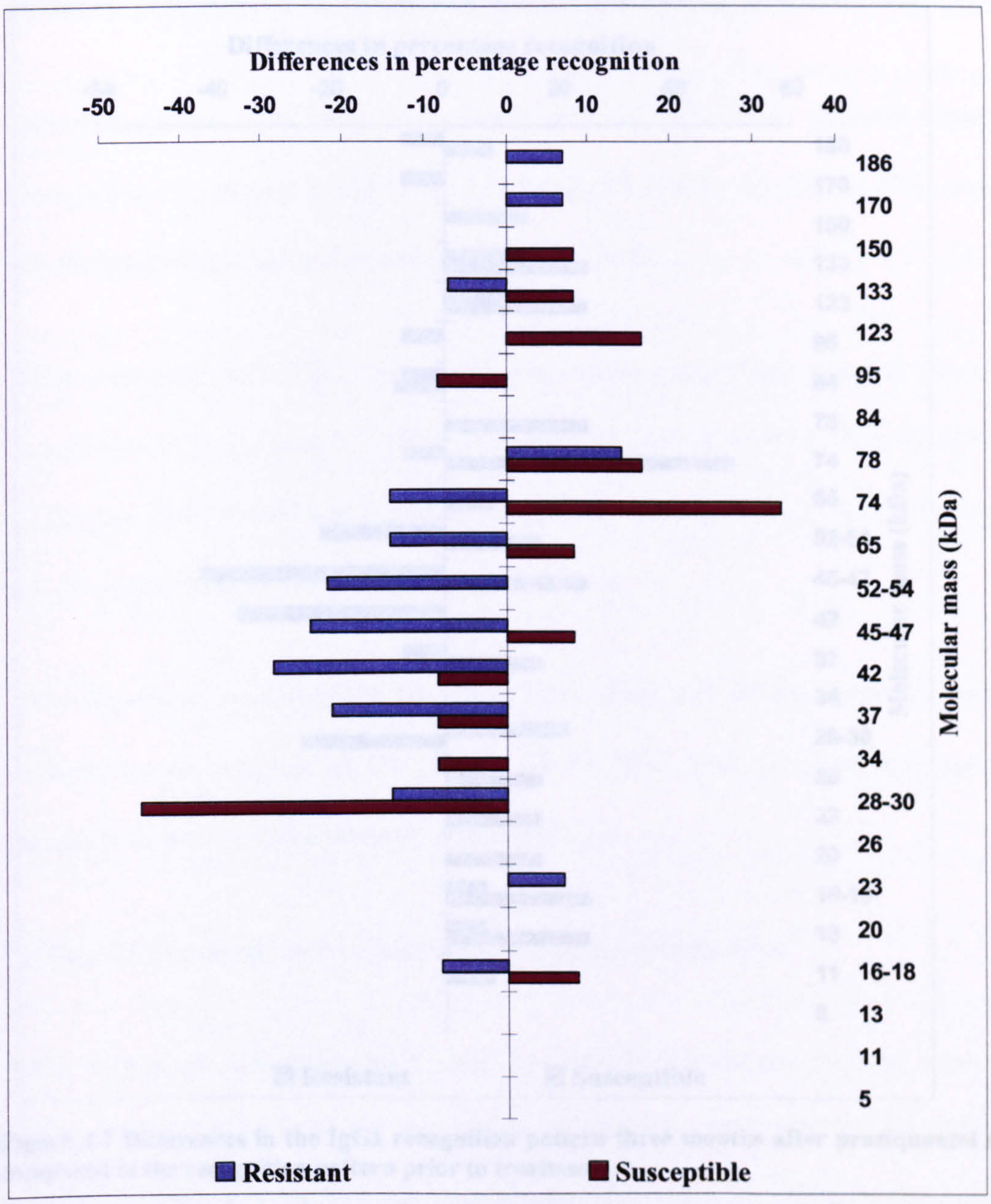

Figure 4.6 Differences in the IgG1 recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

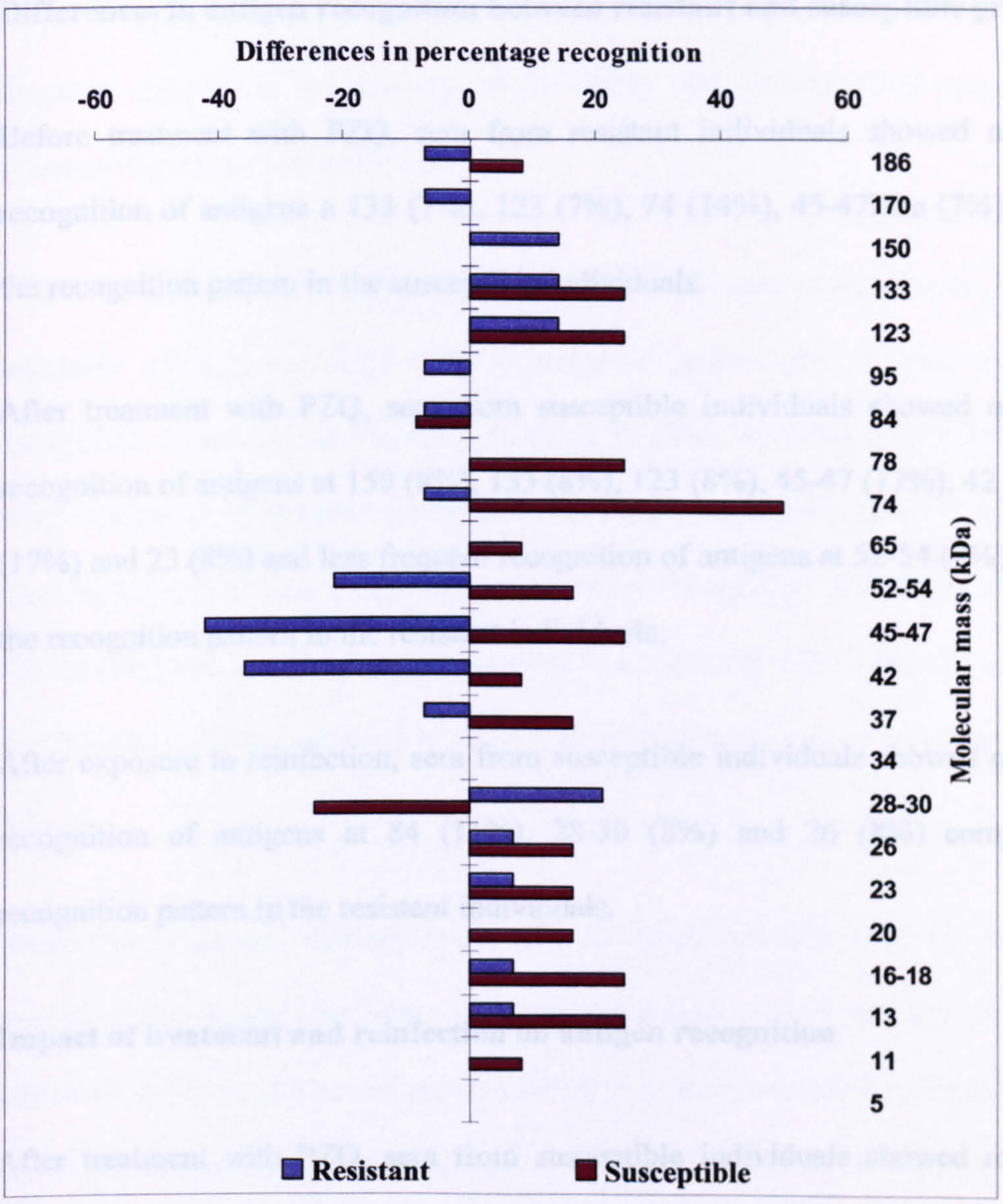

Figure 4.7 Differences in the IgG1 recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.3.5 Antigen recognition by IgG2 antibodies

Few proteins from the parasite were recognised by IgG2 in the serum samples from the resistant and susceptible groups. IgG2 antibodies in sera from control individuals did not recognise any parasite antigen.

Differences in antigen recognition between resistant and susceptible groups

Before treatment with PZQ, sera from resistant individuals showed more frequent recognition of antigens a 133 (7%), 123 (7%), 74 (14%), 45-47kDa (7%) compared to the recognition pattern in the susceptible individuals.

After treatment with PZQ, sera from susceptible individuals showed more frequent recognition of antigens at 150 (8%), 133 (8%), 123 (8%), 45-47 (17%), 42 (17%), 28-30 (17%) and 23 (8%) and less frequent recognition of antigens at 52-54 (7%) compared to the recognition pattern in the resistant individuals.

After exposure to reinfection, sera from susceptible individuals showed more frequent recognition of antigens at 84 (11%), 28-30 (8%) and 26 (8%) compared to the recognition pattern in the resistant individuals.

Impact of treatment and reinfection on antigen recognition

After treatment with PZQ, sera from susceptible individuals showed more frequent recognition of most antigens particularly of antigens at 78 (17%), 74 (17%), 45-47 (17%), 42 (17%) and 28-30kDa (17%) compared to the recognition patterns of the same individuals prior to treatment. Sera from resistant individuals showed more frequent recognition of antigens at 78 (14%) and 52-54kDa (7%) and less frequent recognition of antigens at 133 (7%) and 123 (7%) compared to the recognition patterns of the same individuals prior to treatment (see figure 4.9).

After exposure to reinfection, sera from resistant individuals showed less frequent recognition of antigens at 78 (7%), 74 (7%), 52-54 (7%), and 45-47kDa (7%) and more frequent recognition of the antigen at 42kDa (7%) compared to the recognition pattern of the same individuals after treatment. Sera from susceptible individuals showed less

frequent recognition of most antigens especially of antigen at 45-47 (17%) and more frequent recognition of antigens at 84 (8%) and 26kDa (8%) compared to the recognition pattern after treatment.

When comparing the recognition pattern after exposure to reinfection with the pre-treatment recognition, sera from susceptible individuals showed more frequent recognition of antigens at 84 (8%), 78 (8%), 74 (8%), 42 (8%), 28-30 (8%) and 26kDa (8%) compared to samples taken from the same individuals prior to treatment. Sera from resistant individuals showed more frequent recognition of antigens at 78 (8%) and 42kDa (8%) and less frequent recognition of antigens at 133 (8%), 123 (8%), 74 (8%) and 45-47kDa (8%) compared to the recognition patterns prior to treatment (see figure 4.8).

These results showed that while the effect of treatment with PZQ on the recognition pattern of the resistant individuals was heterogeneous, there was a general pattern of increased IgG2 recognition of parasite antigens in the susceptible individuals as a direct result of treatment with praziquantel. After exposure to re-infection, sera from susceptible individuals showed more frequent recognition of antigens at 74kDa compared to the recognition patterns prior to treatment. Sera from resistant individuals showed less frequent recognition of the same antigen.

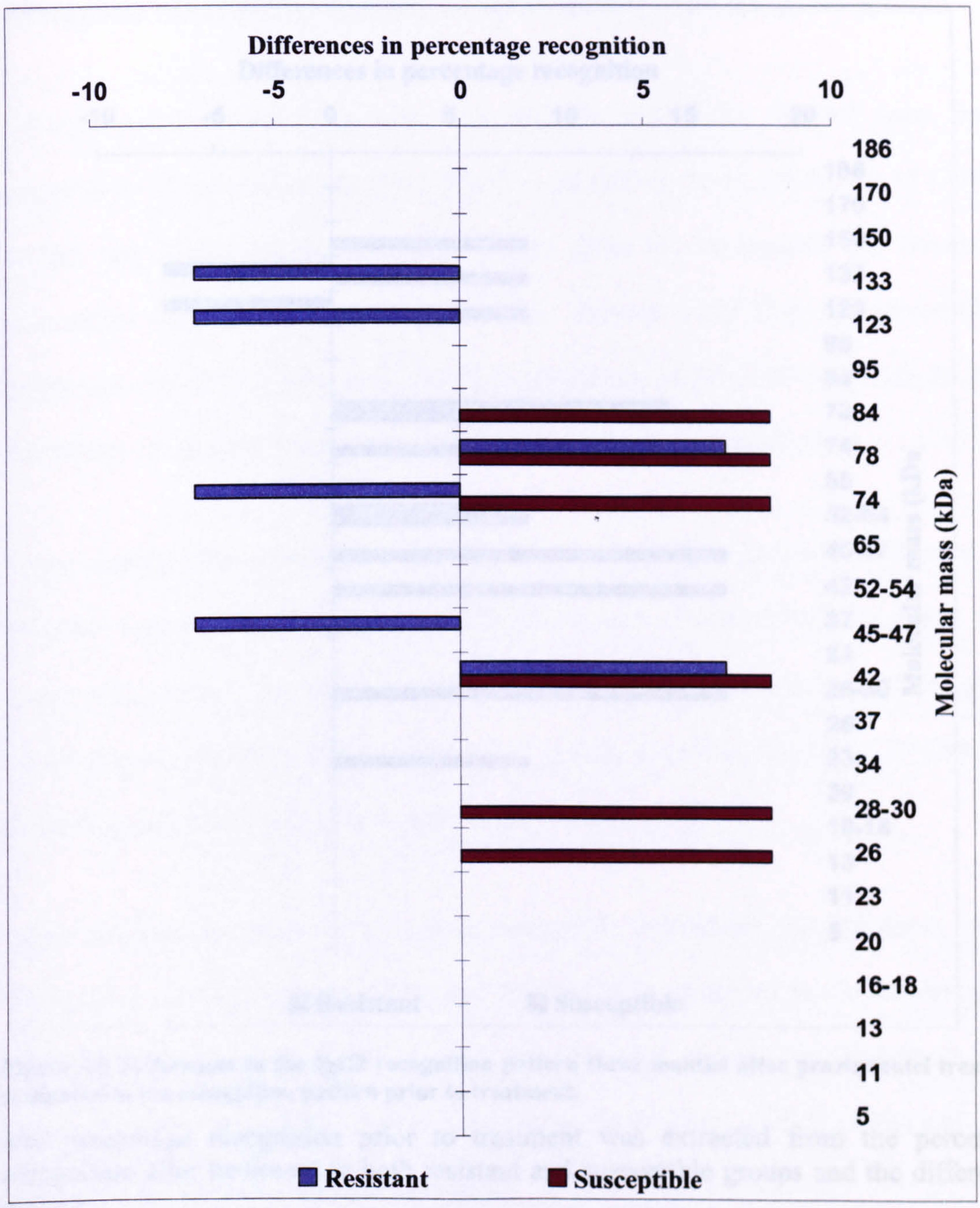

Figure 4.8 Differences in the IgG2 recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

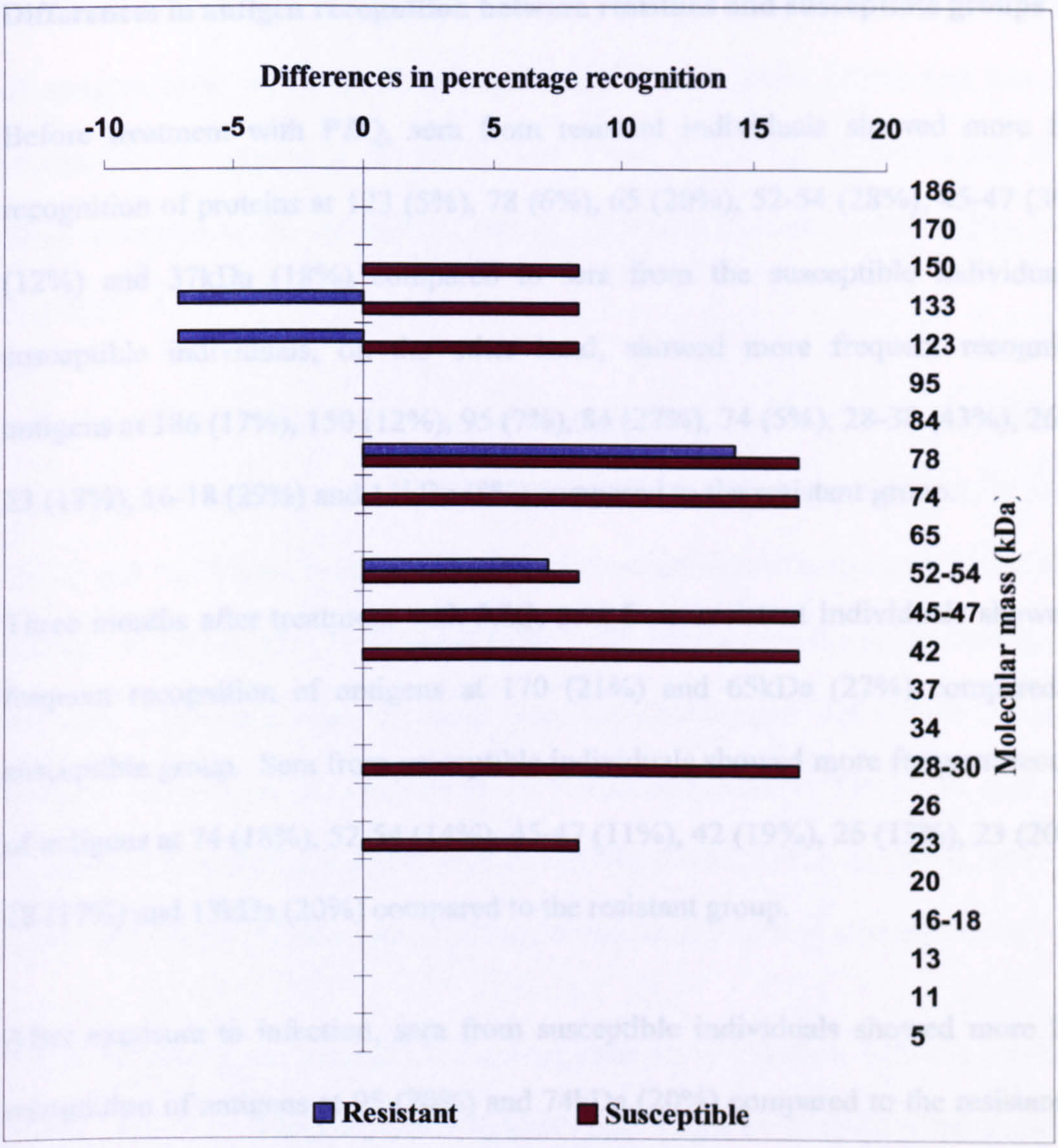

Figure 4.9 Differences in the IgG2 recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.3.6 Antigen recognition by IgG3 antibodies

Most of the proteins from the parasite were recognised by IgG3 in at least some of the serum samples from the resistant and susceptible groups. IgG3 antibodies in sera from control individuals did not recognise any parasite antigen.

Differences in antigen recognition between resistant and susceptible groups

Before treatment with PZQ, sera from resistant individuals showed more frequent recognition of proteins at 123 (5%), 78 (6%), 65 (20%), 52-54 (28%), 45-47 (30%), 42 (12%) and 37kDa (18%) compared to sera from the susceptible individuals. The susceptible individuals, on the other hand, showed more frequent recognition of antigens at 186 (17%), 150 (12%), 95 (7%), 84 (27%), 74 (5%), 28-30 (43%), 26 (12%), 23 (18%), 16-18 (29%) and 11kDa (8%) compared to the resistant group.

Three months after treatment with PZQ, sera from resistant individuals showed more frequent recognition of antigens at 170 (21%) and 65kDa (27%) compared to the susceptible group. Sera from susceptible individuals showed more frequent recognition of antigens at 74 (18%), 52-54 (14%), 45-47 (11%), 42 (19%), 26 (13%), 23 (20%), 16-18 (17%) and 13kDa (20%) compared to the resistant group.

After exposure to infection, sera from susceptible individuals showed more frequent recognition of antigens at 95 (20%) and 74kDa (20%) compared to the resistant group. Sera from resistant individuals showed more frequent recognition of antigens at 186 (7%), 170 (7%), 84 (13%), 78 (14%), 65 (14%), 52-54 (31%), 45-47 (11%) and 23kDa (19%) compared to the susceptible group.

Impact of treatment and reinfection on antigen recognition

IgG3 recognition of *S. haematobium* adult worm antigens was altered in both resistant and susceptible groups after treatment with praziquantel. Three months after the treatment, sera from resistant individuals showed more frequent recognition of antigens at 186 (14%), 170 (14%), 28-30 (43%) and 23kDa (14%) and less frequent recognition of antigens at 95 (14%), 78 (14%), 74 (21%), 52-54 (42%), 45-47 (27%), 42 (14%) and 37kDa (14%) compared to the frequency of recognition patterns of the same individuals

prior to treatment. Sera from susceptible individuals showed more frequent recognition of antigens at 45-47 (8%), 42 (17%), 23 (17%) and 13kDa (17%) and less frequent recognition of antigens at 186 (8%), 150 (17%), 95 (17%), 84 (33%) and 28-30kDa (8%) compared to the recognition patterns observed with sera taken before treatment (see figure 4.11).

After exposure to re-infection, sera from resistant individuals showed more frequent recognition of antigens at 78 (14%), 74 (14%), 52-54 (28%), 45-47 (21%), 37 (14%), 23 (14%) and 16-18kDa (14%) and less frequent recognition of antigens at 170 (14%), 150 (14%), 133 (21%), 123 (21%) and 65kDa (21%) compared to the recognition patterns of the same individuals after treatment. Sera from susceptible individuals showed more frequent recognition of antigens at 74 (17%) and 37kDa (17%) and less frequent recognition of antigens at 133 (17%), 123 (17%), 52-54 (16%), 42 (17%), 26 (17%), 23 (25%) and 13kDa (17%) compared to the recognition observed after treatment.

When comparing the recognition after exposure to re-infection with the pre-treatment values, sera taken from resistant individuals after exposure to re-infection showed more frequent recognition of antigens at 186 (7%), 84 (7%), 28-30 (35%) and 23kDa (29%) and less frequent recognition of antigens at 150 (14%), 133 (14%), 123 (14%), 95 (21%), 74 (7%), 65 (14%) and 45-47kDa (14%) compared to the recognition patterns of the same individuals prior to treatment. Sera taken from susceptible re-infected individuals showed more frequent recognition of antigens at 74 (8%) and 37kDa (17%) and less frequent recognition of antigens at 186 (17%), 150 (25%), 133 (17%), 84 (33%), 52-54 (17%), 28-30 (8%), 23 (8%) and 16-18kDa (17%) compared to the recognition patterns prior to treatment (see figure 4.10).

These results illustrate that the effect of praziquantel treatment on the humoral immune response to schistosome antigens is heterogeneous with much variation among

individuals. However, sera from resistant individuals showed a trend of decreased recognition against antigens of medium molecular masses (37 to 123kDa) while sera from susceptible individuals showed a trend of decreased recognition against antigens of higher molecular masses (74 to 186kDa). After 12 months of exposure to re-infection, sera from resistant individuals showed increased recognition of antigens at 28-30 (35%) and 23kDa (29%) compared to the recognition prior to treatment. Sera from susceptible individuals showed less frequent recognition of the same antigens after the same period of exposure.

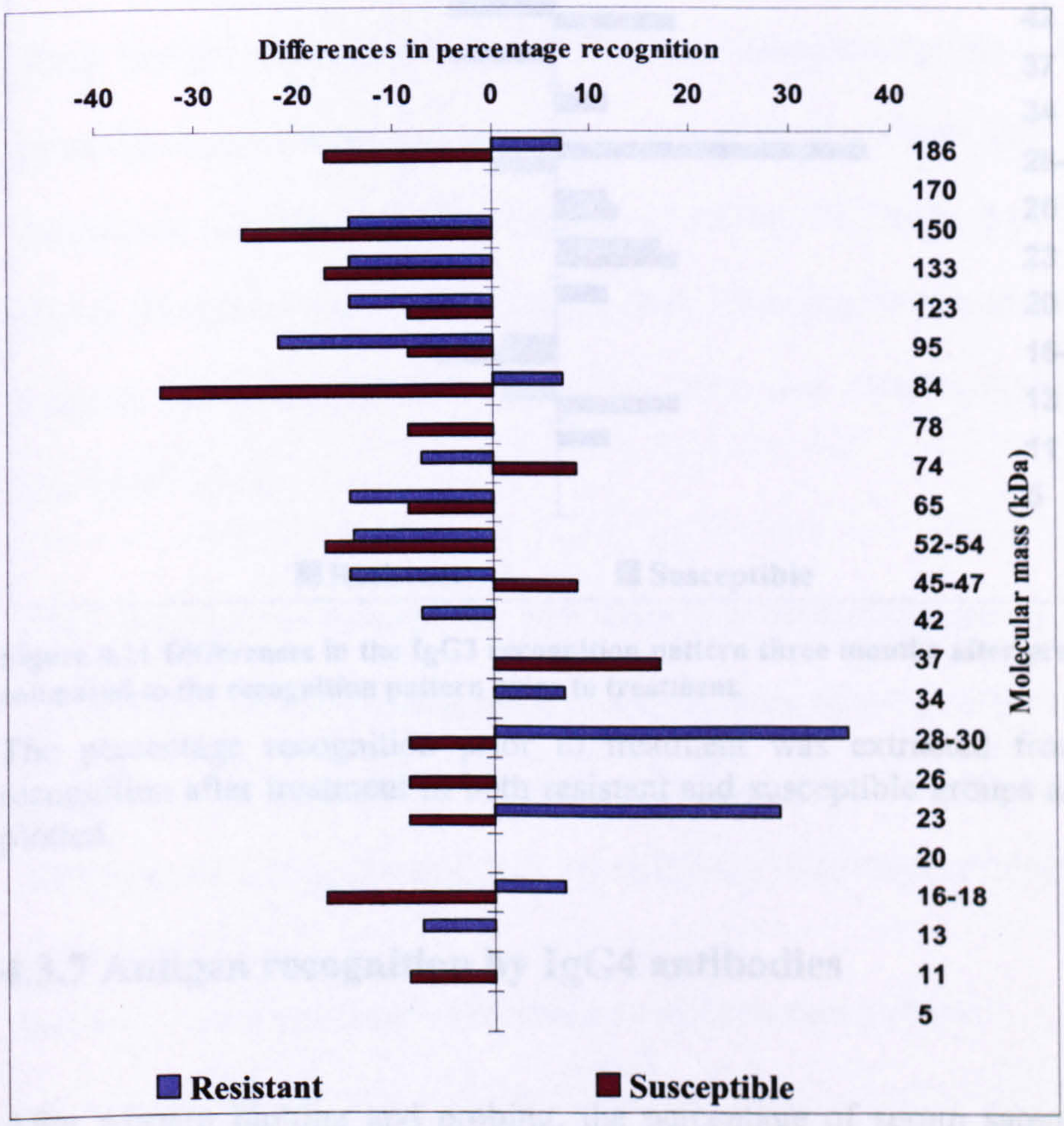

Figure 4.10 Differences in the IgG3 recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

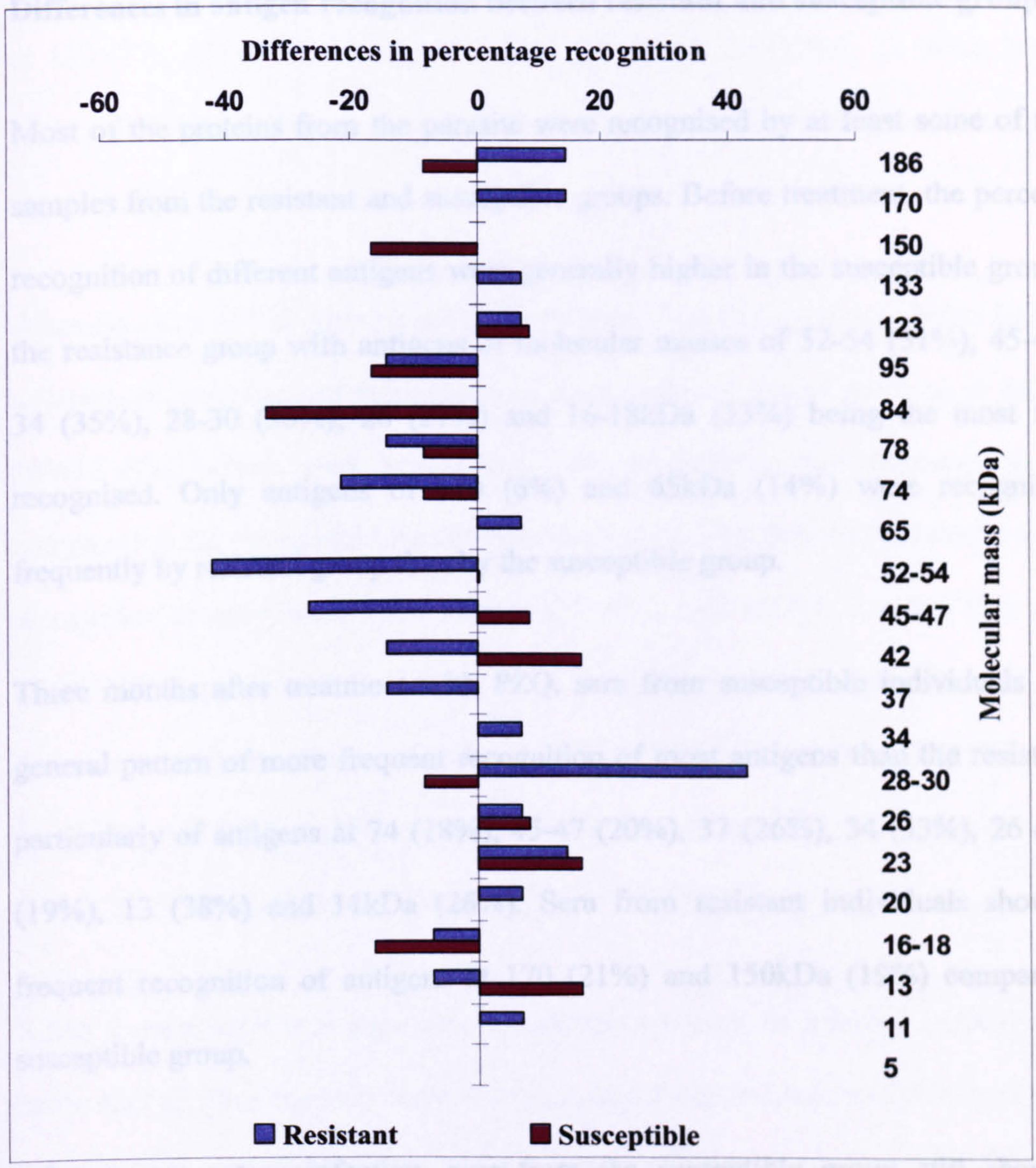

Figure 4.11 Differences in the IgG3 recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.3.7 Antigen recognition by IgG4 antibodies

After western blotting and probing, the percentage of serum samples of each group recognising each antigen band was calculated. Sera from control individuals did not recognise any parasite antigen.

Differences in antigen recognition between resistant and susceptible groups

Most of the proteins from the parasite were recognised by at least some of the serum samples from the resistant and susceptible groups. Before treatment, the percentages of recognition of different antigens were generally higher in the susceptible group than in the resistance group with antigens of molecular masses of 52-54 (31%), 45-47 (52%), 34 (35%), 28-30 (30%), 26 (27%) and 16-18kDa (33%) being the most frequently recognised. Only antigens of 170 (6%) and 65kDa (14%) were recognised more frequently by resistant group than by the susceptible group.

Three months after treatment with PZQ, sera from susceptible individuals showed a general pattern of more frequent recognition of most antigens than the resistant group particularly of antigens at 74 (18%), 45-47 (20%), 37 (26%), 34 (33%), 26 (37%), 20 (19%), 13 (38%) and 11kDa (26%). Sera from resistant individuals showed more frequent recognition of antigens at 170 (21%) and 150kDa (19%) compared to the susceptible group.

After exposure to reinfection, sera from the susceptible group still showed more frequent IgG4 antibody recognition of a wide range of antigens than the resistant group. However the greatest differences were in the recognition of antigens at 74 (33%), 45-47 (40%) and 42 (33%), 28-30 (25%), 16-18 (30%), 13 (54%) and 11kDa (33%).

Impact of treatment and reinfection on antigen recognition

After treatment with PZQ, sera from resistant individuals showed an increased frequency of recognition of antigens at 170 (7%), 150 (21%), 133 (14%), 123 (7%), 95 (14%), 45-47 (7%), 42 (14%), 26 (7%) and 16-18kDa (22%) and a decreased frequency of recognition of antigens at 84 (7%), 74 (14%), 34 (7%) and 13kDa (14%) compared to the frequency of recognition of antigens by sera from the same patients prior to

treatment. Sera from susceptible patients showed more frequent recognition of antigens at 133 (17), 123 (8%), 65 (8), 42 (17%), 37 (25%), 26 (17%), 23 (8%), 20 (8%), 13 (8%) and 11kDa (17%) compared to the recognition pattern of the resistant group (see figure 4.13).

After exposure to infection after PZQ treatment, sera from resistant individuals showed a decreased frequency of recognition of most antigens except antigens at 84 (7%) and 26kDa (7%) which were recognised more frequently after exposure to infection compared to the recognition pattern after treatment. The greatest reductions were in the recognition of antigens at 150 (36%), 133 (29%), 123 (21%), 45-47 (37%), 42 (21%), 28-30 (29%) and 16-18kDa (37%). Sera from susceptible patients showed decreased frequency in the recognition of antigens at 133 (25%), 123 (17%), 37 (25%), 34 (17%), 26 (33%) and 23kDa (25%) and increased recognition of antigens at 170 (8%), 84 (17%), 74 (8%), 42 (8%) and 13kDa (8%).

When comparing the recognition of antigens by sera of infected individuals before treatment and after exposure to re-infection, sera from resistant individuals maintained a reduced frequency of recognition of most antigens after exposure to re-infection except the antigens at 95 (7%) and 26kDa (14%) which showed an increase in the percentage recognition. On the other hand, sera from susceptible individuals showed reduction in the percentage recognition of all protein bands except those of 84 (8%), 42 (25%), 13 (17%) and 11kDa (17%) (see figure 4.12).

These results showed that after treatment of *S. haematobium* infection with praziquantel there was heterogeneity in the effect of treatment on IgG4 isotype reactivity. After treatment, some antigen bands were recognised more frequently and others less frequently than by sera taken prior to treatment. After exposure to re-infection, individuals who were not re-infected were considered resistant and their serum samples

showed a decreased percentage recognition of antigens at 42, 13 and 11kDa and increased percentage recognition of the 26kDa (14%) antigens compared to sera from susceptible individuals who were reinfected during the same transmission period.

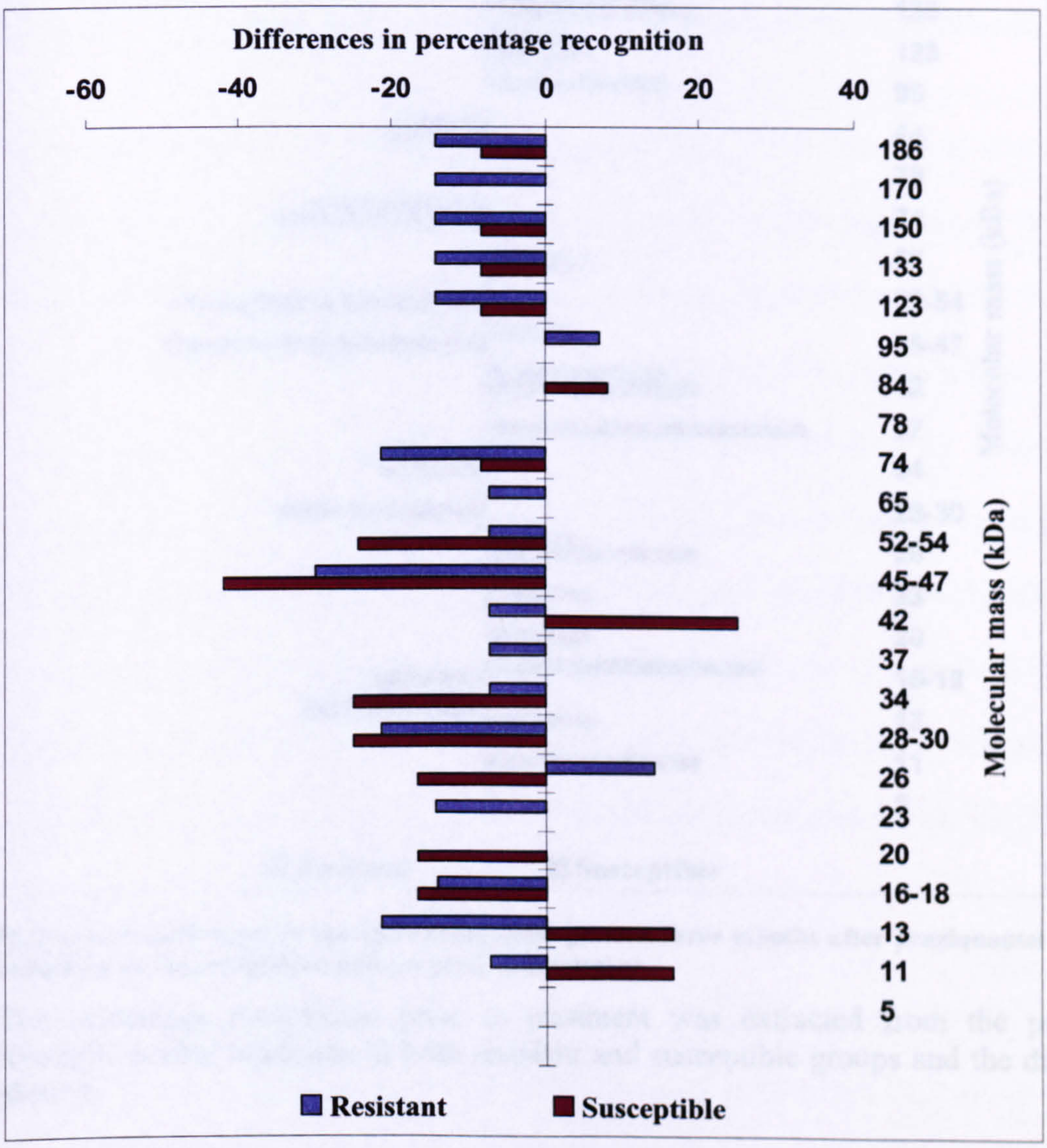

Figure 4.12 Differences in the IgG4 recognition pattern after exposure to reinfection compared to the recognition pattern prior to praziquantel treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after exposure to infection in both resistant and susceptible groups and the differences plotted.

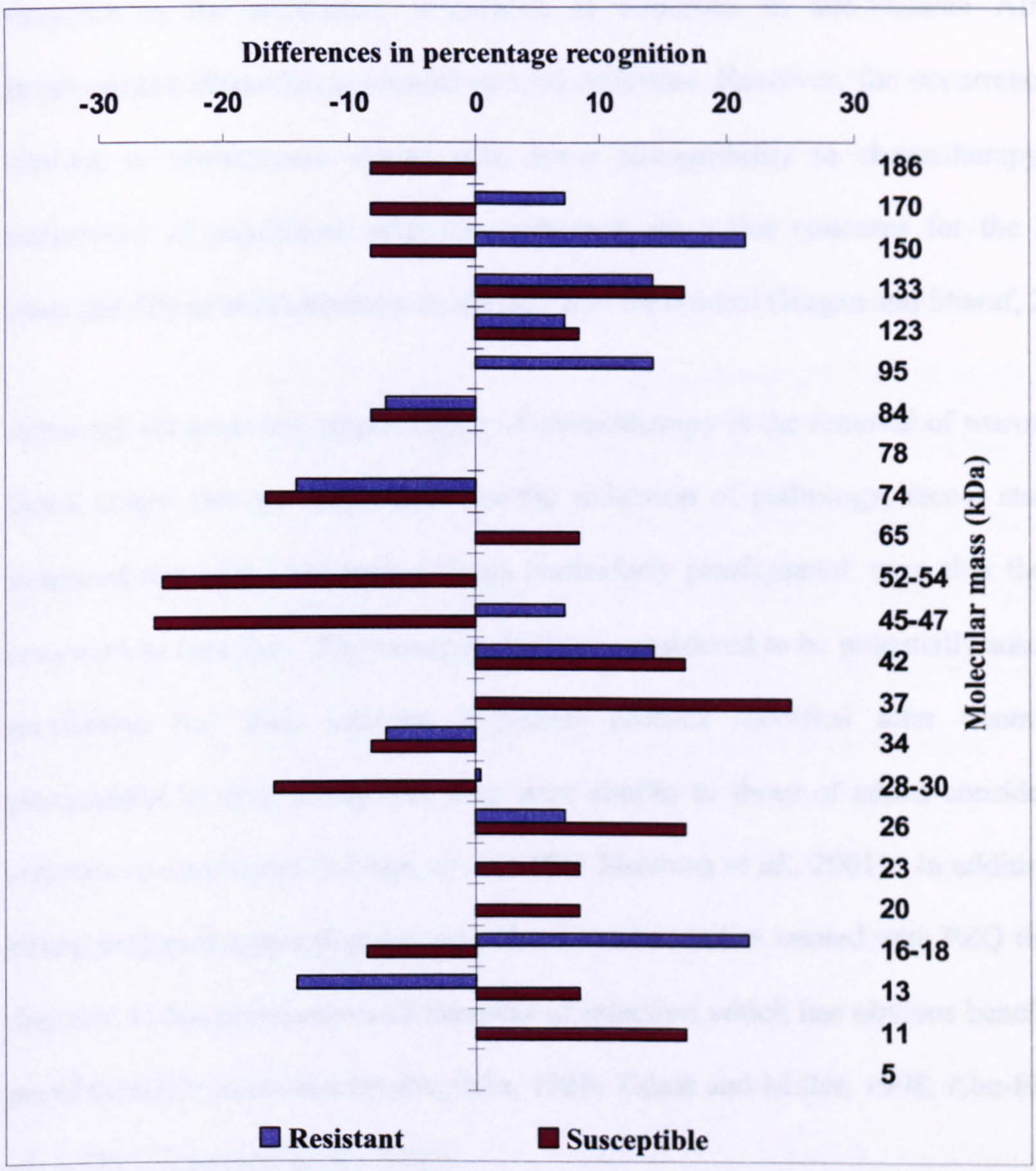

Figure 4.13 Differences in the IgG4 recognition pattern three months after praziquantel treatment compared to the recognition pattern prior to treatment.

The percentage recognition prior to treatment was extracted from the percentage recognition after treatment in both resistant and susceptible groups and the differences plotted.

4.4 Discussion:

While several control measures have been exploited to control schistosome infection chemotherapy now underpins virtually all major control programmes. Concern for the relatively limited use of anthelminthics in areas that are endemic for the major parasitic worms has led the World Health Assembly to adopt resolution 54.19 to encourage WHO member states to seek to achieve 75% deworming of school children by 2010. In

response to the resolution, a number of countries in sub-Saharan Africa have implemented chemotherapy-based control activities. However, the occurrence, though limited, of schistosome strains with lower susceptibility to chemotherapy and the occurrence of reinfection after chemotherapy are major concerns for the long-term sustainability of chemotherapy as the sole tool for control (Hagan and Sharaf, 2003).

Although the most important impact of chemotherapy is the removal of worms, eggs or larval stages that are responsible for the induction of pathology, recent studies have indicated that anti-schistosomal drugs particularly praziquantel, may alter the immune responses to infection. For example children, considered to be potentially susceptible to reinfection had their immune responses profiles modified after treatment with praziquantel in such a way that they were similar to those of adults considered to be resistant to reinfection (Mutapi *et al.*, 1998; Mduluza *et al.*, 2001). In addition there is strong evidence supporting the view that in communities treated with PZQ there was a decrease in the prevalence and intensity of infection which has obvious benefits for the population (Gryseels and Nkulikyinka, 1989; Talaat and Miller, 1998; Abu-Elyazeed *et al.*, 1998; Campagne *et al.*, 2001).

It is generally accepted that in order to achieve satisfactory, long-term and sustainable control of schistosome infection, several control measures will have to be employed. Vaccination has been used as a very powerful tool for the control of many bacterial and viral diseases. Over the last two decades, several attempts have been made to develop and test an efficacious anti-schistosome vaccine. The complex life cycle and the antigenic complexity of each life stage as well as the complex network of immune responses elicited by schistosome infection are major obstacles to the development of such a vaccine. Nevertheless, a number of promising vaccine antigens have been identified. Some have been tested in animal models, and at least one has reached phase II clinical trials for safety and immunogenicity in humans. Vaccine candidate antigens

have been identified in a number of ways, by screening DNA libraries with serum from infected animals or animals that had been immunised against infection with irradiated parasites. Serum from such animals, or from humans naturally exposed to infection (and considered to be resistant to infection), has also been used to screen for parasite antigen recognition by immunoprecipitation using protein A or protein G Sepharose or that have been separated by SDS-PAGE, in one or two dimensions and blotted to nitrocellulose. The identification of vaccine candidate antigens using human serum has been facilitated by attempts to determine immunological correlates of resistance to reinfection in immunoepidemiological studies in humans (Butterworth *et al.*, 1996). Serum samples from studies that have demonstrated a degree of resistance to infection in naturally exposed individuals has obvious value for screening schistosome antigens.

The serum samples exploited in the present study were obtained from an immunoepidemiological study by Hagan and his colleagues (1991) that gave the first clear immune correlation between the parasite specific IgE and IgG4 and the resistance or susceptibility to reinfection with *S. haematobium*. They reported that parasite specific IgE correlated with resistance to reinfection while IgG4 correlated with susceptibility to reinfection (Hagan *et al.*, 1991). The present study was designed to investigate the differences in antigen recognition between potentially resistant individuals and potentially susceptible individuals after exposure to reinfection with *S. haematobium*. The effect of treatment with praziquantel on the immune responses of resistant and susceptible individuals was also investigated.

Before discussing the results of the present study, it is important to note that the molecular weights of the proteins reported herein may vary slightly from the reported molecular weights obtained in other studies. This is due to the limitations of the SDS-PAGE technique in making precise determinations of molecular weights of proteins. Even with the two dimensional SDS-PAGE and mass spectrometry, which is a far

superior technique in terms of accuracy of molecular weight determinations there is an error factor of up to 2% of the molecular weight of the identified protein (Berg *et al.*, 2002b). It is worth mentioning that schistosomes share most of their antigens as evidenced by their cross reactivity. Several studies confirmed the reactivity of sera or lymphocytes obtained from hosts infected with one schistosome species with antigenic preparations from the other schistosome species (Abdel-Hafez *et al.*, 1983; Barral-Netto *et al.*, 1983; Amanor *et al.*, 1996). Using immunoprecipitation techniques, it was reported that the immune responses of patients infected with *S. mansoni* or *S. haematobium* were so similar that differentiation could not be established easily (Norden and Strand, 1984). However, some species-specific antigens were identified for each schistosome species (Kelly *et al.*, 1987; Amanor *et al.*, 1996).

The effect of treatment with PZQ on the recognition pattern of *S. haematobium* SWAP by IgM antibodies was heterogeneous in both resistant and susceptible individuals with some antigens being recognised more frequently and others less frequently, three months after treatment, compared to the recognition pattern prior to treatment. However when the same individuals were exposed to reinfection, sera from infected individuals maintained more frequent recognition of antigens at 45-47, 42, 37, 34 and 28-30kDa. Sera from resistant individuals recognised these antigens less frequently compared to the recognition three months after treatment or prior to treatment. One possible interpretation of these data is that the reduction in IgM recognition may be a contributory factor in rendering these individuals resistant to reinfection. In the susceptible individuals, the enhanced recognition of these antigens by IgM may compete with potentially-protective antibodies rendering these individuals susceptible to reinfection. These results and this interpretation are consistent with the reported role of IgM as a blocking antibody (Butterworth *et al.*, 1987; Ndhlovu *et al.*, 1996).

Three months after treatment with PZQ, sera from susceptible individuals showed more recognition of almost all antigens except the 28-30kDa antigen by IgG1 antibody isotype compared to the recognition prior to treatment. When the same individuals were reinfected they failed to maintain the higher levels of recognition and showed a further reduction in the recognition of the antigen of 28-30kDa. The effect of treatment on the recognition pattern of sera from resistant individuals was heterogeneous. The greatest increase in the recognition was the recognition of the antigen at 28-30kDa. However, the resistant individuals failed to maintain this increased recognition after exposure to reinfection. The boost in recognition of the 28-30kDa antigens after treatment may have contributed to the observed protection. While the treatment boosted the responses it seems that it was insufficient to stimulate a long-term increase in antibodies against this antigen. Others have speculated that in order to acquire sufficient levels of protective antibody isotypes, several episodes of schistosome infection (Li *et al.*, 2002) or repeated chemotherapy (Mduluza *et al.*, 2001) may be required.

After treatment with PZQ, the recognition of *S. haematobium* SWAP by IgG3 in sera from resistant and susceptible individuals showed heterogeneous changes. The most striking effects of treatment on the recognition pattern were increased frequency of recognition of antigens of 23 and 28-30kDa of both resistant and susceptible individuals. However, while sera from resistant individuals maintain the increased recognition after exposure to reinfection, sera from susceptible individuals failed to maintain such response. These observations favour the possibility that PZQ may have, albeit temporarily, an immunising effect (Mutapi *et al.*, 1998; Woolhouse and Hagan, 1999; Mutapi *et al.*, 2003). However, although the present study provided an evidence for this hypothesis, other factors may be involved in the difference in the immune responses between resistant and susceptible individuals. The resistant individuals (mean age 35±13.5 years) of the present study are significantly older than the susceptible

individuals (mean age 8.2±3 years). The protective response of the resistant individuals observed in the present study may result from prolonged experience of the immune system of those individuals with parasite antigens released from naturally dying parasites and repeated infection rather than the release of these antigens in response to enhanced parasite death by the chemotherapy (Hagan *et al.*, 1985; Butterworth *et al.*, 1985; Woolhouse *et al.*, 1991; Fulford *et al.*, 1992) or it may be attributed to age *per se* (van dam *et al.*, 1996; Ouma *et al.*, 1998).

In the present study, the majority of antigens were less frequently recognised at all time points by IgG4 antibody isotype of sera from resistant individuals compared to sera from susceptible individuals. Although sera from both resistant and susceptible individuals showed heterogeneous changes in the recognition pattern in response to treatment with PZQ, the recognition of most antigens became less frequent after exposure to reinfection in sera from resistant individuals compared to susceptible individuals. These results agree with the proposed role of IgG4 as a blocking antibody during schistosome infections (Hagan *et al.*, 1991; Demeure *et al.*, 1993; Ndhlovu *et al.*, 1996). Similar effects of treatment with PZQ on the recognition of *S. mansoni* SWAP by IgG4 antibody isotye were reported by Satti and colleagues. They reported that the resistant group maintained the same level of IgG4 to SWAP after treatment compared to a significant increase in the susceptible group (Satti *et al.*, 1996). Furthermore, Mduluza and colleagues treated children aged under six years of age and living in a *S. haematobium* endemic area with praziquantel every eight weeks for two years. Chemotherapy reduced infection prevalence and mean intensity of infection. The immunoglobulin levels changed during the course of treatment with a shift towards 'protective' mechanisms. The significant changes noted in some individuals were the drop in 'blocking' IgG2 and IgG4 whereas the 'protecting' IgA and IgG1 levels

increased. However, the antibody profiles in the rest of the children remained generally unchanged throughout the study (Mduluza *et al.*, 2001).

The recognition of the majority of antigens by total IgG antibodies were more frequent, prior to treatment with PZQ, in sera from resistant individuals compared to sera from susceptible individuals. Three months after treatment, the recognition pattern changed with most antigens being more frequently recognised by sera from susceptible individuals than resistant individuals. Because of the preponderance of IgG4 antibodies in the serum of infected individuals, the pattern of recognition obtained from the blots when assessing the total IgG antibody responses will be dominated by the antigens that are recognised by IgG4. In practice therefore the patterns of antigen recognition obtained for total IgG and IgG4 were virtually similar.

Interestingly, some of the known vaccine candidates were highly immunogenic and frequently recognised by different antibody isotypes from the serum samples employed in the present study. The recognition of these vaccine candidates was different between resistant and susceptible individuals with some antigen specific antibody isotypes being associated with resistance and others associated with susceptibility to reinfection. After exposure to reinfection, sera from resistant individuals showed a higher frequency of recognition of the antigen of 28-30kDa by IgG3 antibody isotype compared to the susceptible individuals. The 28-30kDa antigen band recognised in this study may correspond to glutathione-S-transferase (GST) which has been identified in all schistosome species as a major enzyme acting as an antioxidant and involved in detoxification of xenobiotics. GST is one of the leading vaccine candidate antigens. GST was tested in independent trials in animals that were coordinated by WHO. Like the other vaccine candidate antigens it failed to give greater than the 40% level of protection that had been set as the cut-off for success. Nonetheless in infected human populations, schistosome anti-fecundity immunity has been associated with IgA and

IgG3 responses (Riveau *et al.*, 1998; Remoue *et al.*, 2000) directed against GST. In phase I clinical trials it was found that following 3 injections of 100μg of *S. haematobium* GST in alum hydroxide as adjuvant (Bilhvax), a strong IgG immune response was elicited in immunised individuals. Analysis of isotypic profile revealed high levels of IgG1, IgG3 and low levels of IgG2 and IgA (Capron *et al.*, 2002).

On the other hand, after exposure to reinfection, sera from susceptible individuals showed higher recognition of the 28-30kDa antigen by the IgM, IgG2 and IgG4 antibody isotypes compared to the resistant individuals. These results are in agreement of those of Al-Sherbiny and colleagues (2003). They investigated the cellular and humoral immune responses of peripheral blood lymphocytes from individuals resistant or susceptible to reinfection with *S. mansoni* in response to *in vitro* stimulation with Sm28-GST. They reported that increased levels of antigen specific IgA and IgE were associated with resistance while IgG2 and IgM antibodies were associated with susceptibility to infection (Al Sherbiny *et al.*, 2003). The increased IgG4 recognition in the susceptible group may interfere with the neutralising effects of the protective isotypes rendering them more susceptible to reinfection. It has been demonstrated that IgG4 antibodies may compete with the protective antibodies for the recognition of the same antigens and thus act as blocking antibodies (Butterworth *et al.*, 1987; Hagan *et al.*, 1991; Demeure *et al.*, 1993).

In the present study, sera from resistant individuals exposed to reinfection showed more frequent recognition of antigens at 23kDa by IgG3 antibody compared to sera from susceptible individuals. These results appear to parallel those of Webster and colleagues (1996). They reported that the recognition of the rSm22 (rSm22.6) by human IgG3, IgG4, IgE and IgA was increased in a group of naturally exposed individuals considered to be resistant to reinfection. However, only the IgE antibody response against this antigen was significantly correlated with resistance to reinfection after treatment

(Webster *et al.*, 1996). *In vitro* stimulation of lymphocytes from the peripheral blood of individuals resistant to infection with *S. mansoni* showed preferential production of IgG3 in response to stimulation with multiple antigenic peptides (MAP3) from the integral membrane antigen Sm23 (Al Sherbiny *et al.*, 2003).

In the present study, IgM, IgG3 and IgG1 antibodies of sera from resistant individuals showed more frequent recognition of the antigen of 84kDa compared to the susceptible individuals. The 84kDa antigen could be the *S. haematobium* equivalent of the calcium binding protein (Calpain Sm-p80) which was reported by Hota-Michell and colleagues (1997). They reported that recombinant Sm-p80 provided a 29-39% reduction in worm burden when they immunised mice and challenged them with *S. mansoni.* The recombinant Sm-p80 was recognised by IgA, IgM, IgG1, and IgG3 isotype antibodies found in *S. mansoni*-infected humans (Hota-Mitchell *et al.*, 1997). DNA immunisation using Sm-p80 with or without plasmids encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were tried in mice. The protection achieved with administration of GM-CSF DNA with Sm-p80 DNA was associated with distinct increases in total IgG and IgG1 titers, whereas the coadministration of IL-4 DNA with Sm-p80 DNA resulted in a slight elevation of IgG1 and IgG3 titers (Siddiqui *et al.*, 2003).

After exposure to reinfection, sera from resistant individuals showed more frequent recognition of the antigen of 65kDa by IgM and IgG3 compared to sera from susceptible individuals. It is possible to speculate that this antigen band may correspond to the 68kDa antigen from *S. mansoni* (SmW68) reported by King and colleagues. They found a negative correlation between intensity of infection and the recognition of the SmW68 antigen by IgM antibodies in chronically infected individuals (King *et al.*, 1989).

One of the most frequently recognised antigen bands in the present study was that of 95kDa which is most likely the *S. haematobium* equivalent of paramyosin (97kDa). Paramyosin is a myofibrillar protein found exclusively in invertebrates and has been identified in all schistosome species (McManus, 1999). The 95kDa antigen was highly recognised by all antibody isotypes of sera from both susceptible and resistant individuals. However, sera form resistant individuals showed more frequent recognition of the 95kDa band by IgG1 and IgM and less frequent recognition by total IgG and IgG3 compared to the susceptible individuals, after exposure to reinfection. In a small community infected with *S. japonicum* there was a higher recognition of paramyosin by IgA antibodies in older putatively resistant individuals (Hernandez *et al.*, 1999). Futhermore, oral immunisation of mice with recombinant *S. japonicum* paramyosin elicited significantly increased levels of antigen specific IgG1, IgG2a, IgG2b and IgM (Kalinna and McManus, 1997). *In vitro* stimulation of peripheral blood lymphocytes from infected individuals with Sm97-paramyosin revealed association between antigen specific IgE and resistance and between total IgG, IgG1, IgG2, IgG3, IgG4 and IgA and susceptibility to reinfection (Al Sherbiny *et al.*, 2003).

The antigen band of 52-54kDa was represented as a strong band on the SDS-PAGE. After exposure to reinfection, sera from resistant individuals showed more frequent recognition of the 52-54kDa band by IgG3 and IgM antibodies compared to the susceptible individuals. This antigen band may correspond to the PR52-filamin. In mice vaccinated with DNA of the partial coding sequence of the structural protein, filamin lead to 50% protection. The protection offered was associated with elevated levels of total IgG and IgG2a antibodies (Cook *et al.*, 2004).

The 37kDa band in the present study which may correspond to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was more frequently recognised by IgM and IgG4 antibody isotypes of sera from susceptible individuals than resistant individuals. After

exposure to reinfection, while sera from the susceptible individuals showed more frequent recognition of the 37kDa antigen by total IgG, IgG1 and IgG3 isotypes compared to the recognition prior to treatment, sera from the resistant individuals showed less recognition. However when the percentage recognition of both resistant and susceptible individuals was directly compared, there was a tendency of more frequent recognition of this antigen by IgG1 and IgG3 isotypes for sera taken from the resistant individuals. These results are correspondent to those of El-Ridi and colleagues. They investigated the recognition patterns of the recombinant G3PDH as well as different peptides from this molecule. They used sera from adolescent individuals resistant or susceptible to reinfection with schistosome infection. The antigen was recognised more frequently by IgG4 and IgM antibody isotypes of sera from susceptible individuals while there was more recognition by IgG, IgG1 and IgG3 isotypes of sera from resistant individuals (El Ridi *et al.*, 2001a; El Ridi *et al.*, 2001b). Similarly, IgG1 antibodies from sera of resistant individuals recognised one of the B cell determinants of *S. mansoni* G3PDH (Sm 37-5) more than sera from susceptible individuals (Argiro *et al.*, 2000).

There were some other antigens that were recognised with patterns that might be expected of antigens that were contributing to protection. For instance, the antigen of 16-18kDa was more frequently recognised by the IgG3 isotype of sera from resistant individuals after exposure to reinfection compared to the susceptible group. Sera from the susceptible group showed more frequent recognition of the same antigen by IgG4 and IgM antibodies after exposure to reinfection. This antigen may correspond to PN18-cyclophillin. Al Sherbiny and others investigated the *in vitro* response of peripheral blood lymphocytes from resistant and susceptible individuals after stimulation with PN18-cyclophillin. Resistance to reinfection was correlated with increased levels of antigen specific IgG1antibody isotye (Al Sherbiny *et al.*, 2003).

Furthermore, sera from susceptible individuals showed more recognition of antigen of 45-47kDa by total IgG, IgG1, IgG3 and IgM antibodies after exposure to reinfection compared to sera from resistant individuals. This antigen may correspond to PL45-phosphoglycerate kinase. After *in vitro* stimulation of peripheral blood lymphocytes from resistant and susceptible individuals by PL45-phosphoglycerate kinase revealed correlation of IgE with resistance and IgG1 with susceptibility to reinfection (Al Sherbiny *et al.*, 2003).

It should also be mentioned that there is a consensus that IgE antibody is one of the most important antibody involved in the resistant to schistosome infection in humans and it has been correlated with resistance to human reinfection with schistosome infection in many immunoepidemiological studies including the study from which the sera of the present study were obtained (Hagan *et al.*, 1991). The procedure applied for investigating the recognition patterns of *S. haematobium* SWAP by IgM and different IgG isotypes were used for the IgE antibody. Despite the use of higher concentrations of serum samples and secondary antibodies, no recognition by IgE antibody was achieved in the present study. The recognition patterns by IgA antibody were not investigated in the present study essentially because of the limited quantities of the serum samples.

In conclusion the work presented in this chapter has demonstrated that:

1. The treatment with PZQ can cause alteration of the immune responses from responses characteristic of susceptible individuals to protective responses.

2. Most of the identified vaccine candidates were recognised by sera from putatively resistant individuals by immune responses characteristic of protection.

3. The 28-30kDa antigen (ShGST) was recognised more frequently by IgG3 of sera from resistant individuals and by IgM, IgG2 and IgG4 in susceptible individuals.

4. The 23kDa antigen was recognised more frequently by IgG3 of sera from resistant individuals.

5. The 95kDa (paramyosin) was recognised more frequently by IgM and IgG1 of sera from resistant individuals.

6. The 84kDa (Calpain) was recognised more frequently by IgM, IgG1 and IgG3 of sera from resistant individuals.

Chapter Five

General Discussion

Human schistosomiasis is one of the most important parasitic diseases and it constitutes a major health problem in many areas in the world especially in sub-Saharan Africa. Although several measures have been used to control the infection, chemotherapy is still the cornerstone for the most successful control programs around the world. Praziquantel is now the drug of choice for treatment of all schistosome species infecting humans and has been used in mass chemotherapy scale in control programmes in many areas (Magnussen, 2003; Hagan *et al.*, 2004). The exact mode of action of PZQ is not fully understood. However, the most important effects of PZQ, the tegumental damage and muscular contraction, have been attributed to its effects on calcium influx and redistribution. Other effects including its effects on the metabolic pathways of the parasite have been proposed (Cioli *et al.*, 1995).

However, because of the limited efficacy of praziquantel on the immature stages of schistosomes (Sabah *et al.*, 1986) there is a high probability of missing some of the infected population thus ensuring the continued transmission of the disease. Although repeated doses of praziquantel and combination chemotherapy regimens can help reduce this problem, this appears to be impractical for control programs in the endemic areas. Furthermore, the appearance of schistosome strains with decreased susceptibility to the effects of praziquantel (Ismail *et al.*, 1996) has emphasised the necessity for investigation and discovery of new drugs for treatment of schistosomiasis (Cioli, 2000).

In the last few years, Mirazid®, an extract of myrrh, has been developed and licensed for use for the treatment of human schistosomiasis. Since then, there has been a lot of debate in the scientific communities about the real potential for its efficacy against schistosome infections. In the present study, the effects of MZD against *S. mansoni* were investigated both *in vitro* and *in vivo*, compared to the standard drug PZQ. The effects of both drugs on the hosts' immune responses to schistosome infection were also

investigated. Finally, the effects of *in vitro* exposure to both drugs on the protein expression of the parasite were investigated.

The *in vitro* effects of both drugs on the viability of schistosomula of *S. mansoni* were investigated using the toluidine blue dye exclusion test and the Hoechst test. The principle of these tests is that intact parasite membrane is impermeable to these dyes and hence only damaged or dead parasites will be stained (Modha *et al.*, 1997). The effects on the fluidity of the outer membrane of the parasite, which is important for many biochemical processes, were also investigated using the fluorescent recovery after photobleaching technique. The effects of both drugs were confirmed by virtue of scanning electron microscopy. As shown in chapter 2, the *in vitro* studies revealed that MZD, albeit at high doses, is more effective against the schistosomula of *S. mansoni* than PZQ. These results are not unique since other drugs such as artemisinin derivatives were reported to have more effects against immature stages of schistosome than PZQ (Xiao and Catto, 1989). The possibility of treating schistosomiasis with drugs that affect different life-cycle stages of the parasite has renewed the focus of attention on the potential benefits of combination chemotherapy. This combination of PZQ, against adult worms, and artemether, against immature stages, was tried in mice and hamsters infected with either *S. japonicum* or *S. mansoni* and having different developmental stages present in the animals at the time treatment was administered. The worm reductions obtained with the combination therapy were greater than those obtained with PZQ alone (Utzinger *et al.*, 2001a). If these effects can be repeated *in vivo* one would expect that MZD might be used effectively in combination with PZQ. One important aspect that was not investigated in the present study and needs further investigation is the effect of MZD on the immature stages. However that is because the present study was designed essentially to answer a simple question, that is "Is Mirazid® effective against *S. mansoni*?"

The *in vitro* studies revealed that PZQ was more effective than MZD against the adult worms of *S. mansoni*. A prolonged exposure to high concentrations of MZD was necessary to cause damage to the surface of the parasite. The most striking and earliest effect of MZD was the separation of worm pairs. The unpairing effect started within 15min after exposure. The exact mechanism of worm separation is not clear, however, SEM revealed surface damage and blebs on the interior surface of the gynaecophoric canal of male parasite that extraordinarily occurred after relatively short periods of exposure to lower concentrations of MZD. This preferential surface damage might give a clue to the cause of the observed worm separation. In murine schistosomiasis it was reported that MZD caused unpairing of worm pairs and shifting of separated worms to the livers (Badria *et al.*, 2001). However, the hepatic shift *per se* is not a criterion for drug efficacy since some drugs and even anaesthetics can cause a temporary hepatic shift of schistosomes. Once the effect of the drug is lost they resume their position in their normal habitat (Aboul-Atta *et al.*, 1989). Another interesting observation after exposure of adult worms to MZD was a temporary increase in worm motility. With time, the motility returned to normal and then diminished. Several antischistosome drugs have been shown to increase worm motility including oxamniquine and PZQ at sub-lethal doses (Chavasse *et al.*, 1979). In the case of hycanthone and oxamniquine, the increased worm motility was attributed to the blockage of the acetylcholine receptors, thus removing the inhibitory effects of acetylcholine (Hillman and Senft, 1975).

As one would expect, PZQ caused very early contractions of the parasite musculature followed later by surface damage with the characteristic surface blebbings. TEM revealed that while PZQ caused disruption of the parasite tegument, musculature and parenchyma, MZD apparently caused physical damage only to the surface of the

parasite. Both drugs caused a significant reduction in the fluidity of the parasites' surface membrane.

The present study attempted to shed light on the mode(s) of action of MZD. Its effect on calcium distribution was investigated using the fluo3 dye. Both MZD and PZQ caused a significant increase in the levels of intra-parasite calcium. Taking these results together with the parasite surface damage caused by both drugs, it is possible to speculate that both MZD and PZQ share some of their modes of action. However, the extent of damage caused by MZD might point to a possibility of a more localised action of this drug. In this context, Triton X-100 was shown to cause surface damage of adult schistosome and to increase calcium accumulation inside the parasites (Depenbusch *et al.*, 1983).

The *in vivo* effects of MZD and PZQ were investigated using the murine model. Although PZQ was very effective in causing worm reductions, and reductions of liver and tissue egg burden, MZD did not show any of these effects. The apparent discrepancy between the *in vitro* and the *in vivo* results of the present experiments are not fully understood. The pharmacokinetics of MZD may be one explanation for this discrepancy. It is possible that MZD is poorly absorbed, rapidly metabolised or cleared from the circulation. In any case, the level of MZD in the blood may not be enough to exert the actions that were obtained *in vitro*. In order to get these effects one would predict that very large doses are needed, doses that proved to be toxic to mice (Botros *et al.*, 2004). However, earlier studies in mice reported the efficacy of MZD in reducing worm and egg burdens (Badria *et al.*, 2001). Another possibility is that one or more of the many compounds that are constituents of MZD might be responsible for the anti-schistosome effects while others might have no effects and may even have deleterious effects on the hosts. If these active components could be identified one would predict that efficacy of MZD can be greatly increased. Preliminary attempts to fractionate MZD

by column chromatography and using different concentrations of dichloromethane in hexane, revealed 10 fractions. The potential anti-schistosome effects of these fractions was assessed and revealed that only two of these fractions had anti-schistosomal effects (data not shown). Further fractionation experiments are in progress.

The results of the *in vivo* studies do not agree with the reported efficacy of MZD in human studies. In human studies, MZD was used to treat both *S. haematobium* and *S. mansoni* infected Egyptians with cure rates of more than 90% being reported (Sheir *et al.*, 2001; El Baz *et al.*, 2003). This might be attributed to differences in the drug metabolic pathways in humans and mice. However, other human studies failed to demonstrate any significant anti-schistosomal effects of MZD (Fenwick *et al.*, 2003).

Sheir and colleagues and El Baz and colleagues reported that MZD caused an apparent improvement of the symptoms of infected individuals. The possibility that MZD instead of affecting the worm burden might have anti-pathology effects was investigated in the present study. Although there are some differences between the immunopathology of schistosome in humans and mice, there is enough similarity to allow murine infections to be used to investigate the likely effects on pathology in humans (Cheever *et al.*, 2002). There was no significant difference in the diameter of liver granulomas or their eosinophil contents in MZD-treated, PZQ-treated and un-treated mice. However, in MZD treated mice, the slight decrease in the diameter of liver granulomas and fibrosis was accompanied with a preferential decrease of Th2 type immune response cytokines. Spleen cells and LNs from mice treated with MZD produced lower amounts of IL-10, IL-4 and IL-13 in response to stimulation with either SEA or SWAP compared to the un-treated mice. This might indicate an anti-inflammatory effect of MZD or one or more of its components (Kimura *et al.*, 2001).

The *in vitro* effects of both MZD and PZQ on the soluble proteome of adult worms of *S. mansoni* were investigated. The use of the powerful DIGE technique allowed the comparison between two samples in one gel with the advantage of them being run under identical conditions. Although several protein spots were expressed differently in both drug treated and control parasites, only three proteins were identified using the MALDI ToF and electrospray tandem mass spectrometry. This is partly because the genome sequence of *S. mansoni* has yet to be completed (LoVerde *et al.*, 2004). Some proteins such as actin had reduced expression in MZD-treated worms. Interestingly other proteins were more highly expressed in drug treated parasites. For instance, paramyosin, which is a leading vaccine candidate, showed a 4-fold increased expression in worms exposed to MZD compared to control parasites. In worms exposed to PZQ, there was a 2-fold increase in the expression of fructose 1,6 bisphosphate aldolase compared to control parasites. Whatever the mechanism of this differential expression of such proteins in drug-exposed worms, the increased expression of some proteins might shed some light on the changes of the immune responses that occur after the use of some anti-schistosome drugs.

In addition to its chemotherapeutic effects against schistosomes, PZQ has been proposed to have the capacity to enhance immune protection against reinfection. As reported by others, treatment of infected children can change their immune responses from 'putatively susceptible to reinfection' to immune responses similar to adults that is 'putatively resistant to reinfection' (Mutapi *et al.*, 1998; Mduluza *et al.*, 2001). The hypothesis behind the immune priming effect of PZQ is that, by damaging or killing worms, PZQ might expose previously concealed parasite antigens or simply initiate the release of a considerable amount of parasite antigens that normally would have been released only after a long duration of infection in conditions of normal exposure (Harnett and Kusel, 1986; Woolhouse and Hagan, 1999).

In the present study, the differences in the recognition patterns of SWAP *S. haematobium* prior to PZQ treatment, after treatment and after exposure to reinfection were addressed with an emphasis on the differences between resistant and susceptible individuals and the impact of chemotherapy on the humoral immune responses against different parasite antigens. Not surprisingly, most of the identified vaccine candidates were highly recognised by sera from both resistant and susceptible individuals. However, resistant individuals more frequently recognised some of these antigens by antibody isotypes that have been characterised as those that might be associated with protective immune responses. For instance, an antigen band of 28-30kDa (Sh28GST) was recognised more frequently by the IgG3 antibody isotype and less frequently by IgM, IgG2 and IgG4 antibody isotypes of sera from resistant compared to susceptible individuals.

After treatment with PZQ, many antigens including some of the potential vaccine candidate antigens were recognised by antibody isotypes that favoured protective immune responses compared to their recognition prior to treatment. However, sometimes such immune responses were not maintained when the individuals were exposed to reinfection and thus rendered susceptible to reinfection. It seems reasonable that repeated treatments might be necessary to maintain such protective immune responses (Mduluza *et al.*, 2001).

Conclusion

Although MZD was effective against the schistosomula and adult stages of *S. mansoni*, *in vitro*, the results of the *in vivo* experiments in the murine model do not support the use of MZD for the treatment of schistosomiasis. Further independent investigations of its efficacy in human studies may need to be done. Until this happens, all measures must be taken to ensure that MZD is withheld in order that it is not substituted for the more

effective drug, PZQ, in communities where both drugs are available. Otherwise, there will be a risk of an increase in the prevalence of human schistosomiasis which is now partly under control, in many parts of the world by virtue of the efficacy of PZQ. Another major factor against the use of MZD on a community basis is its cost (double that of PZQ) and the inconvenience of the way of its administration.

The present study supports the efficacy of PZQ for treatment of schistosome infections. It also supports the hypothesis that PZQ, albeit after repeated cycles of treatment, can help the development of protective immune responses by infected individuals. This is perhaps a consequence of an increase in antigen production and expression by the parasites.

In conclusion the work presented in the present thesis has demonstrated that

1. Both MZD and PZQ showed *in vitro* anti-schistosome activities against *S. mansoni*.

2. MZD was more effective against schistosomula than PZQ *in vitro* but its efficacy was less against adult worms.

3. Lengthy exposures to high concentrations were necessary for the effects of MZD to be evident.

4. Although there were similarities in the effects of MZD and PZQ after *in vitro* exposure, the extent of the damage was distinct for each drug.

5. Both MZD and PZQ caused an increase in the intra-parasite calcium levels.

6. MZD had no anti-schistosome activity in infected mice. On the other hand PZQ caused a significant reduction in worm burden and liver and gut egg loads.

7. There were no significant differences in the diameter or cell content of liver granulomas from treated mice.

8. The treatment with PZQ can cause alteration of the immune responses from responses characteristic of susceptible individuals to protective responses.

9. Most of the identified vaccine candidates were recognised by sera from putatively resistant individuals by immune responses characteristic of protection.

10. The 28-30kDa antigen (ShGST) was recognised more frequently by IgG3 of sera from resistant individuals and by IgM, IgG2 and IgG4 in susceptible individuals.

11. The 23kDa antigen was recognised more frequently by IgG3 of sera from resistant individuals.

12. The 95kDa (paramyosin) was recognised more frequently by IgM and IgG1 of sera from resistant individuals.

13. The 84kDa (Calpain) was recognised more frequently by IgM, IgG1 and IgG3 of sera from resistant individuals.

14. *In vitro* exposure of adult worms to PZQ caused increased expression of fructose 1,6 bisphosphate aldolase supporting the immunising hypothesis of some anti-schistosome drugs.

15. *In vitro* exposure of adult worms to MZD caused increased expression of paramyosin and decreased expression of actin.

Suggestions for future work

1. The efficacy of MZD against early schistosome infection should be investigated. From the results of the present *in vitro* experiments, one would expect that if MZD has any anti-schistosome effects it is more likely to be against the immature stages of the parasite.

2. The true potential of MZD in treatment of human schistosomiasis should be independently investigated.

3. Based on the results of the preliminary studies on the effects of MZD on the immunopathology of schistosome infection, the possibility that MZD can affect the immunopathology of human disease needs further investigation.

4. Fractionation of MZD and identification of the potential anti-schistosome components is strongly recommended. One would expect if there is a potential anti-schistosome effect of this mixture of compounds, identification of the active ingredient would greatly improve this efficacy.

5. Further studies on the effects of PZQ and other drugs on the soluble schistosome proteome would greatly increase our understanding of the mode of actions of these drugs. This is possible with the use of the rapidly developing 2-D PAGE and mass spectrometry techniques. It is expected that these techniques would be applicable with more efficiency in the near future especially when the genome of schistosome is completed.

6. Making use of the 2-D PAGE and mass spectrometry techniques it is now possible to study the differences in the recognition patterns of parasite antigens in resistant and susceptible individuals. The impact of chemotherapy on the

recognition patterns of such parasite antigens can be also investigated. The antigens associated with protective responses in resistant individuals can then be identified and investigated as potential vaccine candidates.

Appendix

Reagents used in western blotting

Phospate buffered saline (pH 7.2)

	Amount
$Na_2HPO_4.12H_2O$	60.0g
$Na_2HPO_4.2H_2O$	13.6g
NaCl	8.5g
De-ionised and distilled water	Completed to 1 litre

Coomassie Blue Stain

	Amount
Methanol	250.0ml
Acetic acid	100.0ml
Glycerol	10.0ml
Coomassie Blue	1.0g
De-ionised and distilled water	Completed to 1 litre

Coomassie Blue Destain

	Amount
Methanol	250.0ml
Acetic acid	100.0ml
Glycerol	10.0ml
De-ionised and distilled water	Completed to 1 litre

Tris Buffered Saline (TBS)

	Amount
NaCl	9g
Tris HCl	1.6g
pH	Adjusted to pH 7.6 with HCl
De-ionised and distilled water	Completed to 1 litre

Anode 1 solution

	Amount	Final concentration
Tris- base	36.3g	300mM
Methanol	200ml	20%
DdH_20	800ml	

The Tris- base was dissolved in 800ml of water and then the methanol was added to make a litre.

Anode 2 solution

	Amount	Final concentration
Tris- base	3.0g	25mM
Methanol	200ml	20%
DdH_20	800ml	

Cathode solution

A 40mM of 6-amino-caproic acid was dissolved in 1litre of anode 2 solution to obtain the cathode solution.

Substrate solution for development of the western blotting reaction

	Amount
4-chloro-1-naphthol	90mg
Methanol	20ml
TBS	100ml
H_2O_2 (immediately before development)	200µl

Reagents used in ELISA

Phosphate buffered saline

	Amount
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.21g
DdH_2O	Complete to 1 litre
HCl	Adjust the pH to 7.2-7.4

Wash buffer

To 1 litre of PBS, 0.5ml Tween X20 was added to reach a concentration of 0.05% Tween. PH must be 7.2-7.4.

Block buffer

	Amount
PBS	1 litre
BSA	10g
Sucrose	50g
NaN_3	0.5g

Reagent diluent

Ten grams of BSA were dissolved in 1 litre of PBS to reach a final concentration of 1% BSA. The solution was sterilised through a 0.2μm filter.

Reagents used in the 2D gel electrophoresis

DIGE lysis buffer

	Final concentration
Urea	7M
Thiourea	2M
CHAPS	4%
Tris base	25mM

Sample rehydration stock buffer

	Amount	Final concentration
Urea (FW 60.06)	12g	8M
CHAPS	0.5g	2% (w/v)
IPG Buffer	125µl	0.5% (v/v)
Bromophenol blue	50µl 1% solution	0.002%
Double distilled H_2O	Complete to 25ml	

The stock was aliquoted in 2.5ml aliquots and stored at –20°C. 7mg of DTT was added for each aliquot just prior to use.

Sample equilibration buffer

	Amount	Final concentration
Tris-HCl, pH 8.8	10.0ml	50mM
Urea (FW 60.06)	72.07g	6M
Glycerol (87% v/v)	69ml	30% (v/v)
SDS (FW 288.38)	4.0 g	2% (w/v)
Bromophenol blue	400µl of 1% solution	0.002% (w/v)
Double distilled H_2O	Complete to 200ml	

The stock solution was aliquoted in 10ml aliquots and stored at –20°C. Just prior to use 100mg of DTT was added to 10ml of the stock to prepare the first equilibration buffer. To prepare the second equilibration buffer, 250mg of iodacetamide was added to 10ml of the stock solution.

SDS gel electrophoresis buffer

	Amount	Final concentration
Tris-base (FW 121.1)	30.3 g	25 mM
Glycine (FW 75.07)	144.0 g	192 mM
SDS (FW 288.38)	10.0 g	0.1% (w/v)
Double distilled H_2O	Complete to 10 litre	

Store at room temperature

Preparation of the 12.5% PAGE

	Amount
Acrylamide/Bis 40% (w/v)	281.25ml
Tris (1.5M, pH 8.8)	225ml
10% (w/v) SDS	9.0ml
10% (w/v) Ammonium persulfate (APS)	9.0ml
10% (w/v) TEMED (immediately before use)	1.24ml
Double distilled H_2O	Complete to 900ml

Agarose sealing solution

	Amount	Final concentration
SDS Electrophoresis buffer	100 ml	
Agarose	0.5 g	0.5%
Bromophenol blue	200 µl	0.002% (w/v)

Add all ingredients into a 500 ml Erlenmeyer flask. Swirl to disperse. Heat in a microwave oven on low or a heating stirrer until the agarose is completely dissolved. Do not allow the solution to boil over. Dispense 2 ml aliquots into screw-cap tubes and store at room temperature.

Reference list

Abbas, M.K. and Cain, G.D. 1987. Actine and intermedate-sized filaments of the spines and cytoskeleton of *Schistosoma mansoni*. *Parasitol. Res.* **73**: 66-74.

Abdel-Hafez, S.K., Phillips, S.M. and Zodda, D.M. 1983. *Schistosoma mansoni*: detection and characterization of antigens and antigenemia by inhibition enzyme-linked immunosorbent assay (IELISA). *Exp. Parasitol.* **55**: 219-232.

Abebe, F., Gaarder, P.I., Petros, B. and Gundersen, S.G. 2001. Age- and sex-related differences in antibody responses against *Schistosoma mansoni* soluble egg antigen in a cohort of school children in Ethiopia. *APMIS*. **109**: 816-824.

Abo-Madyan, A.A., Morsy, T.A. and Motawea, S.M. 2004. Efficacy of myrrh in the treatment of schistosomiasis (haematobium and mansoni) in Ezbet El-Bakly, Tamyia Center, El-Fayoum Governorate, Egypt. *J. Egypt. Soc. Parasitol.* **34**: 423-446.

Aboul-Atta, A.M., Hassan, Z.A. and El Ahl, S.S. 1989. Oltipraz treatment in experimental *schistosomiasis mansoni*: III. Effect of high dose oral therapy on hepatic shift of worms and tissue bound ova pattern in infected mice. *J. Egypt. Soc. Parasitol.* **19**: 641-651.

Abu-Elyazeed, R.R., Mansour, N.S. and Campbell, J.R. 1998. The prevalence and intensity of *Schistosoma mansoni* infection among residents in two Egyptian villages, at two and twelve months post-treatment with 40 and 60mg praziquantel per kg body weight at these two villages respectively. *J. Egypt. Soc. Parasitol.* **28**: 9-16.

Al Sherbiny, M., Osman, A., Barakat, R., El Morshedy, H., Bergquist, R. and Olds, R. 2003. *In vitro* cellular and humoral responses to *Schistosoma mansoni* vaccine candidate antigens. *Acta Trop.* **88**: 117-130.

Al-Awadi, F., Fatania, H. and Shamte, U. 1991. The effect of a plants mixture extract on liver gluconeogenesis in streptozotocin induced diabetic rats. *Diabetes Res.* **18**: 163-168.

Al-Mathal, E.M. and Fouad, M.A. 2004. Myrrh (*Commiphora molmol*) in treatment of human and sheep dicrocoeliasis dendriticum in Saudi Arabia. *J. Egypt. Soc. Parasitol.* **34**: 713-720.

Allam, A.F., El-Sayad, M.H. and Khalil, S.S. 2001. Laboratory assesment of the molluscicidal activity of *Commiphora molmol* (Myrrh) on *Biomphalaria alexandrina*, *Bulinus truncatus* and *Lymnaea cailliaudi* . *J. Egypt. Soc. Parasitol.* **31**: 683-690.

Amanor, J.D., Bosompem, K.M., Arishima, T., Assoku, R.K. and Kojima, S. 1996. Characterisation of monoclonal antibodies reactivity with *Schistosoma haematobium*-soluble egg and infected human urinary antigens. *Hybridoma.* **15**: 219-224.

Andersson, M., Bergendorff, O., Shan, R., Zygmunt, P. and Sterner, O. 1997. Minor components with smooth muscle relaxing properties from scented myrrh (*Commiphora guidotti*). *Planta Med.* **63**: 251-254.

Andrews, P. 1981. A summary of the efficacy of praziquantel against schistosomes in animal experiments and notes on its mode of action. *Arzneimittelforschung.* **31**: 538-541.

Andrews, P. 1985. Praziquantel: mechanisms of anti-schistosomal activity. *Pharmacol. Ther.* **29**: 129-156.

Andrews, P., Thomas, H., Pohlke, R. and Seubert, J. 1983. Praziquantel. *Med. Res. Rev.* **3**: 147-200.

Argiro, L., Kohlstadt, S., Henri, S., Dessein, H., Matabiau, V.V., Paris, P., Bourgois, A. and Dessein, A.J. 2000. Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. *Vaccine*. **18**: 2039-2048.

Ashton, P.D., Curwen, R.S. and Wilson, R.A. 2001. Linking proteome and genome: how to identify parasite proteins. *Trends Parasitol.* **17**: 198-202.

Atlas, D., Volsky, D.J. and Levitzki, A. 1980. Lateral mobility of beta-receptors involved in adenylate cyclase activation. *Biochim. Biophys. Acta*. **597**: 64-69.

Auriault, C., Pancre, V., Wolowczuk, I., Asseman, C., Ferru, I. and Verwaerde, C. 1996. Cellular immune response and pathology in schistosomiasis. *Parasite*. **3**: 199-208.

Badria, F., Abou-Mohamed, G., El-Mowafy, A., Massoud, A. and Salama, O. 2001. Mirazid: a new schistosomicidal drug. *Pharmaceutical Biology*. **93**: 127-131.

Barral-Netto, M., Hofstetter, M., Cheever, A.W. and Ottesen, E.A. 1983. Specificity of antibody and cellular immune responses in human schistosomiasis. *Am. J. Trop. Med. Hyg.* **32**: 106-113.

Barsoum, I.S., Gamil, F.M., Al Khafif, M.A., Ramzy, R.M., El Alamy, M.A. and Colley, D.G. 1982. Immune responses and immunoregulation in relation to human schistosomiasis in Egypt. I. Effect of treatment on *in vitro* cellular responsiveness. *Am. J. Trop. Med. Hyg.* **31**: 1181-1187.

Basch, P. 1991. Schistosomes development, reproduction and host relations. Basch, P. New York: Oxford University Press. pp. 34-82.

Becker, B., Mehlorn, H., Andrews, P., Thomas, H. and Eckert, J. 1980. Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. *Z. Parasitenkd.* **63**: 113-128.

Berg, J.M., Tymoczko, J.L. and Stryer, L. 2002a. Glycolysis and gluconeogenesis. In Biochemistry. New York: W. H. Freeman and Company. pp. 425-464.

Berg, J.M., Tymoczko, J.L. and Stryer, L. 2002b. Exploring proteins. In Biochemistry. Berg, J. M, Tymoczko, J. L., and Stryer, L. New York: W.H. Freeman and company. pp. 77-116.

Berkelman, T. and Stenstedt, T. 1998. 2-D electophoresis using immobilised pH gradient: principles and methods Amersham Biosciences.

Bickle, Q.D. and Andrews, B.J. 1985. Resistance following drug attenuation (Ro 11-3128 or oxamniquine) of early *Schistosoma mansoni* infections in mice. *Parasitology.* **90**: 325-338.

Bickle, Q.D., Sacko, M. and Vignali, D.A. 1990. Induction of immunity against *Schistosoma mansoni* by drug (Ro11-3128)-terminated infection: analysis of surface antigen recognition. *Parasite Immunol.* **12**: 569-586.

Bjellqvist, B., EK, K., Righetti, P.G., Gianazza, E., Gory, A., Westermeier, R. and Postel, W. 1982. Isoelectric focusing in immobilized pH gradients, principle, methodology and some applications. *J. Biochem. Biophys. Meth.* **6**: 317-339.

Booth, M., Mwatha, J.K., Joseph, S., Jones, F.M., Kadzo, H., Ireri, E., Kazibwe, F., Kemijumbi, J., Kariuki, C., Kimani, G., Ouma, J.H., Kabatereine, N.B., Vennervald,

B.J. and Dunne, D.W. 2004. Periportal fibrosis in human *Schistosoma mansoni* infection is associated with low IL-10, low IFN-gamma, high TNF-alpha, or low RANTES, depending on age and gender. *J. Immunol.* **172**: 1295-1303.

Boros, D.L. 1989. Immunopathology of *Schistosoma mansoni* infection. *Clin. Microbiol. Rev.* **2**: 250-269.

Boros, D.L. and Warren, K.S. 1970. Delayed hypersensitivity-type granuloma formation and dermal reaction induced and elicited by a soluble factor isolated from *Schistosoma mansoni* eggs. *J. Exp. Med.* **132**: 488-507.

Boros, D.L. and Whitfield, J.R. 1998. Endogenous IL-10 regulates IFN-gamma and IL-5 cytokine production and the granulomatous response in Schistosomiasis mansoni-infected mice. *Immunology*. **94**: 481-487.

Borrmann, S., Szlezak, N., Faucher, J.F., Matsiegui, P.B., Neubauer, R., Binder, R.K., Lell, B. and Kremsner, P.G. 2001. Artesunate and praziquantel for the treatment of *Schistosoma haematobium* infections: a double-blind, randomized, placebo-controlled study. *J. Infect. Dis.* **184**: 1363-1366.

Botros, S., William, S., Ebeid, F., Cioli, D., Katz, N., Day, T.A. and Bennett, J.L. 2004. Lack of evidence for an antischistosomal activity of myrrh in experimental animals. *Am. J. Trop. Med. Hyg.* **71**: 206-210.

Brindley, P.J. and Sher, A. 1987. The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. *J. Immunol.* **139**: 215-220.

Brindley, P.J., Strand, M., Norden, A.P. and Sher, A. 1989. Role of host antibody in the chemotherapeutic action of praziquantel against *Schistosoma mansoni*: identification of target antigens. *Mol. Biochem. Parasitol.* **34**: 99-108.

Brunet, L.R., Finkelman, F.D., Cheever, A.W., Kopf, M.A. and Pearce, E.J. 1997. IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J. Immunol.* **159**: 777-785.

Buckley, S.A. and Evershed, R.P. 2001. Organic chemistry of embalming agents in Pharaonic and Graeco-Roman mummies. *Nature*. **413**: 783-784.

Butterworth, A.E., Bensted-Smith, R., Capron, A., Capron, M., Dalton, P.R., Dunne, D.W., Grzych, J.M., Kariuki, H.C., Khalife, J. and Koech, D. 1987. Immunity in human schistosomiasis mansoni: prevention by blocking antibodies of the expression of immunity in young children. *Parasitology*. **94**: 281-300.

Butterworth, A.E., Capron, M., Cordingley, J.S., Dalton, P.R., Dunne, D.W., Kariuki, H.C., Kimani, G., Koech, D., Mugambi, M., Ouma, J.H., Prentice, M.A., Richardson, B.A., Arap Siongok, T.K., Sturrock, R.F. and Taylor, D.W. 1985. Immunity after treatment of human schistosomiasis mansoni. II. Identification of resistant individuals, and analysis of their immune responses. *Trans. R. Soc. Trop. Med. Hyg.* **79**: 393-408.

Butterworth, A.E., Dalton, P.R., Dunne, D.W., Mugambi, M., Ouma, J.H., Richardson, B.A., Siongok, T.K. and Sturrock, R.F. 1984. Immunity after treatment of human schistosomiasis mansoni. I. Study design, pretreatment observations and the results of treatment. *Trans. R. Soc. Trop. Med. Hyg.* **78**: 108-123.

Butterworth, A.E., Dunne, D.W., Fulford, A.J., Ouma, J.H. and Sturrock, R.F. 1996. Immunity and morbidity in *Schistosoma mansoni* infection: quantitative aspects. *Am. J. Trop. Med. Hyg.* **55**: 109-115.

Caldas, I.R., Correa-Oliveira, R., Colosimo, E., Carvalho, O.S., Massara, C.L., Colley, D.G. and Gazzinelli, G. 2000. Susceptibility and resistance to *Schistosoma mansoni* reinfection: parallel cellular and isotypic immunologic assessment. *Am. J. Trop. Med. Hyg.* **62**: 57-64.

Camacho, M., Alsford, S., Jones, A. and Agnew, A. 1995. Nicotinic acetylcholine receptors on the surface of the blood fluke *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **71**: 127-134.

Campagne, G., Garba, A., Barkire, H., Vera, C., Sidiki, A. and Chippaux, J.P. 2001. Continued ultrasonic follow-up of children infected with *Schistosoma haematobium* after treatment with praziquantel. *Trop. Med. Int. Health.* **6**: 24-30.

Capron, A., Capron, M. and Riveau, G. 2002. Vaccine development against schistosomiasis from concepts to clinical trials. *Br. Med. Bull.* **62**: 139-148.

Capron, M. and Capron, A. 1994. Immunoglobulin E and effector cells in schistosomiasis. *Science.* **264**: 1876-1877.

Caulada-Benedetti, Z., Al-Zamel, F., Sher, A. and James, S. 1991. Comparison of Th1- and Th2-associated immune reactivities stimulated by single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercariae. *J. Immunol.* **146**: 1655-1660.

Caulfield, J.P., Chiang, C.P., Yacono, P.W., Smith, L.A. and Golan, D.E. 1991. Low density lipoproteins bound to *Schistosoma mansoni* do not alter the lipid lateral diffusion or shedding of lipids in the outer surface membrane. *J. Cell Sci.* **99**: 167-173.

Cetre, C., Pierrot, C., Maire, E., Capron, M., Capron, A. and Khalife, J. 2000. Interleukin-13 and IgE production in rat experimental schistosomiasis. *Eur. Cytokine Netw.* **11**: 241-249.

Chappell, L.H. 1974. Methionine uptake by larval and adult *Schistosoma mansoni*. *J. Parasitol.* **4**: 361-369.

Chavasse, C., Brown, M.C. and Bell, D.R. 1978. Short term effects of oxamniquine on the activity *in vitro* of *Schistosoma mansoni*. *Ann. Trop. Med. Parasitol.* **73**: 241-249.

Chavasse, C., Brown, M.C. and Bell, D.R. 1979. *Schistosoma mansoni*: activity responses *in vitro* to praziquantel. *Z. Parasitenkd.* **58**: 169-174.

Cheever, A.W., Finkelman, F.D., Caspar, P., Heiny, S., Macedonia, J.G. and Sher, A. 1992. Treatment with anti-IL-2 antibodies reduces hepatic pathology and eosinophilia in *Schistosoma mansoni*-infected mice while selectively inhibiting T cell IL-5 production. *J. Immunol.* **148**: 3244-3248.

Cheever, A.W., Lenzi, J.A., Lenzi, H.L. and Andrade, Z.A. 2002. Experimental models of *Schistosoma mansoni* infection. *Mem. Inst. Oswaldo Cruz.* **97**: 917-940.

Chiaramonte, M.G., Donaldson, D.D., Cheever, A.W. and Wynn, T.A. 1999. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J. Clin. Invest.* **104**: 777-785.

Cioli, D. 2000. Praziquantel: is there real resistance and are there alternatives? *Curr. Opin. Infect. Dis.* **13**: 659-663.

Cioli, D., Pica-Mattoccia, L. and Archer, S. 1995. Antischistosomal drugs: past, present ... and future? *Pharmacol. Ther.* **68**: 35-85.

Coles, G.C. 1984. Recent adavances in schistosome biochemistry. *Parasitology.* **89**: 603-637.

Colley, D.G. and Wikel, S.K. 1974. *Schistosoma mansoni*: Simplified method for the production of schistosomules. *Exp. Parasitol.* **35**: 44-51.

Cook, R.M., Carvalho-Queiroz, C., Wilding, G. and LoVerde, P.T. 2004. Nucleic acid vaccination with *Schistosoma mansoni* antioxidant enzyme cytosolic superoxide dismutase and the structural protein filamin confers protection against the adult worm stage. *Infect. Immun.* **72**: 6112-6124.

Curwen, R.S., Ashton, P.D., Johnston, D.A. and Wilson, R.A. 2004. The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages. *Mol. Biochem. Parasitol.* **138**: 57-66.

Czaja, M.J., Weiner, F.R., Flanders, K.C., Giambrone, M.A., Wind, R., Biempica, L. and Zern, M.A. 1989. *In vitro* and *in vivo* association of transforming growth factor-beta 1 with hepatic fibrosis. *J. Cell Biol.* **108**: 2477-2482.

Davis, A. 1993. Antischistosomal drugs and clinical practice. In Human Schistosomiasis. Jordan, P., Webbe, G., and Sturrock, R. F. Wallingford: CAB International. pp. 367-404.

Davis, A. and Bailey, D.R. 1969. Metrifonate in urinary schistosomiasis. *Bull. World Health Organ.* **41**: 209-224.

Day, T.A., Bennet, J.L. and Pax, R.A. 1992. Praziquantel: the enigmatic antiparasitic. *Parasitol. Today.* **8**: 342-344.

De Clercq, D., Vercruysse, J., Verle, P., Kongs, A. and Diop, M. 2000. What is the effect of combining artesunate and praziquantel in the treatment of *Schistosoma mansoni* infection? *Trop. Med. Int. Health.* **5**: 744-746.

Demeure, C.E., Rihet, P., Abel, L., Ouattara, M., Bourgois, A. and Dessein, A.J. 1993. Resistance to *Schistosoma mansoni* in humans: influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. *J. Infect. Dis.* **168**: 1000-1008.

Deng, J., Gold, D., LoVerde, P.T. and Fishelson, Z. 2003. Inhibition of the complement membrane attack complex by *Schistosoma mansoni* paramyosin. *Infect. Immun.* **71**: 6402-6410.

Depenbusch, J.W., Thompson, D.P., Pax, R.A. and Bennet, J.L. 1983. Tegumental disruption with Triton X-100 and its effects on longitudinal muscle function in male *Schistosoma mansoni*. *Parasitology.* **87**: 61-73.

Dessein, A., Butterworth, A.E., Vadas, M.A. and David, J.R. 1983. Maturation *in vivo* of *Schistosoma mansoni* schistosomula after culture *in vitro* with granulocytes and antibody. *Infect. Immun.* **39**: 225-232.

Dessein, A.J., Begley, M., Demeure, C., Caillol, D., Fucri, J., Dos Reis, M.G., Andrade, Z.A., Prata, A. and Bina, J.C. 1988. Human resistance to *Schistosoma mansoni* is

associated with IgG reactivity to a 37-kDa larval surface antigen. *J. Immunol.* **140:** 2727-2736.

Doenhoff, M., Chiodini, P.L. and Hamilton, J.V. 2004. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? *Trends Parasitol.* **20: 35-39.**

Doenhoff, M.J., Hassounah, O., Murare, H., Bain, J. and Lucas, S. 1986. The schistosome egg granuloma: immunopathology in the cause of host protection or parasite survival? *Trans. R. Soc. Trop. Med. Hyg.* **80: 503-514.**

Doenhoff, M.J., Kusel, J.R., Coles, G.C. and Cioli, D. 2002. Resistance of *Schistosoma mansoni* to praziquantel: is there a problem? *Trans. R. Soc. Trop. Med. Hyg.* **96: 465-469.**

Dolara, P., Corte, B., Ghelardini, C., Pugliese, A.M., Cerbai, E., Menichetti, S. and Lo Nostro, A. 2000. Local anaesthetic, antibacterial and antifungal properties of sesquiterpenes from myrrh. *Planta Med.* **66: 356-358.**

Dunne, D.W., Butterworth, A.E., Fulford, A.J., Kariuki, H.C., Langley, J.G., Ouma, J.H., Capron, A., Pierce, R.J. and Sturrock, R.F. 1992. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur. J. Immunol.* **22: 1483-1494.**

Eberl, M., Al Sherbiny, M., Hagan, P., Ljubojevic, S., Thomas, A.W. and Wilson, R.A. 2002. A novel and sensitive method to monitor helminth infections by faecal sampling. *Acta Trop.* **83: 183-187.**

El Ashry, E.S.H., Rashed, N., Salama, O.M. and Saleh, A. 2003. Components, therapeutic value and uses of myrrh. *Pharmazie.* **58: 163-168.**

El Baz, M.A., Morsy, T.A., El Bandary, M.M. and Motawea, S.M. 2003. Clinical and parasitological studies on the efficacy of Mirazid in treatment of schistosomiasis haematobium in Tatoon, Etsa Center, El Fayoum Governorate. *J. Egypt. Soc. Parasitol.* **33**: 761-776.

El Dabaa, E., Mei, H., el Sayed, A., Karim, A.M., Eldesoky, H.M., Fahim, F.A., LoVerde, P.T. and Saber, M.A. 1998. Cloning and characterisation of *Schistosoma mansoni* fructose-1,6-bisphosphate aldolase isoenzyme. *J. Parasitol.* **84**: 954-960.

El Garem, A.A. 1998. Schistosomiasis. *Digestion.* **59**: 589-605.

El Ridi, R., Mahrous, A., Afifi, A., Montash, M., Velek, J. and Jezek, J. 2001a. Human and murine humoral immune recognition of multiple peptides from *Schistosoma mansoni* glyceraldehyde 3-P dehydrogenase is associated with resistance to Schistosomiasis. *Scand. J. Immunol.* **54**: 477-485.

El Ridi, R., Shoemaker, C.B., Farouk, F., El Sherif, N.H. and Afifi, A. 2001b. Human T- and B-cell responses to *Schistosoma mansoni* recombinant glyceraldehyde 3-phosphate dehydrogenase correlate with resistance to reinfection with *S. mansoni* or *Schistosoma haematobium* after chemotherapy. *Infect. Immun.* **69**: 237-244.

El-sayed, N.M.A., Bartholomeu, D., Ivens, A., Johnston, D.A. and Loverde, P. 2004. Advances in schistosome genomics. *Trends Parasitol.* **20**: 154-157.

Eshete, F. and Bennett, J.L. 1991. The schistosomicidal compound Ro 15-5458 causes a reduction in the RNA content of *Schistosome mansoni*. *Mol. Biochem. Parasitol.* **45**: 1-8.

Evengard, B., Hammarstrom, L., Smith, C.I. and Linder, E. 1990. Early antibody responses in human schistosomiasis. *Clin. Exp. Immunol.* **80**: 69-76.

Falcao, P.L., Malaquias, L.C., Martins-Filho, O.A., Silveira, A.M., Passos, V.M., Prata, A., Gazzinelli, G., Coffman, R.L. and Correa-Oliveira, R. 1998. Human Schistosomiasis mansoni: IL-10 modulates the *in vitro* granuloma formation. *Parasite Immunol.* **20**: 447-454.

Fallon, P.G. 2000. Immunopathology of schistosomiasis: a cautionary tale of mice and men. *Immunol. Today.* **21**: 29-35.

Fallon, P.G., Cooper, R.O., Robert, A.J. and Doenhoff, M.J. 1992. Immune-dependent chemotherapy of schistosomiasis. *Parasitology.* **105**: S41-S48.

Fallon, P.G., Fookes, R.E. and Wharton, G.A. 1996. Temporal differences in praziquantel- and oxamniquine-induced tegumental damage to adult Schistosoma mansoni: implications for drug-antibody synergy. *Parasitology.* **112**: 47-58.

Fallon, P.G., Richardson, E.J., McKenzie, G.J. and McKenzie, A.N. 2000a. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J. Immunol.* **164**: 2585-2591.

Fallon, P.G., Richardson, E.J., Smith, P. and Dunne, D.W. 2000b. Elevated type 1, diminished type 2 cytokines and impaired antibody response are associated with hepatotoxicity and mortalities during Schistosoma mansoni infection of CD4-depleted mice. *Eur. J. Immunol.* **30**: 470-480.

Fenwick, A., Savioli, L., Engels, D., Robert, B.N. and Todd, M.H. 2003. Drugs for the control of parasitic diseases: current status and development in schistosomiasis. *Trends Parasitol.* **19**: 509-515.

Fitzsimmons, C.M., Joseph, S., Jones, F.M., Reimert, C.M., Hoffmann, K.F., Kazibwe, F., Kimani, G., Mwatha, J.K., Ouma, J.H., Tukahebwa, E.M., Kariuki, H.C., Vennervald, B.J., Kabatereine, N.B. and Dunne, D.W. 2004. Chemotherapy for schistosomiasis in Ugandan fishermen: treatment can cause a rapid increase in interleukin-5 levels in plasma but decreased levels of eosinophilia and worm-specific immunoglobulin E. *Infect. Immun.* **72**: 4023-4030.

Flores-Villanueva, P.O., Zheng, X.X., Strom, T.B. and Stadecker, M.J. 1996. Recombinant IL-10 and IL-10/Fc treatment down-regulate egg antigen-specific delayed hypersensitivity reactions and egg granuloma formation in schistosomiasis. *J. Immunol.* **156**: 3315-3320.

Foley, M., Kusel, J.R. and Garland, P.B. 1988. Changes in the organization of the surface membrane upon transformation of cercariae to schistosomula of the helminth parasite *Schistosoma mansoni*. *Parasitology*. **96**: 85-97.

Foley, M., MacGregor, A.N., Kusel, J.R., Garland, P.B., Downie, T. and Moore, I. 1986. The lateral diffusion of lipid probes in the surface membrane of *Schistosoma mansoni*. *J. Cell Biol.* **103**: 807-818.

Ford, M.J., Bickle, Q.D. and Taylor, M.G. 1987. Immunity to *Schistosoma mansoni* in congenitally athymic, irradiated and mast cell-depleted rats. *Parasitology*. **94**: 313-326.

Foster, R. 1987. A review of clinical experience with oxamniquine. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 55-59.

Fulford, A.J., Butterworth, A.E., Sturrock, R.F. and Ouma, J.H. 1992. On the use of age-intensity data to detect immunity to parasitic infections, with special reference to *Schistosoma mansoni* in Kenya. *Parasitology*. **105**: 219-227.

Ghandour, A.M., Banaja, A.A. and Shalaby, I.M. 1990. Effects of praziquantel and oxamniquine on a Saudi Arabian strain of *Schistosoma mansoni* in mice. *J. Helminthol.* **64**: 62-64.

Gold, D. 1997. Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. *Parasitol. Res.* **83**: 163-169.

Gonnert, R. and Andrews, P. 1977. Praziquantel, a new broad-spectrum antischistosomal agent. *Z. Parasitenkd.* **52**: 129-150.

Greenfield, E.A., Nguyen, K.A. and Kuchroo, V.K. 1998. CD28/B7 co-stimulation: a reveiw. *Crit Rev. Immunol.* **18**: 389-418.

Grogan, J.L., Kremsner, P.G., van dam, G.J., Metzger, W., Mordmuller, B., Deelder, A.M. and Yazdanbakhsh, M. 1996. Antischistosome IgG4 and IgE responses are affected differentially by chemotherapy in children versus adults. *J. Infect. Dis.* **173**: 1242-1247.

Gryseels, B. 1991. The epidemiology of schistosomiasis in Brundi and its consequences for control. *Trans. R. Soc. Trop. Med. Hyg.* **85**: 626-633.

Gryseels, B. and Nkulikyinka, L. 1989. Two-year follow-up of *Schistosoma mansoni* infection and morbidity after treatment with different regimens of oxamniquine and praziquantel. *Trans. R. Soc. Trop. Med. Hyg.* **83**: 219-228.

Gryseels, B., Stelma, F.F., Talla, I., van dam, G.J., Polman, K., Sow, S., Diaw, M., Sturrock, R.F., Doehring-Schwerdfeger, E., Kardorff, R., Decam, C., Niang, M. and Deelder, A.M. 1994. Epidemiology, immunology and chemotherapy of *Schistosoma mansoni* infections in a recently exposed community in Senegal. *Trop. Geogr. Med.* **46**: 209-219.

Grzych, J.M., Grezel, D., Xu, C.B., Neyrinck, J.L., Capron, M., Ouma, J.H., Butterworth, A.E. and Capron, A. 1989. IgA antibodies to a protective antigen in human schistosomiasis mansoni. *J. Immunol.* **150**: 527-535.

Guirguis, F.R. 2003. Efficacy of praziquantel and Ro 15-5458, a 9-acridanone-hydrazone derivative, against *Schistosoma haematobium*. *Arzneimittelforschung.* **53**: 57-61.

Hagan, P. 1987. The human immune response to schistosome infection. In The biology of schistosomes from genes to latrines. Rollinson, D. and Simpson, A. J. San Diego: Academic Press Limited. pp. 295-320.

Hagan, P., Appleton, C.C., Coles, G.C., Kusel, J.R. and Tchuem-Tchuente, L.A. 2004. Schistosomiasis control: keep taking the tablets. *Trends Parasitol.* **20**: 92-97.

Hagan, P., Blumenthal, U.J., Chaudri, M., Greenwood, B.M., Hayes, R.J., Hodgson, I., Kelly, C., Knight, M., Simpson, A.J., Smithers, S.R. and Wilkins, H.A. 1987. Resistance to reinfection with *Schistosoma haematobium* in Gambian children: analysis of their immune responses. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 938-946.

Hagan, P., Blumenthal, U.J., Dunne, D., Simpson, A.J. and Wilkins, H.A. 1991. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature.* **349**: 243-245.

Hagan, P. and Sharaf, O. 2003. Schistosomiasis vaccines. *Expert. Opin. Biol. Ther.* **3**: 1271-1278.

Hagan, P., Wilkins, H.A., Blumenthal, U.J., Hayes, R.J. and Greenwood, B.M. 1985. Eosinophilia and resistance to *Schistosoma haematobium* in man. *Parasite Immunol.* **7**: 625-632.

Hamilton, J.V., Klinkert, M. and Doenhoff, M.J. 1998. Diagnosis of schistosomiasis: antibody detection, with notes on parasitological and antigen detection methods. *Parasitology*. **117**: S41-S57.

Haridy, F.M., El Garhy, M.F. and Morsy, T.A. 2003. Efficacy of Mirazid (*Commiphora molmol*) against fascioliasis in Egyptian sheep. *J. Egypt. Soc. Parasitol.* **33**: 917-924.

Harnett, W. and Kusel, J.R. 1986. Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. *Parasitology*. **93**: 401-405.

Harrison, R.A. and Doenhoff, M.J. 1983. Retarded development of *Schistosoma mansoni* in immunosuppressed mice. *Parasitology*. **86**: 429-438.

Hassan, M., El Motaiem, M., Afify, H., Abaza, B., El Shafei, M. and Massoud, A.M. 2003. *In vitro* effect of Mirazid on *Schistosoma mansoni* worms. *J. Egypt. Soc. Parasitol.* **33**: 999-1008.

Hayat, M.A. 1989. Principles and Techniques of Electron Microscopy London: The Mac Millan Press Ltd.

Henri, S., Chevillard, C., Mergani, A., Paris, P., Gaudart, J., Camilla, C., Desscin, H., Montero, F., Elwali, N.E., Saeed, O.K., Magzoub, M. and Desscin, A.J. 2002. Cytokine

regulation of periportal fibrosis in humans infected with *Schistosoma mansoni*: IFN-gamma is associated with protection against fibrosis and TNF-alpha with aggravation of disease. *J. Immunol.* **169**: 929-936.

Hernandez, H.J., Rutitzky, L.I., Lebens, M., Holmgren, J. and Stadecker, M.J. 2002. Diminished immunopathology in *Schistosoma mansoni* infection following intranasal administration of cholera toxin B-immunodominant peptide conjugate correlates with enhanced transforming growth factor-beta production by CD4 T cells. *Parasite Immunol.* **24**: 423-427.

Hernandez, M.G., Hafalla, J.C., Acosta, L.P., Aligui, F.F., Aligui, G.D., Ramirez, B.L., Dunne, D.W. and Santiago, M.L. 1999. Paramyosin is a major target of the human IgA response against *Schistosoma japonicum*. *Parasite Immunol.* **21**: 641-647.

Hesse, M., Piccirillo, C.A., Belkaid, Y., Prufer, J., Mentink-Kane, M., Leusink, M., Cheever, A.W., Shevach, E.M. and Wynn, T.A. 2004. The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J. Immunol.* **172**: 3157-3166.

Hillman, G.R., Gibler, W.B. and Anderson, J.B. 1977. Comparative effects of hycanthone in *Schistosoma mansoni* and *Schistosoma japonicum*. *Am. J. Trop. Med. Hyg.* **26**: 238-242.

Hillman, G.R. and Senft, A.W. 1975. Anticholinergic properties of the antischistosomal drug hycanthone. *Am. J. Trop. Med. Hyg.* **24**: 827-834.

Hillman, G.R., Senft, A.W. and Gibler, W.B. 1978. The mode of action of hycanthone revisited. *J. Parasitol.* **64**: 754-756.

Hockley, D.J. 1973. Ultrastructure of the tegument of *Schistosoma*. *Adv. Parasitol.* **11**: 233-305.

Hockley, D.J. and McLaren, D.J. 1973. *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. *Int. J. Parasitol.* **3**: 13-25.

Hota-Mitchell, S., Siddiqui, A.A., Dekaban, G.A., Smith, J., Tognon, C. and Podesta, R.B. 1997. Protection against *Schistosoma mansoni* infection with a recombinant baculovirus-expressed subunit of calpain. *Vaccine*. **15**: 1631-1640.

Hudson, L. and Hay, F. 1989. Immunological techniques in clinical medicine. In Practical Immunology. Hudson, L. and Hay, F. Oxford London: Blackwell Scientific Publications. pp. 443-445.

Ismail, M., Metwally, A., Farghaly, A., Bruce, J., Tao, L.F. and Bennett, J.L. 1996. Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. *Am. J. Trop. Med. Hyg.* **55**: 214-218.

Jacob, W., Bogers, J., Deelder, A., Wery, M. and Van Marck, E. 1997. Adult *Schistosoma mansoni* worms positively modulate soluble egg antigen-induced inflammatory hepatic granuloma formation *in vivo*. Stereological analysis and immunophenotyping of extracellular matrix protein, adhesion molecules, and cytokines. *Am. J. Pathol.* **150**: 2033-2045.

Jankovic, D., Cheever, A.W., Kullberg, M.C., Wynn, T.A., Yap, G., Caspar, P., Lewis, F.A., Clynes, R., Ravetch, J.V. and Sher, A. 1998. CD4+ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J. Exp. Med.* **187**: 619-629.

Jankovic, D., Kullberg, M.C., Dombrowicz, D., Barbieri, S., Caspar, P., Wynn, T.A., Paul, W.E., Cheever, A.W., Kinet, J.P. and Sher, A. 1997. Fc epsilonRI-deficient mice infected with *Schistosoma mansoni* mount normal Th2-type responses while displaying enhanced liver pathology. *J. Immunol.* **159**: 1868-1875.

Jankovic, D., Kullberg, M.C., Noben-Trauth, N., Caspar, P., Ward, J.M., Cheever, A.W., Paul, W.E. and Sher, A. 1999a. Schistosome-infected IL-4 receptor knockout (KO) mice, in contrast to IL-4 KO mice, fail to develop granulomatous pathology while maintaining the same lymphokine expression profile. *J. Immunol.* **163**: 337-342.

Jankovic, D., Wynn, T.A., Kullberg, M.C., Hieny, S., Caspar, P., James, S., Cheever, A.W. and Sher, A. 1999b. Optimal vaccination against *Schistosoma mansoni* requires the induction of both B cell- and IFN-gamma-dependent effector mechanisms. *J. Immunol.* **162**: 345-351.

Joseph, A.L. and Boros, D.L. 1993. Tumour necrosis factor plays a role in *Schistosoma mansoni* egg-induced granulomatous inflammation. *J. Immunol.* **151**: 5461-5471.

Joseph, S., Jones, F.M., Walter, K., Fulford, A.J., Kimani, G., Mwatha, J.K., Kamau, T., Kariuki, H.C., Kazibwe, F., Tukahebwa, E., Kabatereine, N.B., Ouma, J.H., Vennervald, B.J. and Dunne, D.W. 2004. Increases in human T helper 2 cytokine responses to *Schistosoma mansoni* worm and worm-tegument antigens are induced by treatment with praziquantel. *J. Infect. Dis.* **190**: 835-842.

Junqueira, L.C., Bignolas, G. and Brentani, R.R. 1979. Picrosirius staining plus polarisation microscopy, a specific method for collagen detection in tissue sections. *Histochemistry*. **84**: 426-431.

Kabatereine, N.B., Kemijumbi, J., Ouma, J.H., Sturrock, R.F., Butterworth, A.E., Madsen, H., Ornbjerg, N., Dunne, D.W. and Vennervald, B.J. 2003. Efficacy and side effects of praziquantel treatment in a highly endemic *Schistosoma mansoni* focus at Lake Albert, Uganda. *Trans. R. Soc. Trop. Med. Hyg.* **97**: 599-603.

Kalinna, B.H. and McManus, D.P. 1997. A vaccine against the Asian schistosome, *Schistosoma japonicum*: an update on paramyosin as a target of protective immunity. *Int. J. Parasitol.* **27**: 1213-1219.

Kao, J.P.Y., Harootunian, A.T. and Tsien, R.Y. 1989. Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. *J. Biol. Chem.* **264**: 8179-8184.

Kaplan, M.H., Whitfield, J.R., Boros, D.L. and Grusby, M.J. 1998. Th2 cells are required for the *Schistosoma mansoni* egg-induced granulomatous response. *J. Immunol.* **160**: 1850-1856.

Kato, K. and Miura, M. 1954. On the comparison of stool examination methods. *Jpn. J. Parasitol.* **3**: 35-42.

Katz, N., Chaves, A. and Pellegrino, J. 1972. A simple device for quantitative stool thick-smear technique in *Schistosomiasis mansoni*. *Rev. Inst. Med. Trop. Sao Paulo.* **14**: 397-400.

Kaviratne, M., Hesse, M., Leusink, M., Cheever, A.W., Davies, S.J., McKerrow, J.H., Wakefield, L.M., Letterio, J.J. and Wynn, T.A. 2004. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J. Immunol.* **173**: 4020-4029.

Kelly, C., Hagan, P., Knight, M., Hodgson, J., Simpson, A.J., Hackett, F., Wilkins, H.A. and Smithers, S.R. 1987. Surface and species-specific antigens of *Schistosoma haematobium*. *Parasitology*. **95**: 253-266.

Khalil, H.M., Abd el Baki, M.H., Abd el Mawla, M.M., Maklad, K.M., Sharaf, S.A. and Saad, A.E. 1999. Interleukin-4, immunoglobulin E and immunoglobulin A and resistance to re-infection with *Schistosoma haematobium* before and after chemotherapy. *J. Egypt. Soc. Parasitol.* **29**: 395-408.

Khalil, H.M., Bebars, M.A., El Badawy, N.M., Abdallah, H.M., Khalil, N.M., el Zayyat, E.A. and Din Mohamed, M.S. 1995. Effect of praziquantel treatment on hepatic pathology of *Schistosoma mansoni* experimentally infected mice. *J. Egypt. Soc. Parasitol.* **25**: 269-277.

Kimura, I., YOSHIKAWA, M., Kobayashi, S., Sugihara, Y., Suzuki, M., Oominami, H., Murakami, T., Matsuda, H. and Doiphode, W. 2001. New triterpenes, myrrhanol A and myrrhanone A, from guggul-gum resins, and their potent anti-inflammatory effect on adjuvant-induced air-pouch granuloma of mice. *Bioorg. Med. Chem.* **11**: 985-989.

King, C.H., El Ibiary, S., El Nawawi, M., Sawyer, J., Griffin, A., El Hawey, A. and Mahmoud, A.A. 1989. Intensity of *Schistosoma mansoni* infection in a human population is inversely correlated with antibody response to SmW68, a protective parasite antigen. *J. Infect. Dis.* **160**: 686-691.

King, C.H., Keating, C.E., Muruka, J.F., Ouma, J.H., Houser, H., Siongok, T.K. and Mahmoud, A.A. 1988. Urinary tract morbidity in schistosomiasis haematobia: associations with age and intensity of infection in an endemic area of Coast Province, Kenya. *Am. J. Trop. Med. Hyg.* **39**: 361-368.

King, C.L. 2001. Initiation and regulation of disease in schistosomiasis. In Schistosomiasis. Mahmoud, A. F. London: Imperial College Press. pp. 213-264.

King, C.L., Xianli, J., Malhotra, I., Liu, S., Mahmoud, A.A. and Oettgen, H.C. 1997. Mice with a targeted deletion of the IgE gene have increased worm burdens and reduced granulomatous inflammation following primary infection with *Schistosoma mansoni*. *J. Immunol.* **158**: 294-300.

Kohn, A.B., Anderson, P.A., Roberts-Misterly, J.M. and Greenberg, R.M. 2001a. Schistosome calcium channel beta subunits. Unusual modulatory effects and potential role in the action of the antischistosomal drug praziquantel. *J. Biol. Chem.* **276**: 36873-36876.

Kohn, A.B., Lea, J., Roberts-Misterly, J.M., Anderson, P.A. and Greenberg, R.M. 2001b. Structure of three high voltage-activated calcium channel alpha1 subunits from *Schistosoma mansoni*. *Parasitology*. **123**: 489-497.

Kohn, A.B., Roberts-Misterly, J.M., Anderson, P.A., Khan, N. and Greenberg, R.M. 2003. Specific sites in the Beta Interaction Domain of a schistosome Ca2+ channel beta subunit are key to its role in sensitivity to the anti-schistosomal drug praziquantel. *Parasitology*. **127**: 349-356.

Kojima, S. 2004. Overview: from the horse experimentation by Prof. Akira Fujinami to paramyosin. *Parasitol. Int.* **53**: 151-162.

Kusel, J. and Hagan, P. 1999. Praziquantel--its use, cost and possible development of resistance. *Parasitol. Today*. **15**: 352-354.

La Flamme, A.C., Patton, E.A., Bauman, B. and Pearce, E.J. 2001. IL-4 plays a crucial role in regulating oxidative damage in the liver during schistosomiasis. *J. Immunol.* **166**: 1903-1911.

Lescano, S.Z., Chieffi, P.P., Canhassi, R.R., Boulos, M. and Amato, N., V 2004. Antischistosomal activity of artemether in experimental Schistosomiasis mansoni. *Rev. Saude Publica.* **38**: 71-75.

Levy, M.G. and Read, C.P. 1975. Purine and pyrimidine transport in *Schistosoma mansoni. J. Parasitol.* **61**: 257-266.

Li, Y., Sleigh, A.C., Ross, A.G., Li, Y., Zhang, X., Williams, G.M., Yu, X., Tanner, M. and McManus, D.P. 2001. Human susceptibility to *Schistosoma japonicum* in China correlates with antibody isotypes to native antigens. *Trans. R. Soc. Trop. Med. Hyg.* **95**: 441-448.

Li, Y., Yu, D.B., Li, Y.S., Luo, X.S., Liang, Y.S., Bartley, P.B. and McManus, D.P. 2002. Antibody isotype responses to *Schistosoma japonicum* antigens in subjects from a schistosomiasis area with repeated praziquantel chemotherapy compared with a new endemic zone in Hunan Province, P.R. China. *Trans. R. Soc. Trop. Med. Hyg.* **96**: 210-215.

Lima, S.F., Vieira, L.Q., Harder, A. and Kusel, J.R. 1994. Effects of culture and praziquantel on membrane fluidity parameters of adult *Schistosoma mansoni. Parasitology.* **109**: 57-64.

Llewellyn, B.D. 1970. An improved sirius red method for amyloid. *J. Med. Technol.* **27**: 308-309.

LoVerde, P.T., Hirai, H., Merrick, J.M., Lee, N.H. and El Sayed, N. 2004. *Schistosoma mansoni* genome project: an update. *Parasitol. Int.* 53: 183-192.

Lukacs, N.W. and Boros, D.L. 1992. Utilization of fractionated soluble egg antigens reveals selectively modulated granulomatous and lymphokine responses during murine schistosomiasis mansoni. *Infect. Immun.* 60: 3209-3216.

MacGregor, A.N. and Kusel, J.R. 1989. Isolation and characterisation of a surface membrane glycoprotein from adult *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 34: 237-244.

Magnussen, P. 2003. Treatment and re-treatment strategies for schistosomiasis control in different epidemiological settings: a review of 10 years' experiences. *Acta Trop.* 86: 243-254.

Mann, M., Hendrickson, R.C. and Pandey, A. 2001. Analysis of proteins and proteomes by mass spectrometry. *Ann. Rev. Biochem.* 70: 437-473.

Marguerite, M., Gallissot, M.C., Diagne, M., Moreau, C., Diakkhate, M.M., Roberts, M., Remoue, F., Thiam, A., Decam, C., Rogeric, F., Cottrez, F., Neyrinck, J.L., Butterworth, A.E., Sturrock, R.F., Piau, J.P., Daff, B., Niang, M., Wolowczuk, I., Riveau, G., Auriault, C. and Capron, A. 1999. Cellular immune responses of a Senegalese community recently exposed to *Schistosoma mansoni*: correlations of infection level with age and inflammatory cytokine production by soluble egg antigen-specific cells. *Trop. Med. Int. Health.* 4: 530-543.

Massoud, A., El Sisi, S., Salama, O. and Massoud, A. 2001a. Preliminary study of therapeutic efficacy of a new fasciolicidal drug derived from *Commiphora molmol* (myrrh). *Am. J. Trop. Med. Hyg.* 65: 96-99.

Massoud, A., El-Ebiary, F.H. and Abd El Salam, N.F. 2004. Effect of myrrh extract on the liver of normal and bilharzially infected mice: an ultrastructural study. *J. Egypt. Soc. Parasitol.* **34**: 1-21.

Massoud, A.M. and Habib, F.S. 2003. The effects of myrrh (*Commiphora molmol*) on the infected snails of *Schistosoma* Sp. and their egg masses: effect on shedding of cercariae and on the snail fecundity. *J. Egypt. Soc. Parasitol.* **33**: 585-596.

Massoud, A.M., Labib, I.M. and Rady, M. 2001b. Biochemical changes of *Cules pipiens* larvae treated with oil and oleo-resin extracts of Myrrh *Commiphora molmol. J. Egypt. Soc. Parasitol.* **31**: 517-529.

Mathew, R.C. and Boros, D.L. 1986. Anti-L3T4 antibody treatment suppresses hepatic granuloma formation and abrogates antigen-induced interleukin-2 production in *Schistosoma mansoni* infection. *Infect. Immun.* **54**: 820-826.

Mathew, R.C., Ragheb, S. and Boros, D.L. 1990. Recombinant IL-2 therapy reverses diminished granulomatous responsiveness in anti-L3T4-treated, *Schistosoma mansoni*-infected mice. *J. Immunol.* **144**: 4356-4361.

Matsumoto, Y., Perry, G., Levine, R.J., Blanton, R., Mahmoud, A.A. and Aikawa, M. 1988. Paramyosin and actin in schistosomal tegument. *Nature*. **333**: 76-78.

McLaren, D.J. 1980. *Schistosoma mansoni*: the parasite surface in relation to host immunity Research Studies Press.

McLaren, D.J., Hockley, D.J., Goldring, O.L. and Hammond, B.J. 1978. A freeze fracture study of the developing tegumental outer membrane of *Schistosoma mansoni*. *Parasitology*. **76**: 327-348.

McManus, D.P. 1999. The search for a vaccine against schistosomiasis--a difficult path but an achievable goal. *Immunol. Rev.* **171**: 149-161.

Mduluza, T., Ndhlovu, P.D., Madziwa, T.M., Midzi, N., Zinyama, R., Turner, C.M., Chandiwana, S.K., Nyazema, N. and Hagan, P. 2001. The impact of repeated treatment with praziquantel of schistosomiasis in children under six years of age living in an endemic area for *Schistosoma haematobium* infection. *Mem. Inst. Oswaldo Cruz.* **96**: 157-164.

Mehlhorn, H., Becker, B., Andrews, P., Thomas, H. and Frenkel, J.K. 1981. *In vivo* and *in vitro* experiments on the effects of praziquantel on *Schistosoma mansoni*. A light and electron microscopic study. *Arzneimittelforschung.* **31**: 544-554.

Mehlhorn, H., Frenkel, J.K., Andrews, P. and Thomas, H. 1982. Light and electron microscopic studies on *Schistosoma mansoni* Granulomas of mouse livers following treatment with praziquantel. *Tropenmed. Parasitol.* **33**: 229-239.

Mentink-Kane, M.M., Cheever, A.W., Thompson, R.W., Hari, D.M., Kabatereine, N.B., Vennervald, B.J., Ouma, J.H., Mwatha, J.K., Jones, F.M., Donaldson, D.D., Grusby, M.J., Dunne, D.W. and Wynn, T.A. 2004. IL-13 receptor alpha 2 down-modulates granulomatous inflammation and prolongs host survival in schistosomiasis. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 586-590.

Modha, J., Redman, S., Lima, S., Kennedy, M.W. and Kusel, J. 1997. Studies of the properties, lipophilic proteins and metabolism of parasites by the use of fluorescent and "Caged" compounds. In Analytical Parasitology. Rogan, M. T. Springer-Verlag. pp. 269-303.

Mola, P.W., Farah, I.O., Kariuki, T.M., Nyindo, M., Blanton, R.E. and King, C.L. 1999. Cytokine control of the granulomatous response in *Schistosoma mansoni*-infected baboons: role of exposure and treatment. *Infect. Immun.* **67**: 6565-6571.

Mott, K.E. and Dixon, H. 1982. Collaborative study on antigens for immunodiagnosis of schistosomiasis. *Bull. World Health Organ.* **60**: 729-753.

Mutapi, F., Hagan, P., Woolhouse, M.E., Mduluza, T. and Ndhlovu, P.D. 2003. Chemotherapy-induced, age-related changes in antischistosome antibody responses. *Parasite Immunol.* **25**: 87-97.

Mutapi, F., Ndhlovu, P.D., Hagan, P., Spicer, J.T., Mduluza, T., Turner, C.M., Chandiwana, S.K. and Woolhouse, M.E. 1998. Chemotherapy accelerates the development of acquired immune responses to *Schistosoma haematobium* infection. *J. Infect. Dis.* **178**: 289-293.

N'Goran, E.K., Utzinger, J., Gnaka, H.N., Yapi, A., N'Guessan, N.A., Kigbafori, S.D., Lengeler, C., Chollet, J., Shuhua, X. and Tanner, M. 2003. Randomized, double-blind, placebo-controlled trial of oral artemether for the prevention of patent *Schistosoma haematobium* infections. *Am. J. Trop. Med. Hyg.* **68**: 24-32.

Ndhlovu, P., Cadman, H., Vennervald, B.J., Christensen, N.O., Chidimu, M. and Chandiwana, S.K. 1996. Age-related antibody profiles in *Schistosoma haematobium* infections in a rural community in Zimbabwe. *Parasite Immunol.* **18**: 181-191.

Neva, F.A. and Brown, H.W. 1994. Blood flukes of human beings. In Basic clinical parasitology. Prentice-Hall International Inc. pp. 245-262.

Norden, A.P. and Strand, M. 1984. *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*: identification of genus- and species-specific antigenic egg glycoproteins. *Exp. Parasitol.* **58**: 333-344.

Nordgren, I., Holmstedt, B., Bengtsson, E. and Finkel, Y. 1980. Plasma levels of metrifonate and dichlorvos during treatment of schistosomiasis with bilarcil. *Am. J. Trop. Med. Hyg.* **29**: 426-430.

Nyindo, M., Kariuki, T.M., Mola, P.W., Farah, I.O., Elson, L., Blanton, R.E. and King, C.L. 1999. Role of adult worm antigen-specific immunoglobulin E in acquired immunity to *Schistosoma mansoni* infection in baboons. *Infect. Immun.* **67**: 636-642.

O'Boyle, C., Lambe, R. and Darragh, A. 1985. Central effects in man of the novel schistosomicidal benzodiazepine meclonazepam. *Eur. J. Clin. Pharmacol.* **29**: 105-108.

O'Farrell, P.H. 1975. High resolution two-dimensional polyacrylamide of proteins. *J. Biol. Chem.* **250**: 4007-4021.

Otubanjo, O.A. 1981. *Schistosoma mansoni*: Astiban-induced damage to tegument and the male reproductive system. *Exp. Parasitol.* **52**: 161-170.

Ouma, J.H., Fulford, A.J., Kariuki, H.C., Kimani, G., Sturrock, R.F., Muchemi, G., Butterworth, A.E. and Dunne, D.W. 1998. The development of schistosomiasis mansoni in an immunologically naive immigrant population in Masongaleni, Kenya. *Parasitology*. **117**: 123-132.

Ouma, J.H., Wijers, D.J. and Arap Siongok, T.K. 1985. The effect of repeated targeted mass treatment on the prevalence of schistosomiasis mansoni and the intensity of infection in Machakos, Kenya. *Ann. Trop. Med. Parasitol.* **79**: 431-438.

Parizade, M., Arnon, R., Lachmann, P.J. and Fishelson, Z. 1994. Functional and antigenic similarities between a 94-kD protein of *Schistosoma mansoni* (SCIP-1) and human CD59. *J. Exp. Med.* **179**: 1625-1636.

Patton, E.A., Brunet, L.R., La Flamme, A.C., Pedras-Vasconcelos, J., Kopf, M. and Pearce, E.J. 2001. Severe schistosomiasis in the absence of interleukin-4 (IL-4) is IL-12 independent. *Infect. Immun.* **69**: 589-592.

Pax, R.A., Bennet, J.L. and Fetterer, R. 1978. A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. *Naunyn Schmiedebergs Arch. Pharmacol.* **304**: 309-315.

Pearce, E.J., Caspar, P., Grzych, J.M., Lewis, F.A. and Sher, A. 1991. Downregulation of Th 1 cytokine production accompanies induction of Th 2 responses by a parasitic helminth *Schistosoma mansoni*. *J. Exp. Med.* **173**: 159-166.

Pearce, E.J., James, S.L., Hieny, S., Lanar, D.E. and Sher, A. 1988. Induction of protective immunity against *Schistosoma mansoni* by vaccination with schistosome paramyosin (Sm97), a nonsurface parasite antigen. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 5678-5682.

Pellegrino, J., Lima-Costa, F.F., Carlos, M.A. and Mello, R.T. 1977. Experimental chemotherapy of schistosomiasis mansoni. XIII. Activity of praziquantel, an isoquinoline-pyrazino derivative, on mice, hamsters and Cebus monkeys. *Z. Parasitenkd.* **52**: 151-168.

Pica-Mattoccia, L. and Cioli, D. 2004. Sex- and stage-related sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. *Int. J. Parasitol.* **34**: 527-533.

Pontes, L.A., Dias-Neto, E. and Rabello, A. 2002. Detection by polymerase chain reaction of *Schistosoma mansoni* DNA in human serum and feces. *Am. J. Trop. Med. Hyg.* **66**: 157-162.

Popiel, I. and Erasmus, D.A. 1984. *Schistosoma mansoni*: ultrastructure of adults from mice treated with oxamniquine. *Exp. Parasitol.* **58**: 254-262.

Qadir, K., Metwali, A., Blum, A.M., Li, J., Elliott, D.E. and Weinstock, J.V. 2001. TGF-beta and IL-10 regulation of IFN-gamma produced in Th2-type schistosome granulomas requires IL-12. *Am. J. Physiol Gastrointest. Liver Physiol.* **281**: G940-G946.

Race, G.J., Martin, J.H., Moore, D.V. and Larsh, J.E. 1971. Scanning and transmission electron microscopy of *Schistosoma mansoni* eggs, cercariae and adults. *Am. J. Trop. Med. Hyg.* **20**: 914-924.

Remoue, F., Rogerie, F., Gallissot, M.C., Guyatt, H.L., Neyrinck, J.L., Diakkhate, M.M., Niang, M., Butterworth, A.E., Auriault, C., Capron, A. and Riveau, G. 2000. Sex-dependent neutralizing humoral response to *Schistosoma mansoni* 28GST antigen in infected human populations. *J. Infect. Dis.* **181**: 1855-1859.

Ribeiro-Paes, J.T. and Rodrigues, V. 1997. Sex determination and female reproductive development in the genus *Schistosoma*: a review. *Rev. Inst. Med. Trop. Sao Paulo.* **39**: 337-344.

Rihet, P., Demeure, C.E., Bourgois, A., Prata, A. and Dessein, A.J. 1991. Evidence for an association between human resistance to *Schistosoma mansoni* and high anti-larval IgE levels. *Eur. J. Immunol.* **21**: 2679-2686.

Rimon, G., Hansky, E., Braun, S. and Levitzky, A. 1978. Mode of coupling between hormone receptors and adenylate cyclase elucidated by modulation of membrane fluidity. *Nature*. **276**: 394-396.

Riveau, G., Poulain-Godefroy, O.P., Dupre, L., Remoue, F., Mielcarek, N., Locht, C. and Capron, A. 1998. Glutathione S-transferases of 28kDa as major vaccine candidates against schistosomiasis. *Mem. Inst. Oswaldo Cruz*. **93**: 87-94.

Roberts, S.M., Wilson, R.A., Ouma, J.H., Kariuki, H.C., Koech, D., Arap Siongok, T.K., Sturrock, R.F. and Butterworth, A.E. 1987. Immunity after treatment of human schistosomiasis mansoni quantitative and qualitative antibody responses to tegumental membrane antigens prepared from adult worms. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 786-793.

Ruenwongsa, P., Hutadilok, N. and Yuthavong, Y. 1983. Stimulation of Ca^{2+} uptake in the human liver fluke *Opithorchis viverrini* by praziquantel. *Life Sci.* **32**: 2529-2534.

Sabah, A.A., Fletcher, C., Webbe, G. and Doenhoff, M.J. 1985. Schistosoma mansoni: reduced efficacy of chemotherapy in infected T-cell-deprived mice. *Exp. Parasitol.* **60**: 348-354.

Sabah, A.A., Fletcher, C., Webbe, G. and Doenhoff, M.J. 1986. *Schistosoma mansoni*: chemotherapy of infections of different ages. *Exp. Parasitol.* **61**: 294-303.

Sadler, C.H., Rutitzky, L.I., Stadecker, M.J. and Wilson, R.A. 2003. IL-10 is crucial for the transition from acute to chronic disease state during infection of mice with *Schistosoma mansoni*. *Eur. J. Immunol.* **33**: 880-888.

Sato, H., Kusel, J.R. and Thornhill, J. 2004. Excretion of fluorescent substrates of mammalian multidrug resistance-associated protein (MRP) in the *Schistosoma mansoni* excretory system. *Parasitology*. **128**: 43-52.

Satti, M.Z., Lind, P., Vennervald, B.J., Sulaiman, S.M., Daffalla, A.A. and Ghalib, H.W. 1996. Specific immunoglobulin measurements related to exposure and resistance to *Schistosoma mansoni* infection in Sudanese canal cleaners. *Clin. Exp. Immunol.* **106**: 45-54.

Savioli, L., Renganathan, E., Montresor, A., Davis, A. and Behbehani, K. 1997. Control of schistosomiasis-a global picture. *Parasitol. Today*. **13**: 444-448.

Senft, A.W. and Hillman, G.R. 1973. Effect of hycanthone, niridazole, and antimony tartarate on schistosome motility. *Am. J. Trop. Med. Hyg.* **22**: 734-742.

Shaheen, H., Shalaby, K., Farid, Z., Campbell, J. and Kamal, K. 1996. Parasite-specific isotype and subclass antibody profiles during acute prepatent human schistosomiasis. *Exp. Parasitol.* **82**: 222-224.

Shaw, M.K. 1990. *Schistosoma mansoni*: stage-dependent damage after *in vivo* treatment with praziquantel. *Parasitology*. **100**: 65-72.

Shaw, M.K. and Erasmus, D.A. 1983. *Schistosoma mansoni*: the effects of a subcurative dose of praziquantel on the ultrastructure of worms *in vivo*. *Z. Parasitenkd.* **69**: 73-90.

Shaw, M.K. and Erasmus, D.A. 1987. *Schistosoma mansoni*: structural damage and tegumental repair after *in vivo* treatment with praziquantel. *Parasitology*. **94**: 243-254.

Sheir, Z., Nasr, A.A., Massoud, A., Salama, O., Badra, G.A., El Shennawy, H., Hassan, N. and Hammad, S.M. 2001. A safe, effective, herbal antischistosomal therapy derived from myrrh. *Am. J. Trop. Med. Hyg.* **65**: 700-704.

Sher, A., Jankovic, D., Cheever, A. and Wynn, T. 1996. An IL-12-based vaccine approach for preventing immunopathology in schistosomiasis. *Ann. N. Y. Acad. Sci.* **795**: 202-207.

Sheterline, P. and Sparrow, J. 1994. Actin. London: Academic Press.

Shuhua, X., Binggui, S., Utzinger, J., Chollet, J. and Tanner, M. 2002. Ultrastructural changes in adult *Schistosoma mansoni* caused by artemether. *Mem. Inst. Oswaldo Cruz.* **97**: 717-724.

Siddiqui, A.A., Phillips, T., Charest, H., Podesta, R.B., Quinlin, M.L., Pinkston, J.R., Lloyd, J.D., Paz, M., Villalovos, R.M. and Pompa, J. 2003. Induction of protective immunity against *Schistosoma mansoni* via DNA priming and boosting with the large subunit of calpain (Sm-p80): adjuvant effects of granulocyte-macrophage colony-stimulating factor and interleukin-4. *Infect. Immun.* **71**: 3844-3851.

Skelly, P.J. 2004. Intravascular schistosomes and complement. *Trends Parasitol.* **20**: 370-374.

Skelly, P.J. and Shoemaker, C.B. 2001. The *Schistosoma mansoni* host-interactive tegument forms from vesicle eruptions of a cyton network. *Parasitology.* **122**: 67-73.

Smith, G. and Roberts, L.S. 1989. Order Strigeata. In Foundations of parasitology. St Louis: Times Mirror/Mosby. pp. 260-279.

Smithers, S.R. and Terry, R.J. 1965. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. *Parasitology*. **55**: 695-700.

Snellen, W.M. 1981. Therapeutic properties of metrifonate. *Acta Pharmacol. Toxicol. (Copenh)*. **49**: 114-117.

Staudt, U., Schmahl, G., Blaschke, G. and Mehlhorn, H. 1992. Light and scanning electron microscopy studies on the effects of the enantiomers of praziquantel and its main metabolite on *Schistosoma mansoni in vitro*. *Parasitol. Res.* **78**: 392-397.

Strickland, G.T. and Abdel-Wahab, M.F. 1991. Schistosomiasis. In Hunter's tropical medicine. Philadelphia: Saunders. pp. 781-809.

Strickland, G.T. and Ramirez, B.L. 2000. Schistosomiasis. In Hunter's tropical medicine and emerging infectious diseases. Strickland, G. T. Philadelphia: W.B. Saunders Company. pp. 804-832.

Sturrock, R.F. 2001. The schistosome and their intermediate hosts. In Schistosomiasis. Mahmoud, A. F. London: Imperial College Press. pp. 7-83.

Suliaman, S.M., Ali, H.M., Homeida, M.M. and Bennett, J.L. 1989. Efficacy of a new Hoffmann-La Roche compound (RO15-5458) against *Schistosoma mansoni* (Gezira strain, Sudan) in vervet monkeys (*Cercopithecus aethiops*). *Trop. Med. Parasitol.* **40**: 335-336.

Talaat, M. and Miller, F.D. 1998. A mass chemotherapy trial of praziquantel on *Schistosoma haematobium* endemicity in Upper Egypt. *Am. J. Trop. Med. Hyg.* **59**: 546-550.

Tanaka, M., Ohmae, H., Utsunomiya, H., Nara, T., Irie, Y. and Yasuraoka, K. 1989. A comparison of the antischistosomal effect of levo- and dextro-praziquantel on *Schistosoma japonicum* and *S. mansoni* in mice. *Am. J. Trop. Med. Hyg.* **41**: 198-203.

Utzinger, J., Chollet, J., You, J., Mei, J., Tanner, M. and Xiao, S. 2001a. Effect of combined treatment with praziquantel and artemether on *Schistosoma japonicum* and *Schistosoma mansoni* in experimentally infected animals. *Acta Trop.* **80**: 9-18.

Utzinger, J., Keiser, J., Shuhua, X., Tanner, M. and Singer, B.H. 2003. Combination chemotherapy of schistosomiasis in laboratory studies and clinical trials. *Antimicrob. Agents Chemother.* **47**: 1487-1495.

Utzinger, J., Xiao, S., Keiser, J., Chen, M., Zheng, J. and Tanner, M. 2001b. Current progress in the development and use of artemether for chemoprophylaxis of major human schistosome parasites. *Curr. Med. Chem.* **8**: 1841-1860.

Utzinger, J., Xiao, S., N'Goran, E.K., Bergquist, R. and Tanner, M. 2001c. The potential of artemether for the control of schistosomiasis. *Int. J. Parasitol.* **31**: 1549-1562.

van dam, G.J., Stelma, F.F., Gryseels, B., Falcao Ferreira, S.T., Talla, I., Niang, M., Rotmans, J.P. and Deelder, A.M. 1996. Antibody response patterns against *Schistosoma mansoni* in a recently exposed community in Senegal. *J. Infect. Dis.* **173**: 1232-1241.

van den Biggelaar, A.H., Borrmann, S., Kremsner, P. and Yazdanbakhsh, M. 2002. Immune responses induced by repeated treatment do not result in protective immunity to *Schistosoma haematobium*: interleukin (IL)-5 and IL-10 responses. *J. Infect. Dis.* **186**: 1474-1482.

Vendrame, C.M., Carvalho, M.D., Yamamoto, C.R., Nakhle, M.C., Carvalho, S.A. and Chieffi, P.P. 2001. Evaluation of anti-*Schistosoma mansoni* IgG antibodies in patients with chronic schistosomiasis mansoni before and after specific treatment. *Rev. Inst. Med. Trop. Sao Paulo*. **43**: 153-159.

Warren, K.S. 1972. The immunopathogenesis of schistosomiasis: a multidisciplinary approach. *Trans. R. Soc. Trop. Med. Hyg.* **66**: 417-434.

Webster, M., Fulford, A.J., Braun, G., Ouma, J.H., Kariuki, H.C., Havercroft, J.C., Gachuhi, K., Sturrock, R.F., Butterworth, A.E. and Dunne, D.W. 1996. Human immunoglobulin E responses to a recombinant 22.6-kilodalton antigen from *Schistosoma mansoni* adult worms are associated with low intensities of reinfection after treatment. *Infect. Immun.* **64**: 4042-4046.

Webster, M., Roberts, M., Fulford, A.J., Marguerite, M., Gallisot, M.C., Diagne, M., Niang, M., Riveau, G., Capron, A. and Dunne, D.W. 1998. Human IgE responses to rSm22.6 are associated with infection intensity rather than age per se, in a recently established focus of Schistomiasis mansoni. *Trop. Med. Int. Health.* **3**: 318-326.

Wegner, D.H. 1984. The profile of the trematodicidal compound praziquantel. *Arzneimittelforschung.* **34**: 1132-1136.

Wilkins, H.A., Blumenthal, U.J., Hagan, P., Hayes, R.J. and Tulloch, S. 1987. Resistance to reinfection after treatment of urinary schistosomiasis. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 29-35.

Wilkins, H.A., Goll, P.H., Marshall, T.F. and Moore, P.J. 1984. Dynamics of *Schistosoma haematobium* infection in a Gambian community. III. Acquisition and loss of infection. *Trans. R. Soc. Trop. Med. Hyg.* **78**: 227-232.

Wilkins, M.R., Sanchez, J.C., Gooley, A.A., Appel, R.D., Humphery-Smith, I., Hochstrasser, D.F. and Williams, K.L. 1996. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol. Genet. Eng. Rev.* **13**: 19-50.

Wilson, R.A. and Barnes, P.E. 1977. The formation and turnover of the membranocalyx on the tegument of *Schistosoma mansoni*. *Parasitology*. **74**: 61-71.

Wilson, R.A., Curwen, R.S., Braschi, S., Hall, S.L., Coulson, P.S. and Ashton, P.D. 2004. From genomes to vaccines *via* the proteome. *Mem. Inst. Oswaldo Cruz*. **99**: 45-50.

Woolhouse, M.E. and Hagan, P. 1999. Seeking the ghost of worms past. *Nat. Med.* **5**: 1225-1227.

Woolhouse, M.E.J., Taylor, P., Matanhire, D. and Chandiwana, S.K. 1991. Acquired immunity and epidemiology of *Schistosoma haematobium*. *Nature*. **351**: 757-759.

World Health Organisation 1990. Oxamniquine. In: WHO Model Prescribing Information. In Drugs Used in Parasitic Diseases. Geneva: World Health Organisation. pp. 118-119.

Wynn, T.A. 2003. IL-13 effector functions. *Annu. Rev. Immunol.* **21**: 425-456.

Wynn, T.A., Cheever, A.W., Jankovic, D., Poindexter, R.W., Caspar, P., Lewis, F.A. and Sher, A. 1995. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Nature*. **376**: 594-596.

Wynn, T.A., Eltoum, I., Oswald, I.P., Cheever, A.W. and Sher, A. 1994. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma*

mansoni and exogenous IL-12 both inhibits and prophylactically imunizes against egg pathology. *J. Exp. Med.* **179**: 1551-1561.

Wynn, T.A., Morawetz, R., Scharton-Kersten, T., Hieny, S., Morse, H.C., III, Kuhn, R., Muller, W., Cheever, A.W. and Sher, A. 1997. Analysis of granuloma formation in double cytokine-deficient mice reveals a central role for IL-10 in polarizing both T helper cell 1- and T helper cell 2-type cytokine responses *in vivo*. *J. Immunol.* **159**: 5014-5023.

Wynn, T.A., Reynolds, A., James, S., Cheever, A.W., Caspar, P., Hieny, S., Jankovic, D., Strand, M. and Sher, A. 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J. Immunol.* **157**: 4068-4078.

Xiao, S., Shen, B., Chollet, J., Utzinger, J. and Tanner, M. 2000. Tegumental changes in adult *Schistosoma mansoni* harbored in mice treated with artemether. *J. Parasitol.* **86**: 1125-1132.

Xiao, S., Shen, B., Chollet, J., Utzinger, J. and Tanner, M. 2001. Tegumental alterations in juvenile *Schistosoma haematobium* harboured in hamsters following artemether treatment. *Parasitol. Int.* **50**: 175-183.

Xiao, S., Tanner, M., N'Goran, E.K., Utzinger, J., Chollet, J., Bergquist, R., Chen, M. and Zheng, J. 2002. Recent investigations of artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and haematobia. *Acta Trop.* **82**: 175-181.

Xiao, S.H. and Catto, B.A. 1989. *In vitro* and *in vivo* studies of the effect of artemether on *Schistosoma mansoni*. *Antimicrob. Agents Chemother.* **33**: 1557-1562.

Xiao, S.H., Catto, B.A. and Webster, L.T. 1985. Effects of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. *J. Infect. Dis.* **151:** 1130-1137.

Yamashita, T. and Boros, D.L. 1992. IL-4 influences IL-2 production and granulomatous inflammation in murine schistosomiasis mansoni. *J. Immunol.* **149:** 3659-3664.

Yole, D.S., Reid, G.D. and Wilson, R.A. 1996. Protection against *Schistosoma mansoni* and associated immune responses induced in the vervet monkey *Cercopithecus aethiops* by the irradiated cercaria vaccine. *Am. J. Trop. Med. Hyg.* **54:** 265-270.

Zhu, H., Zeng, L., Zhu, D. and Yuan, Y. 2000. The role of TGF-beta 1 in mice hepatic fibrosis by Schistosomiasis japonica. *J. Tongji Med. Univ.* **20:** 320-1, 329.

Zouain, C.S., Gustavson, S., Oliveira, S.C., Azevedo, V., Alves, J.B. and Goes, A.M. 2000. The role of IL-10 and IgG1 in the protection and granulomatous response in *Schistosoma mansoni* P24-immunized mice. *Vaccine*. **19:** 1218-1224.