POSSIBLE MECHANISMS OF THROMBOCYTOPAENIA DURING *SCHISTOSOMA MANSONI* INFECTION IN THE MOUSE AND IN MAN

By

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BEd (Sc), MSc

A thesis submitted in fulfilment of the degree of Doctor of Philosophy in the University of Nairobi.

1997

DECLARATION

I, **Edith Jacobet Wambayi**, hereby declare that this is my original work and has not been presented for a degree in any other university.

10.7.97

EDITH JACOBET WAMBAYI

This thesis has been submitted for examination with our approval as University Supervisors.

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DEDICATION

This thesis is dedicated to my children, Tabitha, Nathan, Grace and Jennifer.

ACKNOWLEDGEMENTS

I am indebted to my supervisors, the late Prof. Edward Kasili (*May his soul rest in eternal peace*) and Prof. Jasper Mumo for their guidance and encouragement during the course of the project. I would also like to acknowledge the financial and technical support I received from Dr. Mike Doenhoff and his staff of the University of North Wales in Bangor, U.K., where I did most of my laboratory experiments. I would like to thank Dr. Davy Koech, the Director, Kenya medical Research Institute (KEMRI) and Dr. John Githure, the Director, Biomedical Sciences Research Centre (BSRC) for giving me the spirit to work with very limited or no funds at times. My thanks go to Mr. Kimani Gachuhi, the head of the schistosomiasis programme in our laboratory for enabling me to carry out studies on schistosomiasis patients from the Machakos project. I would like to thank the technical staff of Clinical Research Centre (CRC), and BSRC for their technical assistance throughout my work.

Lastly, I would like to thank Dr. John Ouma of the Division of Vector-borne Diseases,
Ministry of Health, for his encouragement and financial support for the thesis preparation.

This study received financial support for part of the work from the UNDP/WORLD BANK/WHO special Programme for Research and Training in Tropical Diseases (TDR), through Dr. Mike Doenhoff, U.K. The study also received financial support from the National Council for Science and Technology, Nairobi, Kenya.

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SUMMARY.

Schistosomiasis is a chronic debilitating and sometimes fatal disease which is estimated to affect at least 200 milliom people in the tropical and subtropical countries. There are an estimated 2 million infected people in Kenya. Schistosomes live within the blood vessels of man and other mammalian hosts where they interact with the cellular components of blood including platelets.

This study aimed at investigating the mechanisms that cause thrombocytopaenia during schistosome infections. Literature has been reviewed which indicates that such mechanisms may include immunological reactions. Thrombocytopaenia was induced in mice by injecting them with 25μl of anti-platelet serum and platelet levels determined. Platelet depletion was observed as soon as one hour after injection. It was found that a thrombocytopaenic state in the host enhanced worm survival. Mice were infected with *S. mansoni* and

platelet levels were monitored before and after treatment with praziquantel. The treated mice were cured of the infection and their platelet levels reverted to normal suggesting that loss of infection with *S. mansoni* was associated with recovery from a thrombocytopaenic state.

The study was also designed to investigate the level of thrombocytopaenia in humans infected with *S. mansoni*. Human subjects from Machakos District, Kenya, infected with *S. mansoni* participated. Platelet counts were done before treatment with 30mg praziquantel/kg body weight and three weeks after treatment. 15% of the patients had low platelet counts before treatment. Three weeks after treatment the counts reverted significantly back to normal ($P<0.01$). Therefore there was thrombocytopaenia associated with this infection.

To determine the stage at which thrombocytopaenia develops mice were infected with 25 cercariae/mouse for chronic infection. Stool and liver egg counts

were determined. Platelet levels were monitored during the course of infection. Platelet counts began to decline during the fifth week of infection and by the sixth week, platelet levels were reduced significantly and the counts remained low upto the fifteenth week.

That thrombocytopaenia was linked to the immune responses of the host against the parasite was demonstrated by an analysis of antibodies against platelet and parasite antigens. Antibodies reactive with the surface of mouse platelets and with solubilized *S. mansoni* worm and egg antigens were quantified by the Enzyme-linked Immunosorbent Assay. The antibody titres against both the antigens were significantly higher ($P<0.05$) from week eight post infection onwards suggesting that both the antibodies rose steadily with the course of infection. To investigate the presence of shared epitopes between the parasite and platelets, platelet antigens that are involved in the said depletion were analysed. Whole platelets were

characterised by solubilizing in Sodium Dodecyle Sulphate and subjected to polyacrylamide gel electrophoresis. Antibody reactivity was detected against antigens of approximately 94-100 kD. Some of the sera also reacted with antigens of 70, 67, 43, 33 and 30 kD. A comparison of parasite and platelet antigens revealed that sera reacted with both parasite and platelet antigens of approximately 43 and 33 kD suggesting that schistosomes and platelets may share these epitopes.

Finally the interaction of platelets with the parasite larval stage was investigated to speculate on the possible effector mechanisms in platelet mediated parasite destruction. Schistosomulae were incubated with freshly washed platelets. Platelets attacked schistosomulae within 30 minutes of incubation. The interaction of the chistosomulae with platelets was confirmed by Electron Microscopy where platelets were seen to extend their protruberances between the parasite tubercles.

The killing of schistosomulae by platelets was investigated and it was found that by the third day of incubation 50% of the parasites were dead suggesting that platelets are capable of killing schistosomulae.

From the above findings it was concluded that thrombocytopaenia is a prominent feature of chronic schistosomiasis which enhances worm survival in the host thus contributing to the compromised state of the host. There are antiplatelet antibodies produced by the host during the course of infection and it was shown that parasite and platelet antigens may share some epitopes. It will be useful in future to further analyse the antibodies involved in platelet destruction by the use of monoclonal antibodies and also to analyse toxins produced by platelets using chromatographic techinques among other investigations. The whole network of interactions between schistosomes and platelets and the implications of such interaction have been discussed.

CHAPTER ONE

1 INTRODUCTION AND LITERATURE REVIEW.

1.1. GENERAL INTRODUCTION.

1.1.1. Schistosomiasis.

Schistosomiasis is a chronic debilitating and sometimes fatal disease which is estimated to affect at least 200 million people in the tropical and subtropical countries. It is caused by digenetic trematodes of the genus *Schistosoma* and the three species that principally infect man are *S. mansoni, S. japonicum* and *S. haematobium*. The other species that may parasitise man are *S. mekongi, S. matheei, S. intercalatum, S. margrebowiei* and *S. rodhaini*.

Schistosomiasis is an ancient water-borne disease whose occurence was recorded as early as 4,000 years ago. The disease has spread to new areas around the world through human migration in the form of trade, war, colonization, slave trade and

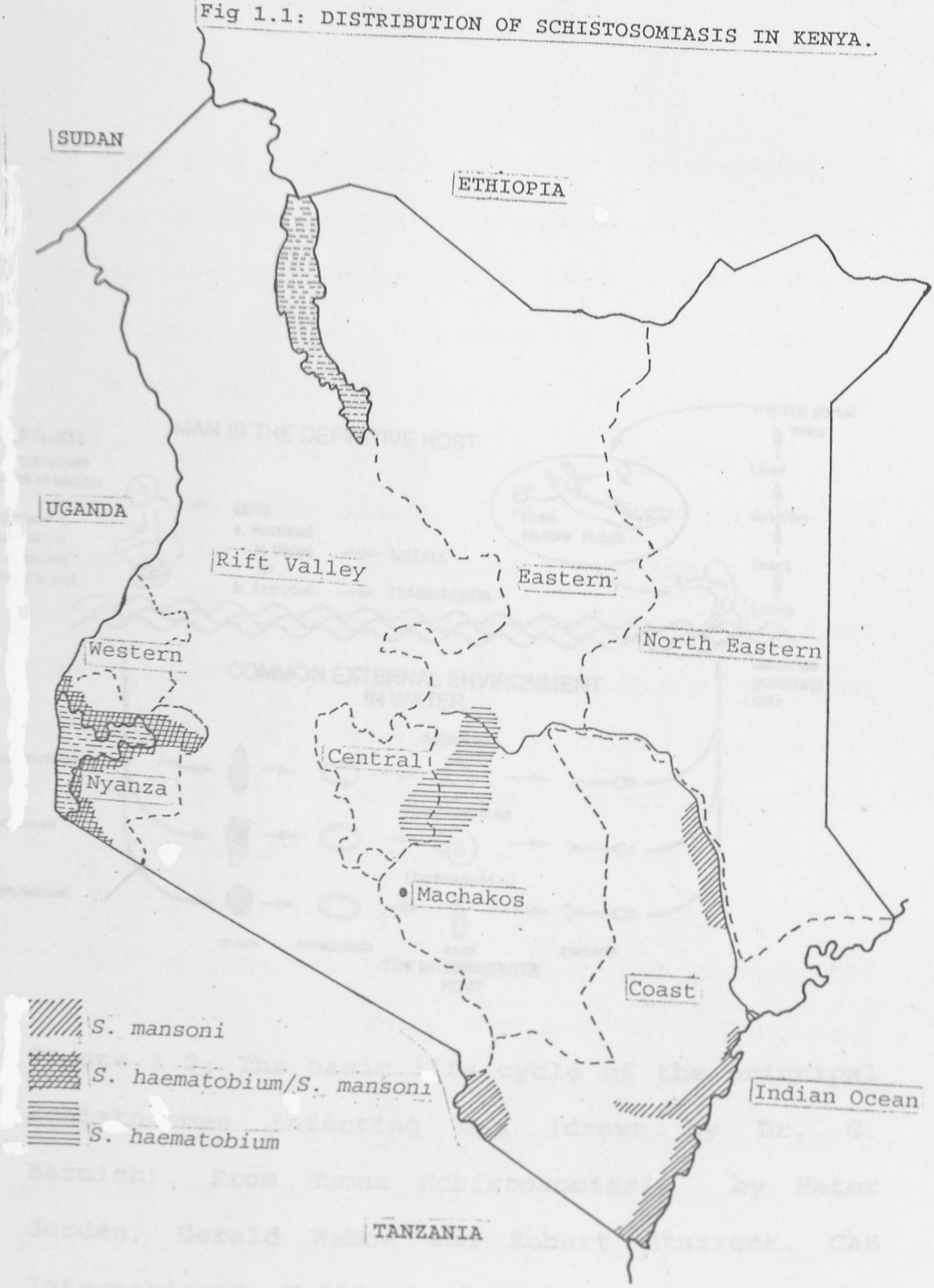
pilgrimages. In recent times the extension of irrigation schemes, the construction of dams and the concentration of human populations have contributed to its spread. In Kenya about 2 million people are infected with schistosomiasis. The two types of human schistosomiasis in Kenya are, urinary schistosomiasis caused by *S. haematobium* and intestinal schistosomiasis caused by *S. mansoni*.

The urinary type is distributed along the coastal region while the intestinal type is distributed in the drier parts of the country. Both types are found in the lake Victoria region (Fig. 1.1).

Figure 1.2 is a diagramatic representation of the schistosome life cycle. It is complex involving alternating parasitic (eggs, schistosomulae and adult worms) and free living (miracidia and cercariae) stages. When eggs are passed into fresh water via urine and/or stool they are viable for 8-12 hours during which time they hatch into

miracidia under the influence of warmth (10-30°C) and light. Free miracidia swim actively and make contact with the appropriate snail hosts. Inside the snails the miracidia develop into mother and daughter sporocysts which develop into cercariae. Mature cercariae emerge from the snail after 4-5 weeks under the influence of light and at temperatures between 10 and 30°C or higher. Cercariae penetrate the skin of man during his water-related activities like swimming and farming and transform into schistosomula. Approximately 5 weeks from the time of skin penetration male and female worms pair up in the liver and are transported to mesenteric venules of the host's gut in the case of *S. mansoni* and *S. japonicum* and to the urinary tract in the case of *S. haematobium*. While in the bloodstream of these sites the female worms produce hundreds of eggs per day and this process can continue for a long time sometimes upto 30 years.

Fig 1.1: DISTRIBUTION OF SCHISTOSOMIASIS IN KENYA.



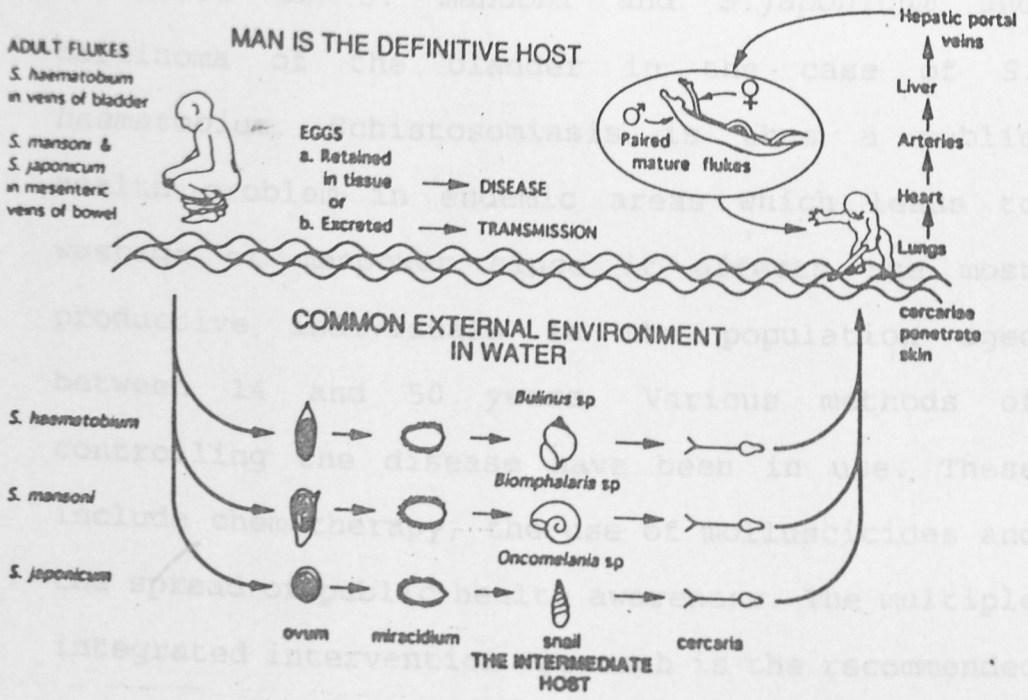


Figure 1.2: The basic life cycle of the principal schistosomes infecting man (drawn by Dr. G. Barnish). From *Human Schistosomiasis* by Peter Jordan, Gerald Webbe and Robert Sturrock. CAB International, Wallingford, U.K.

The eggs that lodge in the organs are responsible for the pathology that is associated with this disease which includes portal hypertension, oesophageal varices, haematemesis and ascites in the case of *S. mansoni* and *S.japonicum* and carcinoma of the bladder in the case of *S. haematobium*. Schistosomiasis is thus a public health problem in endemic areas which leads to wastage of manpower since it affects the most productive individuals in the population aged between 14 and 50 years. Various methods of controlling the disease have been in use. These include chemotherapy, the use of molluscicides and the spread of public health awareness. The multiple integrated intervention approach is the recommended method of control with the aim of controlling morbidity due to schistosomiasis rather than its transmission. However, with the evidence of human subjects developing immunity to schistosomiasis that is age-dependent and the successful immunization of animal models with extracts of various life cycle stages of the parasite, the

development of candidate vaccines for future control of he disease is in progress.

1.1.2. Platelets.

Platelets are small anuclear blood cells and unlike other granule-containing cells, they have a characteristic disc-like appearance rather than the spherical form manifested by lymphocytes, monocytes, neutrophils and other organelle-filled leucocytes (Fig. 1.3).

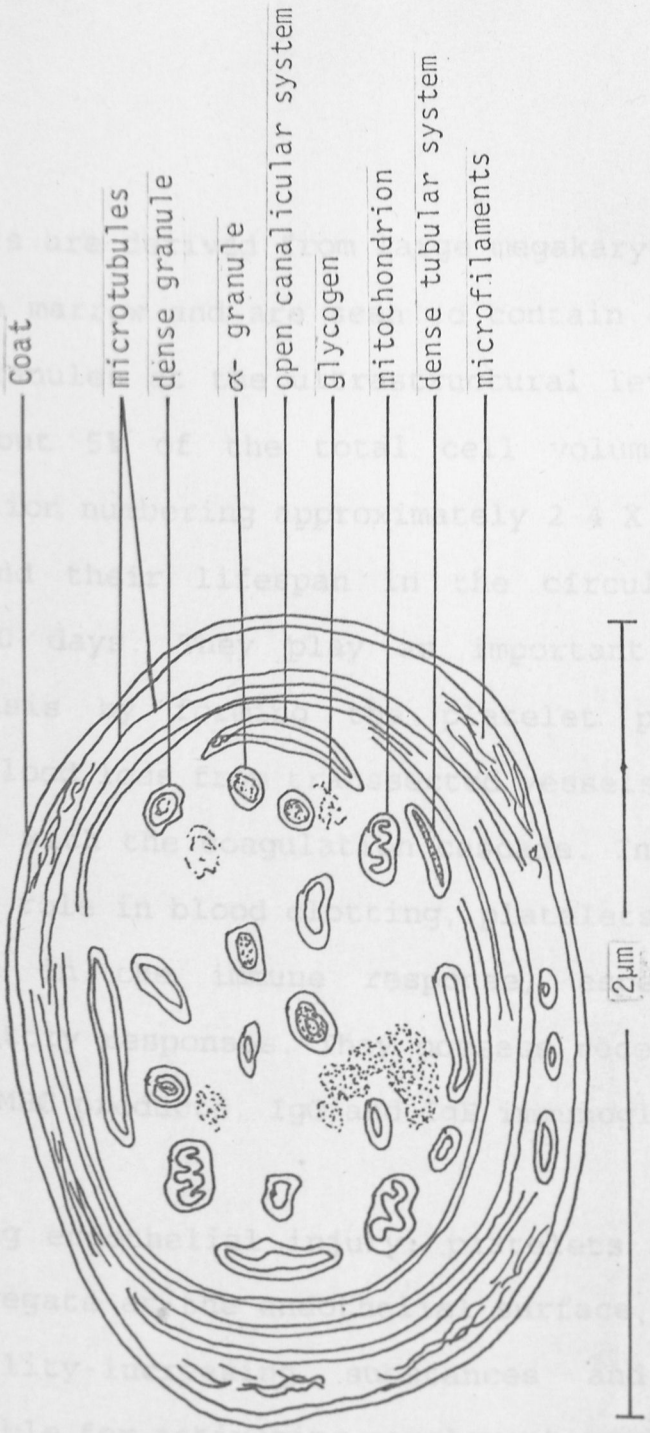


Figure 1.3: Schematic representation of a platelet cut in the equatorial plane.

Platelets are derived from large megakaryocytes in the bone marrow and are seen to contain dense and alpha granules at the ultrastructural level. They form about 5% of the total cell volume in the circulation numbering approximately 2-4 X 10^8/ml of blood and their lifespan in the circulation is about 10 days. They play an important role in homeostasis by forming the platelet plug that limits blood loss from transsected vessels and they interact with the coagulation cascade. In addition to their role in blood clotting, platelets are also involved in the immune response, especally in inflammatory responses. They possess receptors for Class 1 MHC products, IgG and IgE immunoglobulins.

Following endothelial injury, platelets adhere to and aggregate at the endothelial surface releasing permeability-increasing substances and factors responsible for activating complement components to attract leucocytes. Discoid platelets in circulating blood or platelet-rich plasma are not sticky. Contact with subendothelium or other

foreign surfaces results in adhesion and initiation of shape change from the disc shape.

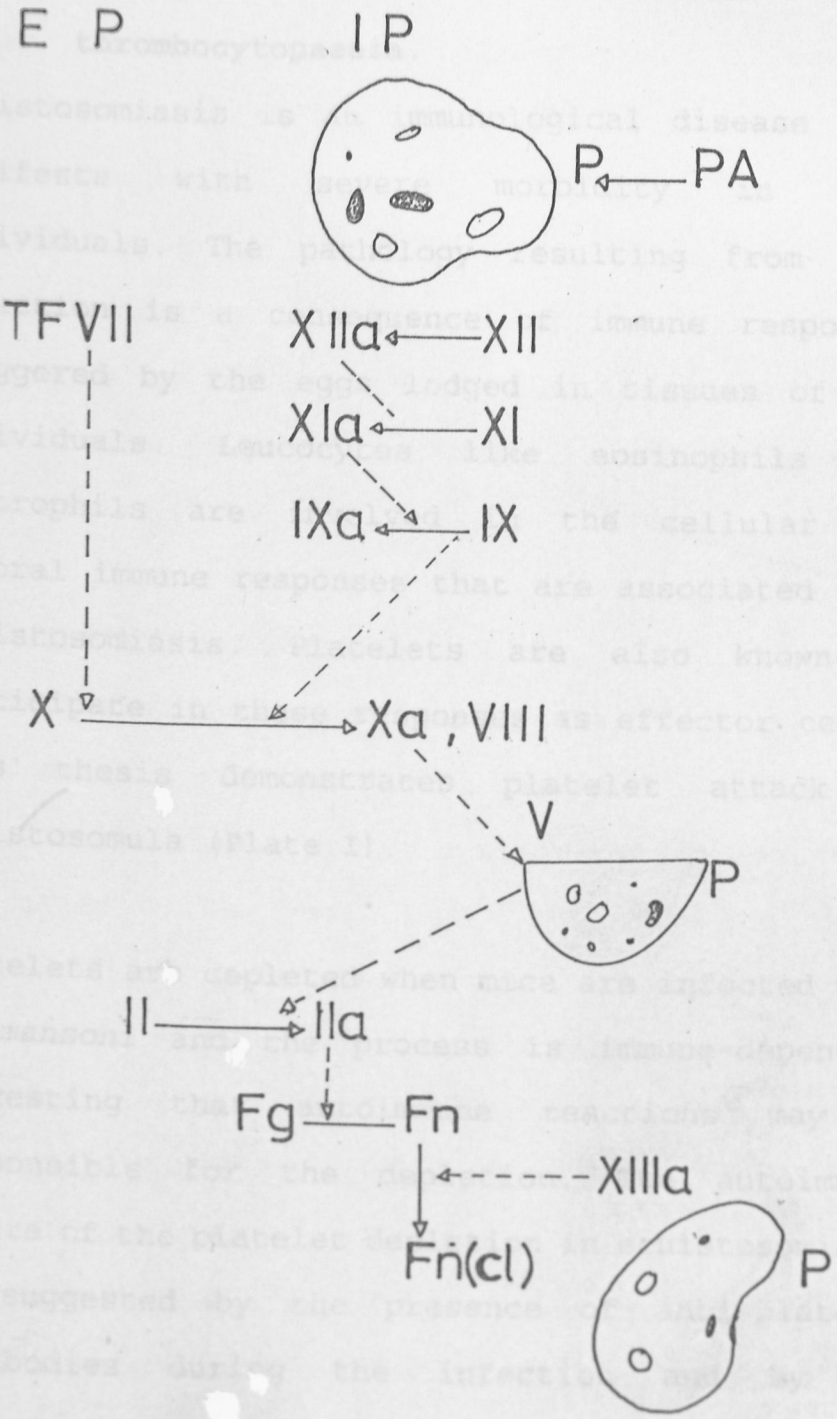
Extensions of long filariform processes from the platelet surface are formed first and these together with the variety of receptors on the surface facilitate the conversion of the cell to a sticky state. The three major platelet functions are adhesion, aggregation and secretion. In all three receptor-ligand interactions are involved with subsequent platelet metabolic responses. Attachment to extracellular matrix protein, von-Willebrand Factor, one of the important clotting factors, is mediated primarily by a family of heterodimeric cell surface proteins known as integrins. These are adhesion receptors that play a major role in platelet function. The clotting process involves an interplay of the intrinsic and extrinsic pathways of blood coagulation (Fig. 1.4). Initiation of the intrinsic coagulation pathway can be significantly enhanced by platelet activation. The sequential activation of a series of

coagulation factors from factor XII to fibrin formation lead to the formation of the blood clot. When fibrin is finally formed more platelets aggregate at the site thus completing the formation of the plug.

Platelets are thus very important in homeostasis and their depletion greatly affects the normal functions of the body. Platelet depletion to levels below the threshold values leads to thrombocytopaenia. In normal humans, platelet counts below 100 X $10^3/mm^3$ are indicative of thrombocytopaenia while in mice normal values range from 150 - 1,000 X $10^3/mm^3$ (see appendix).

PARTICIPATION OF PLATELETS IN BLOOD COAGULATION.

Intrinsic (IP) and extrinsic (EP) pathways of blood coagulation illustrated in simplified form. Coagulation factors displayed in Roman numerals in which an activated factor (a) is shown to activate the subsequent factor. Initiation of the intrinsic coagulation pathway can be significantly enhanced by platelet activation (PA--- P). The assembly of the prothrombinase complex (Xa-V) requires an activated platelet surface (P). Fibrinogen (Fg) is converted to fibrin (Fn) which is later cross-linked (Fn (cl)) with the assistance of Factor XIIIa and activated platelets (P).


E P
I P
P
PA
TF VII
XIIa
XII
XIa
XI
IXa
IX
X
Xa , VIII
V
P
II
IIa
Fg
Fn
XIIIa
Fn(cl)
P

1.1.3. Association of schistosomiasis with thrombocytopaenia.

Schistosomiasis is an immunological disease that manifests with severe morbidity in some individuals. The pathology resulting from this infection is a consequence of immune responses triggered by the eggs lodged in tissues of the individuals. Leucocytes like eosinophils and neutrophils are involved in the cellular and humoral immune responses that are associated with schistosomiasis. Platelets are also known to participate in these responses as effector cells. This thesis demonstrates platelet attack on schistosomula (Plate I).

Platelets are depleted when mice are infected with *S. mansoni* and the process is immune-dependent suggesting that autoimmune reactions may be responsible for the depletion. The autoimmune nature of the platelet depletion in schistosomiasis is suggested by the presence of anti-platelet antibodies during the infection and by the

correlation of the levels of such antibodies with those of anti-parasite antibodies. Platelet depletion also seems to favour survival of schistosome worms.

An understanding of the interaction of cellular components of blood with the worms may shed some light on the complex host-parasite coexistence in schistosomias.

1.2. LITERATURE REVIEW.

1.2.1. Pathology of schistosomiasis.

Based on parasitological, clinical and pathological changes four stages of the schistosomal disease are recognized by the World Health Organization (WHO, 1985). These are invasion, maturation, the establishment of infection and the stages of the complications. Pathology of schistosomiasis results from the delayed hypersensitivity reaction to schistosome eggs in various tissues that is characterised by the formation of granulomas with the severity of disease being mostly related to the

intensity of infection (Jordan *et. al.*, 1993). Schistosomiasis is an ancient disease and direct evidence of infection was demonstrated in Egyptian mummies as early as 4,000 years ago (Farooq, 1973; Woodruff, 1974). Although the eggs are responsible for the severe pathology in this infection, the inflammatory reactions associated with the other stages of infection are also important in the general disease state.

(i). *Cercarial penetration.*

Cercariae penetrate the skin of man during his water-related activities. The stimuli for cercariae penetration include sweat from the definitive host. The cercariae first adhere to the skin then violent bursts of the tail assist the start of penetration. During the penetration the cercarial penetration glands are emptied and the enzymes in the secretion of the postacetabula glands disrupt the ground cement of the epidermis, facilitating mechanical disruption of the cells as cercarial body without the tail moves to the stratum corneum. The syndrome

of 'cercarial dermatitis' (Swimmwer's itch) results from incomplete infection by small mammal or avian schistosomes and occurs in immune populations in endemic areas following heavy re-exposure to *S. mansoni* (Jordan *et. al.*, 1993). In the case of *S. haematobium* infection severe dermatitis may also develop particularly in young children exposed for the first time to large numbers of cecariae (Cowper, 1971).

In initial exposure, the cercariae produce only mild reactions. Macules appear within 12 hours and in non-sensitized individuals, soon disappear. In sensitised individuals macules are followed by papules possibly accompanied by erythema, vesicles, oedema and pruritus that may persist for 5-15 days. Pustules may form if secondary infection occurs. Residual pigmentation at the sites of the lesion may persist for weeks or months. The focal lesion demonstrates oedema and heavy dermal and epidermal eosinophil and mononuclear cell infiltrates. Teixeira *et. al.*, (1993) have demonstrated that

penetration of skin by cercariae of *S. mansoni* is associated with a local inflammatory response induced by cercariae proteases and characterised by eosinophil and neutrophil accumulation.

These lesions do not cause serious pathological conditions and are normally treated by topical agents like corticosteroid creams and more serious cases by oral and paranteral antihistamines.

(ii). *The lung stage.*

At the end of penetration the cercariae are transformed into schistosomulae which are adapted to the isotonic medium within the definitive host. After penetration the trilaminate tegument of cercariae is replaced by a heptalaminate membrane (Rollinson and Simpson, 1987). The newly formed schistosomulae may remain in the skin unaltered for upto 112 hours but within 90 hours they may penetrate a nearby vein discharging futher lytic enzymes from the apical gland vesicles. They may enter a lymphatic vessel but unless trapped here

they eventually reach the venous system via the thoracic duct. Within the vein the worms are carried passively in the blood flow to the heart and on to the pulmonary capillaries. In the next 72 hours the schistosomulae change shape to much slender, longer organisms in order to traverse pulmonary capillaries *en route* to the left heart and the systemic circulation. To develop further they must reach the liver via the mesenteric artery and capillaries and the hepatic portal vein. Those that are unable to reach the liver try a second and a third circuit until they reach (Wilson, 1987).

The lung schistosomulae are the target of protective immunity in mice by T cell-mediated effector responses triggered by the release of antigens from the parasite (Harrop and Wilson, 1993). It is during the evasion of these host defence mechanisms that schistosomulae may cause haematopathological changes to the host, for example, by depleting thrombocytes (Ngaiza and Doenhoff, 1987). The migrating larvae release

proteases that assist them in migration, growth and survival within the vertebrate host. Doenhoff *et. al.*, (1990) have described an elastase-like serine protease of schistosome larvae which has a role in facilitating migration. In the liver the chistsomulae feed and grow with the subsequent development into adult worms.

(iii). *Adult worms.*

The paired adult worms in the liver are transported by hepatic portal vein to its branches around the intestine or vesical plexus in the case of *S. mansoni* infection and venules of the pelvic venous plexus in the case of *S. haematobium* infection. Adult worms provoke no tissue reactions while alive within vessels (Jordan *et. al.*, 1993). However, worm pairs in strategic sites particularly ureters and ureterovesical junction in the case of *S. haematobium* can lead to serious complications (Abdel-Salam and Ehsan, 1978; Cheever *et. al.*, 1978). Dead worms on the other hand cause a severe necrotizing reaction with numerous eosinophils

resulting in eosinophilic abscess formation. Live adult worms like the larvae and the eggs produce proteases and peptidases that may modulate immunological and homeostatic defence mechanisms and thus prolong survival of the parasite (Jordan *et. al.*, 1993).

The consequence of the interactions between schistosomes and host protease inhibitors could be immune modulatory (Doenhoff *et. al.*, 1990). After maturing in a permissive host, the schistosome worm pairs take up residence in the bloodstream of the mesenteric venules of the host's gut in the case of *S. mansoni* and the urinary system in the case of *S haematobium*. The worm pairs engage in permanent copula and egg-laying for many years; their lifespan in humans has been variously estimated to average 3.5-12 years, with some worms surviving for 30 years or longer (Vermund *et. al.*, 1983; Rollinson and Simpson, 1987).

(iv). *Eggs*.

After the worms lay eggs in the above sites most of the eggs pass through the intestinal wall into the gut or bladder and are passed out with the host's faeces or urine. Passage of the eggs through the vein walls and tissues to the lumen of the gut and the bladder is aided by the release through micropores in the shell of histolytic enzymes secreted by the miracidia (Jordan *et. al.*, 1993). The eggs which do not follow this route are trapped in the capillary beds of various organs principally the liver where they undergo interactions with the host's tissues and immune system. Some eggs are trapped in the wall of the viscus or swept on to the portal radicals or lung arterioles (Cheever, *et. al.*, 1969) while afew may bypass these organs via collaterals, ending up in the brain, kidneys or other distal sites. More eggs follow that route after capillary beds have been blocked and additional shunts have opened. The resultant pathology is caused by inflammatory immune and activated responses of the host. Both antibody and

cell-mediated mechanisms are activated by exposure of humans and experimental animals to these eggs.

Eggs are laid immature and miracidial differentiation is completed over several days. Leucocytic responses to immature eggs are modest but enlarge to full blown granulomas as miracidia mature, followed by their lengthy gradual, involution after the eggs die and disintegrate (von Lichenberg, 1962).

(v). *Pathophysiology associated with schistosomiasis*

The granulomatous inflammatory responses induced by the schistosome eggs are mainly responsible for the pathophysiology associated with this infection. Antigenic factors, released from the secretory glands of miracidia enclosed within the egg are considered responsible for granuloma formation. The induction of the egg granuloma is instrumental in causing pathological symptoms. *S. mansoni* and *S. haematobium* egg granulomas are cell-mediated immune

responses with many characteristics of type IV hypersensitivity reactions (Warren *et. al.*, 1975). It has been postulated that schistosomiasis causes haematological changes in the immune status of the host and may be responsible for the anaemia associated with this infection which is thought to be of an autoimmune nature (Mahmoud and Woodruff, 1972). Platelets are also believed to be depleted by an autoimmune process (Ngaiza and Doenhoff, 1987).

Acute schistosomiasis which is known as 'toxemic schistosomiasis' (Brazil) or 'Katayama fever' (Japan), a serum sickness-like syndrome occurs 3-9 weeks after infection. This period coincides with the onset of egg production. Among people living in endemic areas the acute phase may pass undiagnosed (Jordan *et. al.*, 1993). When eggs have lodged in tissues there is severe inflammatory reactions around them (Rollinson and Simpson, 1987). The perivascular granulomas are large, with predominant necrotic-exudative features and appear as

translucid granules disseminated on the serosal surface of liver and intestines with central necrosis and dense eosinophil infiltration. In *S. mansoni* infected mice the size of the granuloma, which may reach 100 times the volume of the egg, decreases with increasing infection. This is followed by reduced organ size and pathology. Domingo and Warren (1968) demonstrated that modulation of granuloma size was dependent on endogenous desensitization of the host rather than on parasite dependent factors. Subsequent studies on *S. japonicum*-infected chimpazees indicated that a similar modulation of host response to eggs also occurs (Smithers and Doenhoff, 1982). The decrease in size of *S. mansoni* hepatic granuloma is paralleled by a decline in reactivity of other cell-mediated immune parameters such as lymphocyte blastogenic response to egg antigens and delayed hypersensitivity (footpad) response to injected egg antigen (SEA) (Boros *et. al.*, 1975).

Only in immune modulated state, as in chronic schistosomiasis, when the granulomas are smaller but still protective, does the host's immunologic response and disease state maintain an acceptable balance (Colley *et. al.*, 1986). Some patients never effectively express this modulation, leaving them to respond vigorously throughout their infection (Goes *et. al.*, 1991). Lesions of chronic schistosomiasis can be divided into those of systemic cellular immune responses (lymphoreticular proliferation) and lesions due to immune complexes.

Egg lesions may affect excretory sites in hollow viscera (gut and urinary tract) as well as downstream parenchimal organs (liver and lung) or ectopic sites like the central nervous system (von Lichtenberg, 1962). Mice lightly infected with *S. mansoni* consistently develop a disease syndrome remarkably similar to that of man exhibiting hepatosplenomegaly, portal hypertension and eosophageal varices (Rollinson and simpson, 1987). In *S. haematobium* the egg granuloma is also a cell-

mediated reaction that can be accelerated and augmented by prior sensitization of the host with eggs. In man and some primates, *S. haematobium* adults reside in the vesicle plexus and are not confined to the mesenteric veins as in rodents. So the granulomatous lesions of *S. haematobium* occur in the urinogenital system of man where extensive submucosal accumulations of calcified eggs are a characteristic feature (Cheever *et. al.*, 1985).

1.2.2. Immunology of schistosomiasis.

(i). *Humoral responses.*

All stages of the life cycle of schistosomes are recognized by antibodies in the serum of man although not all of them can be relevant to protective immunity and some have been shown to prevent the expression of immunity (Butterworth *et. al.*, 1985, 1987; Hagan *et.al.*, 1987). Eggs can be destroyed in the intense granuloma they stimulate but this reaction would be damaging to the host while killing of schistosomulae at any stage would be protective. Any immune response which destroys

schistosomes or reduces their fecundity with minimum pathology is protective to the host (Butterworth *et al.*, 1987). In the absence of complement components or cells, human antibodies appear to have no lethal effect on schistosomes *in vitro*. Macroscopic immune complexes develop around eggs or schistosomula when incubated with antibodies taken from infected people and this reaction is a diagnostic test. In both experimental animals as well as in man anti-schistosome antibodies may be detected soon after exposure to infection (Jordan *et. al.*, 1993).

Cytotoxic antibodies lethal to schistosomulae of *S. mansoni* have been described using *in vitro* assays (Capron *et. al.*, 1977). These antibodies of the IgG class bind to the surface of schistosomulae and activate complement which extensively damages the outer membrane of the parasite and leads to parasite death. Freshly prepared normal human sera has been shown to kill schistosmulae of *S. mansoni* by complement activation via the alternative

pathway (Santoro *et. al.*, 1979). Analysis of sera from school children in Kenya has shown that there is immunity developing after a long time of infection. Although there was no correlation with resistance to reinfection, susceptibility to reinfection was correlated with total anti-egg antibodies and the inhibition of binding of two monoclonal antibodies to a 38 kD antigen on the schistosomulum surface. There was also a correlation between reinfection intensities and both IgM anti-schistosomulum antibodies and IgM and IgG anti-egg antibodies. By depleting serum of IgM it was possible to demonstrate increased activity of IgG-dependent killing of schistosomulae (Khalife *et. al.*, 1986).

Therefore resistance to *S. mansoni* may be in part due to specific anti-parasite antibodies which have a protective function and also to the absence of blocking antibodies (Khalife *et. al.*, 1986; Butterworth *et. al.*, 1987). Early in infection egg antigens promote IgM antibody production or, in

some cases perhaps promote production of antibodies of an IgG isotype which is not protective. These antibodies crossreact with the major glycoproteins on the schistosomulum surface. Potentially protective IgG and IgE responses may also be mounted against the same antigens. *In vitro* studies have indicated that such antibodies may be involved in possible protective mechanisms. As blocking antibodies predominate early in infection, children remain susceptible. Eventually, balance switches to a predominantly protective response and the children become immune to infection (Butterworth *et. al.*, 1987).

A study on anti-schistosome immnoglobulin levels in the serum of Egyptian patients infected with *S. mansoni* and/or *S. haematobium* showed that a high proportion of the IgG antibodies belonged to the IgG_4 subclass. It was concluded that IgG_4 antibodies are inefficient in activating complement and binding to receptors on monocytes and macrophages (Iskander *et. al.*, 1981). These workers suggested

that the presence of IgG_4 antibodies might interfere with complement activation by IgG_1 antibodies. They also suggested that by competing with IgG antibodies for allergenic worm antigens, IgG_4 could also block mast cell degranulation. However, the IgG_4 antibodies were directed predominantly against egg antigens and IgE antibodies against adult worm antigens.

Early experiments by Smithers and Terry (1967) showed that animals bearing a patent primary *S mansoni* infection had the capacity to resist reinfection with a secondary challenge of fresh cercariae. This phenomenon of acquired resistance to reinfection in the presence of a continued primary infection is referred to as concomitant immunity. Experimental studies on immunity have been carried out in primates (rhesus monkeys and baboons) and in various laboratory rodents (mouse, rat, hamster and guinea pig) where resistance to reinfection has been observed. This resistance was not the result of a specific protective immune

response but instead was a non-specific process resulting from an abnormal migration or trapping of schistosomulae as a consequense of egg deposition and granuloma formation in various organs (Dean *et. al.*, 1978, Wilson *et. al.*, 1983).

In order to focus on specific protective immune responses most workers choose to study mice immunized with heavily irradiated cercariae or schistosomulae (Bickle *et. al.*, 1979, 1985; James and Sher, 1983; James *et. al.*, 1984). The other main laboratory rodent model the rat, is a non permissive host in that worms of a primary infection do not mature fully and do not lay eggs, instead they are rejected by a non-immunological process after 5-6 weeks and the animals are then strongly resistant to reinfection (Capron *et. al.*, 1980).

The antigenic properties of the adult worm surface have been examined and it was shown that unlike the surface of schistosomulum that of the worm was not recognised by antibodies in the serum of the host

(Rollinson and Simpson, 1987). The worms passively adsorbed host macromolecules that were mainly glycolipid in nature into their tegumental membrane. Transfer of adult worms from mice into monkeys that had been immunized against mouse antigens resulted in immune destruction of the transferred worms indicating that adult worms were intrinsically susceptible to immune destruction but they 'mask' their antigens during primary infection. In addition to losing surface antigenicity it has also been shown that developing schistosomulae and adult worms become progressively refractory to immune attack even though they can still be damaged (Moser *et. al.*, 1980).

There is a possible link between antibody levels and resistance to infection. It has been shown that high antibody titres are associated with a subsequent drop in egg count in *S. haematobium* infected patients (Wilkins and Capron 1977). If high antibody levels develop only after many years of exposure then this factor alone may explain the

slow development of immunity and may not be necessary to postulate a role for blocking antibodies. There are strong humoral immune responses mounted against schistosome infections and it has been shown that man develops a significant immunity to *S. mansoni* and *S. haematobium* which is age-dependent (Butterworth *et. al.*, 1985; Hagan *et. al.*, 1987).

(ii). Cell-mediated immunity.

All mammalian species appear to have some innate resistance to infection with schistosomes. It has been demonstrated that human monocytes derived from peripheral blood of individuals with no history of exposure to schistosome infection were capable of killing schistosomulae of *S. mansoni* independent of specific antibody and complement (Ellner and Mahmoud (1979).

One of the first cellular mechanisms to be described involved normal rat macrophages together with heat-labile components in sera from infected

animals (Capron *et. al.*, 1975). This mechanism was demonstrated in baboons and humans and was found to require IgE antibodies particularly in immune complexes . It was also shown that macrophages bear low affinity Fc receptors for bound IgE in contrast to the high affinity mast cell receptor (Joseph *et. al.*, 1978). This work provided the first evidence for a specific effector function of IgE in beyond its capacity to mediate immediate hypersensitivity reactions (Jordan *et. al.*, 1993). In the case of IgE-dependent killing by macrophages contact is required between the effector cell and the schistosomulum target. A similar IgE-dependent reaction involving platelets instead of macrophages has recently been described in both rat and man (Joseph *et. al.*, 1983). These workers also showed that transfer of platelets from immune into normal rats will protect the recipients against challenge infection. In this case direct contact is not required, instead killing may depend on the generation and release of toxic oxygen metabolites.

(iii). *Immunopathology.*

Immunological processes contribute to disease processes in schistosomiasis particularly in the context of the egg granuloma. Developing schistosomula also release a variety of moieties such as proteolytic enzymes and lysophospholipase that may inhibit or interfere with host immune effector cell function and therefore lead to irregularities that result in pathological processes (Auriault *et. al.*, 1981). The granulomatious reactions that develop around the eggs in the livers of acutely infected animals which subsequently lead to fibrosis and portal hypertension are T-cell mediated reactions resembling delayed hypersensitivity (Warren *et.al.*, 1967; Boros and Warren 1970). These workers injected eggs intravenously into the lungs of mice bearing a primary infection or previously immunized with egg antigens and showed that the capacity to mount an accelerated and enhanced reaction following acute infection was an immunologically specific event that could be adoptively transferred

to naive animals with T cells, but not with serum. They also reported that granulomas develop normally in mice depleted of B cells.

Chronically infected mice developed smaller granulomas than those with acute infection (immunomodulated)(Boros *et. al.*, 1975). This reduction in size has been attributed either to serum suppressor factors, or to suppressor T cells or to non-specific suppressor macrophages (Fidel and Boros 1990; 1991). This may suggest that florid granuloma formation with extensive fibrosis may be a feature of early infections as seen in children. Some evidence for this suggestion has come from examination of granulomatous reactions in rectal snips (Rocklin *et. al.*, 1980). It has further been shown that not only the granulomatous reaction but also the subsequent fibrosis is a T cell-dependent event attributable to the release from T cells of fibroblast stimulating factors (Wyler *et. al.*, 1987; Prakash and Wyler, 1991;).

The granuloma not only damages the host leading to subsequent fibrosis but also protects it against the more serious hepatotoxic effets of certain toxic egg products. Thus, mice depleted of T cells fail to mount a granulomatous reaction to *S. mansoni* eggs deposited in the liver. Instead they show an early light mortality, with areas of fatty deposits and necrosis in liver cells surrounding the eggs. A potent hepatotoxin has been isolated from eggs of *S. mansoni* but not *S. haematobium* and *S. japonicum* and antisera raised against this protein prevented the hepatotoxic effect (Dunne *et. al.*, 1981). The granuloma has also been found to promote egg extravasation through the intestinal lumen into the faeces and in T cell deprived mice, the egg output was reduced (Ngaiza *et. al.*, 1990).

More T cell studies have demonstrated their association with anti-idiotypic specificity. These cells are found in chronically infected patients without severe disease or in those treated up to several years previously . These cells recognize

idiotypic epitopes of antibodies against egg antigens and respond by proliferating *in vitro* and are found less frequently in patients with hepatosplenic disease suggesting that they may be involved in 'normal' regulation of the granulomatous response that is seen in the majority of patients. They can also be found in neonates born of infected mothers (Montesano *et. al.*, 1990; Para *et. al.*, 1991).

The granulomatous reaction around the eggs which is characterised by extensive infiltration of lymphocytes and macrophages, is suggestive of a Th_1 type of response. However, the granuloma also contains a high percentage of eosinophils which is a feature of Th_2 response. The onset of egg laying during murine infection is associated with a switch from a predominantly Th_1 to a predominantly Th_2 response. Depletion of interferon-Γ has been shown to have no effect on granuloma formation whereas depletion of IL-5 causes a significant, although small, reduction (Roberts *et. al.*, 1993). Mice with

severe combined immunodeficiency (SCID) are a good model for examining the role of individual components of the immune system. It has been shown that SCID mice are unable to mount a granulomatous reaction to eggs deposited in the liver during infection. They also show increased mortality with hepatotoxicity, and reduced egg output in the faeces. Normal granulomatous reactions in such mice can be reconstituted by adoptive transfer of spleen cells from infected mice. Also the entire granuloma can be reconstituted by injection of a single cytokine, tumour necrosis factor (TNF)-α which restores egg laying by adult female worms and leads to recruitment of other cellular components of the granuloma, including macrophages and eosinophils. These cells secrete their own cytokines which are capable of maintaining and extending the granulomatous reaction (Amiri *et. al.*, 1992). Therefore, the granuloma formation and modulation are important immunological events in mice and even human subjects and are a measure of disease in schistosomiasis.

1.2.3. Platelets and thrombocytopaenia.

(i). *Platelet structure and function.*

Platelets are flattened or slightly oval, disc-shaped cells in criculating blood (White and Escolar, 1993). The surface of the platelets is corrugated resembling that of the brain. These convolutions are important in the platelets' interactions with other surfaces. Extensions of long, filariform processes is the first response of platelets to foreign surfaces. These psuedopods help in the spread process when platelets restore vascular integrity at damaged areas (White, 1987). Platelets adhere to sites of injury in damaged vessel walls or exposed foreign surfaces.

Discoid platelets in circulating blood, in platelet-rich plasma and washed platelets are not sticky. Contact with subendothelium or other foreign surfaces results in adhesion and initiation of the above shape change. There is a variety of receptors on the platelet surface that facilitate the conversion of the cell to a sticky state

(Kunicki and Peter, 1992). Attachment to extracellular matrix proteins is mediated primarily by a family of heterodimeric cell surface proteins (integrins), each composed of an α and a β subunit. These are adhesion receptors that play a leading role in platelet function. The existence of this family was first suggested by the identification of functional, gross structural and immunologic similarities among adhesion receptors (Ginsberg *et. al.*, 1993).

The major platelet integrin is αIII-β3 (Glycoprotein IIb-IIIa complex). About 50,000 copies af it are detectable on resting platelets. The complex is not expressed until the cell is activated in suspension or by foreign surfaces (White and Escolar, 1993). The morphology of platelet receptor interactions with surfaces has been difficult to visualise by electron microscopy. However, the use of immunocytochemical methods with electron microscopy revealed that platelets in suspension exposed first to monoclonal antibodies

against GPIIb-IIIa and then fluorescein tagged anti-immunoglobulin revealed a sequence of clustering, patching, capping and endocytosis similar to events observed on lymphocytes exposed to immune reactions (Santoro, 1987; Kakaiya *et. al.*, 1988).

The platelet membrane is structurally suited for interactions with a variety of agonists and surfaces. Its carbohydrate-rich external coat is especially designed for binding to subendothelial microfibrils and the extracellular matrix protein, von Willebrand Factor (vWf) (George *et. al.*, 1984). Platelet membrane glycoproteins GpIb and GpIIb-IIIa bind soluble vWf after stimulation with ristocetin (Gp Ib), thrombin or ADP (GpIIb-IIIa) (Danton *et. al.*, 1994). The high content of sialic acid in the major glycoprotein (Gp Ib) of the external platelet membrane maintains the negative charge which prevents undesired 'stickiness' in unactivated platelets. The platelet response to agonists is a remarkably choreographed cascade of molecular

events, one of these being the reorganization of the actin cytoskeleton and the mechanisms of regulating the extent of actin polymerization (Furman *et. al.*, 1993).

Activation of newly adherent platelets results in the exposure of the fibrinogen receptor which is located on the Gp IIb-IIIa complex. During the early phase of platelet activation, both Gp Ib and Gp IIb-IIIa complex (Polley *et. al.*, 1981) are rearranged on the platelet membrane and fibrinogen, fibronectin and the endogenous platelet lectin, thrombospondin, are detectable on the platelet surface (Asch *et. al.*, 1985). All three adhesive glycoproteins are known to bind to GpIIb-IIIa on activated platelets. These adhesive molecules have been shown to sustain interplatelet adhesiveness and may be crucial in the binding of platelets to microoganisms (Hawinger, 1987).

Activated platelets display P-selectin on their surfaces which is one of the receptors that

initiate rolling of leucocytes on activated platelets or endothelium through Ca^{2+}-dependent recognition of cell surface carbohydrates. Adhesion of platelets is enhanced by this selectin (McEver, 1994). P-selectin and PAF are key molecules in the accumulation of leucocytes within damaged tissues by promoting the initial adhesion of PMN cells to endothelial cells (Coughlan *et. al.*, 1994).

The significance of these adhesive glycoproteins in platelet aggregation has been reported. Thrombin-induced platelet aggregation was inhibited by monoclonal antibodies raised against fibronectin (Hawinger, 1987). The fibrinogen receptor is located on the Gp IIb-IIIa complex after platelets are activated. In resting platelets, Gp IIb and IIIa have separate epitopes which are recognized by different monoclonal antibodies against Gp IIb-IIIa. This complex has been described as a member of the Arg-Gly-Asp-Ser (RGDS) tetrapeptide sequence containing adhesive molecules (Pytela *et. al.*, 1986). Stimulation by RGDS like that by agonists

may play an important role in inducing activation of platelets that results in futher platelet aggregation (Mohri and Ohkubo, 1994). This tetrapeptide has also been identified on the Gp IIb-IIIa complex as one of the recognition sites for vWf when platelets are stimulated by thrombin or ADP (Williams and Gralnick, 1987). The peptides when purified were used to analyse the recognition specificities for the platelet binding of vWf. Platelet-specific surface molecules detected with antibodies are essential for the biological function of platelets (Hayashi *et.al.*, 1986).

Since monoclocal antibodies react with single epitopes, those reacting with surface molecules of platelets can be used to determine the functions of these molecules (Furukawa *et. al.*, 1986). Adhesive glycoproteins like fibronectin have been shown to bind to cells via RGDS, thereby facilitating cell attachment and migration. Preincubation of platelets with RGDS inhibits binding of fibrinogen, fibronectin and vitronectin on platelets (Pytela

et. al., 1986). Platelets develop a secure hold of the damaged vessel wall through interaction of specific ligands in the subendothelial matrix while during aggregation, platelets develop a secure hold of one another through non-covalent cross-linking of adhesion receptors on adjacent platelets by soluble fibrinogen or vWf (Shattil, 1993).

Platelet membrane antigens with a defined role for platelet activation include receptors for collagen and thrombospondin (Santoro and Cowan, 1986). However, the relationships between these antigens and Gp IIb-IIIa in platelet function have yet to be elucidated. It may be assumed, however, that the ADP or collagen receptors are activated before fibrinogen binds to Gp IIb-IIIa and facilitate platelet aggregation.

(ii). *Platelet depletion in other diseases.*

Platelet depletion has been reported in other disease forms as well as infections. Most of the thrombocytopaenic states result in immune related

platelet depletion that involves autoimmune processes. A number of syndromes have been described including autoimmune thrombocytopaenia purpura, post transfusion purpura, neonatal alloimmune thrombocytopaenia and drug-induced thrombocytopaenia purpura which fit into the category of immunologic thrombocytopaenias (Lau, 1987). It was also suggested that thrombocytopaenia in patients with elevated PAIgG and/or PAIgM is most probably of immune origin in such diverse disorders as systemic lupus erythematosus (SLE), cirrhosis of the liver, lymphoma, cancer or septic conditions as well as immune thrombocytopaenia (ITP) (Szal and Blumberg, 1988). There is also evidence of immune complexes associated with PAIgG in disease states.

Correlation between PAIgG, platelet count and plasma polyethlene glycol (PEG) precipitable IgG immune complex (IC)-like material was tested in normal subjects and patients with ITP, SLE and various types of liver disease. A significant

direct correlation between platelet count and IC was found in SLE patients (Vijayalakshmi *et. al.*, 1984). It has also been shown that anti-phospholipid antibodies bind to the phospholipids of the platelet membrane resulting in immune destruction of the platelets (Galli *et. al.*, 1994).

Thrombocytopaenia has been reported in other infections where the PAIgG levels have been found to be in inverse relation with the platelet counts linking the IgG with the platelet depletion (Kelton *et. al.*, 1979). The association of immunoglobulins with platelet surfaces is a feature which is commonly identified with immunological destruction of platelets, a common complication of microbial infections (Kelton *et. al.*, 1979; Kelton and Gibbons, 1982; Kelton, 1984). IgG is capable of binding to platelets via the Fab terminus, Fc receptors or non-specifically due to the 'stickiness' of the platelet membrane. Specific binding can be masked by this nonspecific binding making the assessment of PAIgG difficult. The

platelet Fc receptor has been shown to be the mechanism through which antigen-antibody complex-induced platelet injury occurs. Antigen-antibody complex unrelated to platelet antigens may produce thrombocytopaenia *in vitro* (Israels *et. al.*, 1973). Platelet aggregation induced by 7S immune complexes or aggregated immunoglobulin was blocked by prior exposure of the platelet to isolated antibody Fc. A 210,000 mw platelet glycoprotein (Gp 210) which is the GPIb-IX platelet receptor that is missing in Bernard-Soulier Syndrom (BSS) was identified, and it was found that an antibody against it inhibited ristocetin-induced platelet agglutination.

Purified Fc fragments inhibited binding of anti-Gp210 antibody to intact platelets and to Gp 210 on immunoblots (Miragliotta *et. al.*, 1988). Therefore, platelets may bind immunologically to antibodies and be depleted from the system a phenomenon that may be beneficial to the organisms that live in the vascular system like the schistosomes.

Bacteria and their products have been shown to aggregate human and rabbit platelets *in vitro* (Clawson and White, 1971). These interactions may involve platelets only but may also be complicated into activation of the intrinsic coagulation pathway. It has been reported that a mechanism for natural resistance to *Lesteria monocytogenes* that is in turn promoted by the *Lesteria* organisms themselves exists. Rats infected with *Lesteria* organisms with administration of heparin developed bacteremia suggesting that natural immunity to the organisms is partly due to a platelet-dependent lysin which is activated during clotting and is in turn promoted by the organisms themselves. (Wayne *et. al.*, 1981). Thrombocytopaenia is frequently observed during malaria infections (Dennis *et. al.*, 1967; Borochovitz *et. al.*, 1970). However, it has been shown that platelets induce a dose-related growth inhibition of *Plasmodium falciparum* (Peyron *et. al.*, 1989).

The role of platelets in metastasis is well known. Tumour cells have been shown to require a component of complement pathway in order to activate platelets however, the requirements for platelet activation have been shown to be heterogenous in that different tumour cell lines seem to involve different pathways of platelet activation (Karpatkin *et. al.*, 1980).

In vitro studies involving other organisms have shown that platelets are cytotoxic to them. Human platelets, in the absence of antibody, are cytotoxic to tachyzoites of *Toxoplasma gondii* as determined by vital staining, transmission electron microscopy and the failure of organisms to survive and replicate in mice after *in vitro* interaction with platelets. The role of thromboxane B_2 (TXB_2) may be important in this cytolytic process (Yong *et. al.*, 1991). It was later reported that in addition to TXB_2 human platelets after incubation with *T. gondii* released oxygenated products of both arachdonic acid and linoleic acid, 12 -

hydroxyheptadecatrienoic acid (12 - HHT), 12-hydroxyeicosatetraenoic acid (12- HETE) and an identified peak (UV_{max} 234 nm) as determined by reverse-phase high performance liquid chromatography which may be important in the response to *T. gondii.* (Henderson *et. al.*, 1992).

(iii). *Platelet depletion in schistosomiasis.*

A platelet count of less than 100 X $10^3/mm^3$ is indicative of thrombocytopenia. In Kenya the mean platelet count in healthy adults is 211 X $10^3/mm^3$ of blood (Mukiibi *et. al.*, 1981). Platelets maintain the integrity of injured vessels via two pathways the intrinsic pathway involving the activation of Factors XII and IX and the extrinsic pathway involving the activation of Factor VII (Walsh, 1987).

Foreign bodies are known to induce platelet activation. For example, intravascular parasites like schistosomes, provide an ideal surface for activation of the intrinsic pathway of coagulation

which could result in intravascular thrombosis or disseminated intravascular coagulation (DIC), both of which could be deleterious to the host and parasite but this does not happen yet the worm surface is negatively charged and could readily attract platelets (Ngaiza, 1988). The worms and intravascular eggs either produce an inhibitor of coagulation pathway and/or initiate fibrinolysis whenever a thrombus forms. Extracts and secretory products of adult *S. mansoni* have been shown to contatin a potent inhibitor of the activation of the Hageman Factor suggesting that schistosomses are capable of evading the coagulation pathways as a survival mechanism (Foster *et. al.*, 1992).

Platelets are pivotal in the initiation and promotion of clot formation and their fate during these interactions may be of importance in explaining how the parasite evades formation of potentially harmful blood clots by destroying platelets. This characteristic of platelets makes them suitable for the study of host-parasite

coexistence. Immune-dependent platelet depletion that is associated with *S. mansoni* infection in mice has been reported where infected mice had 43-52% fewer platelets than the uninfected matched controls (Ngaiza and Doenhoff 1987). This thrombocytopaenia was T-cell dependent suggesting that immunoglobulins may initiate or promote this reduction in platelet numbers perhaps due to autoimmune reactivity against platelet antigens. The amount of platelet-associated IgG (PAIgG) was measured by ELISA and was found to rise as the infection progressed (Wambayi, 1987). The rise in PAIgG levels began at about 5 weeks post infection a time when production of anti-egg antibodies begins (Dunne *et. al.*, 1981).

It has been postulated that egg antigens may resemble platelet antigens. A cathodally migrating antigen k_3 that was associated with the production of antibodies which inhibit antibody dependent cell cytotoxicity in *S. mansoni* infections was strongly precipitated on reacting soluble egg antigen

extract (SEA) with pooled chronic infection sera (Dunne *et. al.*, 1987). In immunoblot analysis IgG from mouse chronic infection sera recognized antigens having similar molecular weight in both mice and humans suggesting that the antigens are well conserved (Ngaiza, 1988).

These observations suggest that locallized *in vivo* antigen/antibody reactions may play a significant role in the pathogenesis of schistsosomiasis. Schistosomes possess a unique membrane made of closely adherent lipid bilayers while other trematodes which do not reside in the blood have a conventional trilaminate outer membrane which may be unsuitable for survival within an intravascular environment (McLaren and Hockely, 1977). The cercarial membrane is also trilaminate and converts to the lipid bilayer of larval form within 3 hours of transformation. Cercarial glycocalyx is unsuitable for intravascular habitation. It was demonstrated that cercarial homogenate induced clot formation in platelet poor plasma (Modha, personal

communication). However, worm homogenate had been shown to prolong the clotting time (Tsang and Damian, 1977). Platelet activation is initiated when there is endothelial damage which may occur when migrating schistosomes traverse blood vessels in the skin and in so doing attract leucocytes/platelets aggregates (Bloch, 1984). Platelets are also effector cells known to attack parasite larvae. Several killing mechanisms against schistosomes *in vitro* involving cellular and humoral factors have been described. Neutrophils, Eosinophils with an accessory role for mast cells, Monocytes and Macrophages have been shown to exhibit cytotoxic properties against *S. mansoni* larvae in association with antibodies of variuos isotypes or with Complement (Capron *et. al.*, 1982; Kimani *et. al.*, 1991).

1.3 AIM AND OJECTIVES.

1.3.1 *General Aim.*

The aim of this study was to investigate the interaction between platelets and schistosomes

which results in immune dependent depletion of platelets.

1.3.2 *Specific objectives.*

1. To investigate mechanisms that cause thrombocytopaenia in mice infected with *Schistosoma mansoni*.
2. To investigate the level of thrombocytopaenia in humans infected with *S. mansoni*.
3. To determine the stage of *S. mansoni* infection at which thrombocytopaenia occurs.
4. To correlate platelet levels with levels of anti-platelet antibodies in mice and in humans infected with *S. mansoni*.
5. To investigate the presence of shared epitopes between *S. mansoni* and platelets of either human or mouse origin.
6. To speculate on possible effector mechanisms in platelet mediated parasite destruction.

CHAPTER TWO

MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Parasite strains.

A Kenyan strain of *S. mansoni* maintained at the Kenya Medical Research Institute (KEMRI) laboratories was used to infect laboratory animals for the experiments performed in Kenya while two strains one originally from Pueto Rico, West Indies, and another from Egypt but now maintained in the laboratory in the University of Wales were used for the experiments carried out in Wales.

2.1.2. Intermediate hosts.

The Kenyan strain of *S. mansoni* was maintained in *Biomphalaria pfeifferi* snails in Kenya. For the studies in Wales the parasite was maintained in *Biomphalaria glabrata*.

2.1.3. Definitive hosts.

C57 and Balb/c mice bred and maintained at KEMRI laboratory were used in the experiments carried out in Kenya. CBA and TO mice also bred and maintained in the laboratory at the University of Wales were used in experiments there.

The human subjects involved in this study were from a *S. mansoni* endemic area of Machakos District, Kenya (see map Fig. 1.1). Other studies on schistosomiasis have been carried out in this area and the transmission patterns are known. Water contact and snail population studies have also been carried out. The individuals came from Kangundo area where a relatively mild form of the disease has been associated with *S. mansoni* infections. They visited the Machakos district hospital where they were seen at the schistosomiasis clinic.

2.1.4. Drugs.

Praziquantel, the drug of choice for the treatment of *S. mansoni* and *S. haematobium* was used in the treatment of mice and of human subjects during the study. The drug made by Bayer AG, Leverkusen and E. Merck, Darmstadt, Germany is locally supplied by Bayer E.A. Ltd. Company.

2.1.5. Antigens.

(i). *Platelets.*

Whole platelets were isolated from fresh human and murine blood. Uninfected laboratory workers volunteered to be bled as controls. The mice that were bled for platelet antigen were also uninfected. Fresh blood was drawn in 3.2% trisodium citrate. It was then centrifuged at 900g for 5 minutes and the platelet rich plasma (PRP) separated. The PRP was resuspended in 1ml tyroides buffer (pH 6.5) washed 3X by the same buffer and finally resuspended in hepes buffered modified tyroides solution (PBS, pH 7.4). The platelets were aliquoted and frozen at -70°C for later use. Whole

platelets (not solubilised) were used in the enzyme-linked immunosorbent assay (ELISA). Platelets for ELISA were fixed with 4% paraformaldehyde prior to storage. Solubilised platelets (in SDS - see appendix) were used in the "Western Blotting" test.

(ii). *Crude schistosome antigens.*

Fresh or frozen adult worms or eggs were thawed and frozen repeatedly for upto ten times. They were homogenized manually with pestle and motor. The homogenate was centrifuged at 6,000g for 45 minutes at 4°C. The supernatant was separated from the pellet which was discarded. Protein estimation of the supernatant was done using the Bio-rad Protein Assay (1-30ug, Bio-rad Laboratories Ltd. U.K.) (see appendix). The worm and egg antigens were used at protein concentrations of 1.8mg/ml and 0.95mg/ml repectively. The antigen preparations were aliqouted and frozen at -20°C for short time storage and -70°C for long time storage. Crude worm homogenate (schistosome worm antigen (SWA)) was

used in all experiments as the adult worm antigen. Crude egg antigen was used as the schistosome egg antigen (SEA).

2.1.6. Antisera.

(i). *Anti-platelet serum* (APS).

Anti-platelet serum was raised in rabbits. Platelets were isolated from fresh mouse blood as in section 2.1.5 above. 20 X 10^9 platelets/ml were resuspended in 5ml PBS (pH 7.4). The suspension was injected into a white New Zealand rabbit intravenously in the marginal ear vein. Two injections were given over 14 days and the rabbit exsanguinated seven days after the last injection. The rabbit was bled by cardiac puncture and the blood drawn in test tubes. The blood was clotted at 37°C for one hour and kept at 4°C overnight. Serum was separated, aliquoted and stored at -20°C for short storage and -70°C for long storage.

(ii). *Anti-schistosome antigens sera.*

Antisera to schistosome worm and egg antigens were

obtained from infected human subjects and from experimentally infected mice. These antisera were then used in ELISA and in "Western Blotting" assays. A maximum of 0.2ml of blood was drawn from mice by the retroorbital sinus method while 5ml was drawn from human subjects from the arm vein each time they were bled. Some of this blood was drawn in Ethylenediamine tetra-acetic acid (EDTA) for platelet counts and haemogramme. The rest of the blood that was not drawn in anticoagulant was clotted at 37°C for one hour and kept at 4°C overnight. Serum was separated, aliquoted and stored at -20°C for short storage and -70°C for long storage.

2.1.7. *Reagents for serological procedures.*

All the reagents: Buffers, solutions and other chemicals used in performing the ELISA and "Western Blotting" tests are listed in the appendix.

2.2. METHODS.

2.2.1. *Schistosoma mansoni* infections.

(i). *Laboratory infection of mice.*

Groups of CBA and Balb/c mice were matched for age, sex and weight before they were used in the experiments. Two month-old mice were used in all experiments. Mice were infected by the ring method of Smithers and Terry (1967). Briefly 10-20 infected snails were placed in a beaker and left to shed cercariae under light. After one hour the shed cercariae were counted and the required number of cercariae per experiment taken. For chronic infections a dose of 25 cercariae/mouse was given while the number varied for acute infections depending on the experiment. Mice were anaesthetised with sagatal given intraperitonealy at a dose of 1mg/100g, shaven on the belly and a metal ring placed on the shaven part. A cercarial suspension with the required number of cercariae was then placed in the ring and the mouse left undisturbed for 30 minutes.

(ii). *Human patients.*

The human subjects recruited in the study were individuals from Machakos district infected with schistosomiasis. 140 patients were randomly recruited at the Machakos district hospital. The inclusion criteria were age between 13 and 45 years and a positive stool test for *S. mansoni*.

The exclusion criteria were age below 13 years and above 45 years, anaemia and pregnancy.

2.2.2. Parasitological and clinical examinations.

(i) *In mice.*

Weekly stool examinations for ova in the stool of infected mice were carried out. Stool was collected from each mouse then emulsified in double strength saline (1.8%) and sieved through a 300um wire mesh on Whatman's filter paper. A drop of Ninhydrin was placed on the filter. The filter paper was kept at 37°C and left to dry overnight. The eggs on the filter paper were then counted under the microscope at X40. The count was adjusted to egg count per gramme (EPG) of faeces. For liver egg counts,

livers were placed in trypsin overnight at 37°C for digesting liver tissue. The emulsion was washed with PBS (pH 7.2) until clear. Three replicates of the clean suspension were observed under the microscope and the eggs counted. The counts were adjusted to EPG of tissue.

(ii). *In human patients.*

Stool samples from the human subjects at the Machakos district hospital were examined using the "formal ether" concentration method as described by Ritchie (1948) and modified by Ridley and Hawgood (1956) to determine the presence of ova of *S. mansoni* in the stool. Positive patients were referred to the schistosomiasis clinic where they were examined. Relevant history: age, sex and water contact activities was recorded. The presence of other infections was also noted and finally an abdominal examination was done to check for hepatosplenomegaly. Another stool sample was taken for the "Kato thick smear" test as modified by Martin and Beaver (1968) to determine the EPG of

faeces. The patients were then treated with a single dose of 30mg praziquantel/kg body weight.

2.2.3. Haematological procedures.

Haemogramme levels were determined by Coulter Counter (Model M5 30, Coulter Electronics Ltd.). Whole blood was diluted at 1:500 in isotone and the values automatically calibrated. The eosinophil counts were determined from differential counts done microscopically on slide smears after staining with Giemsa stain and the counts adjusted to counts/μl. For platelet counts a 1:3,000 dilution of platelet rich plasma was electronically counted on the Coulter Thrombocytometer C (Coulter Electronics Ltd.). The sample haematocrit was set on the thrombocytometer and the instrument automatically converted the raw platelet count into the reportable platelet count. For confirmation platelets were counted microscopically according to the method by Ashman (1976). Briefly, 50μl of blood were added to 1ml of Baar's solution (3.5g sodium citrate, 0.25g saponin, 1ml formalin in 100ml

distilled water) to make a 1:20 dilution. The mixture was charged in a Neubaur haemocytometer, left in a moist chamber at 37°C for 40 minutes and counted at low power (X40). Alternatively 25μl of noncoagulated blood were added to 0.5ml of 1g% oxalic acid (to lyse the red blood cells) to make a 1:20 dilution (Ngaiza, 1988). The rest of the blood that was not drawn in anticoagulant was left to clot at 37°C for one hour, kept at 4°C overnight and sera separated as described in 2.1.6.

2.2.4. Treatment of mice with praziquantel.

Normal C57 mice bred and maintained at the KEMRI animal house were infected with 300 cercariae/mouse. A total of eight mice were infected for the experiment. Another eight mice were kept and used as the controls. Seven weeks after infection four of the infected mice were treated with praziquantel at a dose of 1,000 mg/kg body weight orally in a suspension. The remaining four mice in the same group were not treated. All mice were bled prior to infection and every week

after infection. Some of the blood was collected in EDTA for platelet counts and the rest clotted for serum. The results of the platelet counts in all the groups of mice with the course of infection are given in section 3.2.1.

2.2.5. Induction of thrombocytopaenia.

Anti-platelet antiserum (2.1.6.) was injected into mice at a dose of 25μl/mouse. At this dose thorombocytopaenia was induced without causing deleterious effects on leucocytes. A group of four mice was given APS and bled one hour later, then two days, seven days, nine days and fourteen days after infection. The control mice were bled equally. Platelets were isolated and counted as in section 2.1.5. The results are given in section 3.1.3.

(i). ***Effect of thrombocytopaenia on the longevity of mice.***

In this experiment mice were injected with 25μl APS as above to determine their longevity after becoming thrombocytopaenic. Four mice were given multiple doses of APS every day upto day twelve. Another group of four mice was given APS once only. Four mice were kept as the controls. Platelet counts were done every day and the results are given in section 3.1.3.

(ii). ***Thrombocytopaenia and infection with S. mansoni.***

The effect of thrombocytopaenia on the worm burden was investigated. APS was given to mice prior to infection with 200 cercariae/mouse. A booster injection was given at the same time of infection, another booster one day after infection and the last one on day three after infection. Platelet counts were done prior to infection and at every booster injection of APS. The mice were perfused on day 42 after infection and the worm load

determined. Liver egg counts were done to determine the egg load. The results of platelet counts, worm load and egg load are given in section 3.1.3.

(iii). *Effect of thrombocytopaenia on different stages of infection.*

The effect of thrombocytopaenia on different stages of infection with *S. mansoni* was investigated. One group of mice was infected with 200 cercariae/mouse. Another group was infected with the same dose of cercariae but given APS one day before infection, one day after infection and the last booster three days after infection. The third group was infected as above and given APS on days four, six and eight after infection. The mice were bled for platelet counts every time the APS was administered. The worm burden was determined on day 42 after infection. The results of the platelet counts and worm counts are given in section 3.1.3.

(iv). *Effect of platelets on the initial stages of infection.*

Mice were infected with 3,000 cercariae/mouse to investigate the effect of invading cercariae on platelets. The mice were bled and platelets counted before infection and every day after infection. The results of this experiment are given in section 3.1.3.

2.2.6. Detection of antibody by the enzyme-linked immunosorbent assay (ELISA).

Polyestyrene flatbottomed microtitre plates (Dynatech Laboratories, U.K) were coated with platelets and schistosome antigens. For anti-platelet antibodies the plates were coated with 25,000 platelets/well in 100μl coating buffer. (see appendix). For anti-worm and anti-egg antibodies, the plates were coated with 1.8 X 10^{-4}mg/well and 9.5 X 10^{-5}mg/well respectively. The plates were incubated at 37°C for one hour and then at 4°C overnight. The coated plates were washed 3X with washing buffer (with Tween, see appendix). They

were blocked with 1% Bovine Serum Albumin (BSA) for one hour at room temperature. The plates were again washed. 100μl serum at a dilution of 1:100 was added to each well and the plates incubated for 3 hours at 37°C.

The washing step was repeated and enzyme conjugate: (peroxidase-labelled goat anti-mouse IgG) was added to mouse sera wells while goat anti-human IgG was added to human sera wells. The plates were incubated for 2 hours at 37°C then washed again. 50μl substrate (Orthophenylene diamine (OPD) or 2-Azinobis(3-Ethylbenz-Thiazoline-6 sulphonic acid (ABTS)) (see appendix) was added and the plates left at room temperature for 15-20 minutes for the reaction to take place in the case of OPD substrate and stopped by 12% sulphuric acid. In the case of ABTS the reaction was left for 5 minutes and stopped by 1% SDS.

The plates were then read by a microplate reader (Dynatech Laboratories, U.K.). The ELISA results are given in section 3.3.

2.2.7. Detection of platelet antigens by sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) .

Platelet antigens which are reacted against by antibodies during infection by *S. mansoni* were characterised by electrophoresis. Whole platelets from mouse and human blood were solubilised in 0.2% SDS for 2 minutes and subjected to polyacrylamide gel electrophoresis on 8% and 12.5% gels (see appendix). The resolving gel was prepared and de-gassed. SDS, ammonium persulphate and tetramethylenediamine (TEMED) added to it. The gel was cast onto the glass plates then levelled off with N-butanol or distilled water. The resolving gel was rinsed with distilled water before the stacking gel was added and left to settle. The samples and markers were mixed with loading buffer (see appendix) at 20μl of sample: 5μl of loading buffer. The appropriate volumes of sample solution and markers were then drawn in a micro syringe and carefully injected into the wells through the reservoir buffer. The electrophoresis was run at

200 volts for 45 minutes or until the antigens and markers went through the gel. The gels were removed and placed in a basin with nitrocellulose and electroblotted for four hours at 40 mAmps or overnight at 16 mAmps.

The nitrocellulose paper was blocked with milk (1g% powder in 100ml tween transblotting solution, TTBS (see appendix) for one hour and kept at 4°C overnight. The markers were washed 2X in PBS (pH 7.2), incubated in PBS+0.3% tween for 30 minutes at 37°C, washed 3X in PBS tween at room temperature on a roller and then incubated overnight in protogold. The following day they were washed 2X with distilled water. Alternatively, after transblotting the nitrocellulose paper was stained with Ponceau solution (see appendix) diluted at 1:10 for 30 minutes. The paper was rinsed once with PBS and the markers cut out. The rest of the paper was rinsed 3X with PBS prior to blocking. The test nitrocellulose paper was cut into strips, incubated

with the test sera in troughs for four hours and washed 3X with TTBS. The strips were incubated with conjugate (Goat anti-mouse IgG for mouse sera and goat anti-human IgG for human sera) at a dilution of 1:1,000 for 2 hours then washed 3X with TTBS. Finally to observe the reaction the strips were placed in a solution containing 20mg of 4-chloro-1 naphthol in 4ml methanol for 10 minutes or when the colour change was observed. The strips were rinsed with distilled water, mounted on a clean sheet of paper alongside the molecular weight markers and the respective molecular weights marked. The results of this test are shown in the next chapter (section 3.4).

2.2.8. Parasite / platelet interactions.

(i). *Preparation of schistosomulae.*

Cercariae were shed from infected snails and concentrated with a vacuum pump. The cercariae were placed on ice in a universal tube to settle to the bottom. 0.5ml of medium 199 with antibiotics (100ml of medium: 3ml of penicillin/streptomycin was added

to the cercariae. The cercariae were mechanically transformed by drawing them up and down gently 10-14X in a 1ml syringe. The transformed cercariae were washed of the remaining cercariae with the above medium several times by placing the universal tube under the light, letting the schistosomulae settle under gravity and discarding the supernatant containing floating cercariae and tails. Newborn Bovine Serum (NBS) was added to the final wash and the schistosomulae counted under a low power microscope (X40).

(ii). *Incubation of schistosomulae with platelets.*
100 schistosomulae/well were placed in a 24 well polysterene plate (Dynatech Laboratories U.K.). Freshly washed platelets were resuspended in PBS Hepes (pH 7.4) and counted as described above. Platelet interaction with schistosomulae was observed from the time of incubation, 30 minutes later, 1 hour, 2 hours and 24 hours later. The schistosomulae were observed under low power

microscope (X40). The results of this observation are reported in section 3.5.1.

(iii). *Scanning electron microscopy of schistosomulae.*

Schistosomulae from the above preparation were fixed for 1.5 hours at 4°C in 4% grutaraldehyde buffered to pH 7.2 with phosphate buffer. They were washed overnight in fresh 0.1M phosphate buffer, dehydrated through a series of graded ethanol (25%, 50%, 75%, 100%) then alcohol aceton mixture (75% alcohol: 25% aceton, 50% alcohol: 50% aceton, 25% alcohol: 75% aceton, 100% aceton). The schistosomulae were critical point dried using a polaron critical point drier with CO_2 as the critical point liquid. The dried schistosomulae were removed from the sieve under a binocular microscope, mounted onto an aluminium stub and gold-coated in a polaron E500 sputter coating unit. The schistosomulae were then examined in a Hitachi-S-520 SEM operated in 2° electron mode. Electron micrographs of the schistosomulae with platelets

were prepared at various magnifications. The results of these preparations are given in section 3.5.2.

(iv). *Schistosomulae killing assay.*

Schistosomulae were cultured in 24 well polysterene plates in 199 medium at a concentration of 100 schistosomulae: 10^5 platelets/well. The plates were kept at 37°C in 5% CO_2 and the medium changed every day for three days. The degree of adherence of platelets onto schistosomulae was recorded daily and the rate of parasite death scored by the trypan Blue Dye exclusion test. All the schistosomulae from one well were drawn and examined each day, thus one separate well per day. The percentage death of the schistosomulae was recorded. The results of these experiments are given in section 3.5.3.

CHAPTER THREE.

RESULTS.

3.1. PLATELET DEPLETION AND PARASITOLOGICAL FINDINGS.

3.1.1. In mice.

Platelet counts were done every two weeks. Table 3.1 shows platelet counts before infection compared with six and fifteen weeks post infection. Six weeks after infection platelet levels began to decline and by the fifteenth week they were significantly low ($P<0.01$). The number of eggs found in the stools and livers were indicative of a typical chronic infection showing that the changes in platelet levels observed occured in the course of a chronic *Schistosoma mansoni* infection.

3.1.2. In human patients.

Platelet counts were done before and after treatment of human patients from Machakos District

Hospital who had been confirmed to have *S. mansoni* by faecal examination (2.2.1.). The results are represented by Table 3.2. 22 out of 140 (15%) of the patients had low platelet counts before treatment. Three weeks after treatment the counts had significantly reverted back to normal ($P<0.01$, by the 'Paired *t*-test'). Only 7 patients turned up for the 3 weeks follow up after treatment. The observed thrombocytopaenia was not related to the age or sex of the individuals. There also did not seem to be a correlation between egg output and thrombocytopaenia. The haemoglobin levels of the subjects remained within normal ranges of 14-18g/dL for males and 12-16g/dL for females. However, all but two subjects had eosinophil levels of 0.250 x 10^9/l or above. The non-thrombocytopaenic patients also had raised platelet levels after treatment compared to those before treatment suggesting that treatment with praziquantel enhances the general health status of the patients.

	Weeks after infection					
	0		6		15	
Platelet count	NM	IM	NM	IM	NM	IM
$\bar{X}$	946	723	944	632	948	384
±	±	±	±	±	±	±
SD	89	91	78	127	82	329
		NS		***		***

TABLE 3.1:

Mean Platelet counts in groups of mice, before infection, at six and fifteen weeks post infection with *S. mansoni*.

NM = normal mice

IM = infected mice

NS = not significant.

*** = significant ($P<0.01$), n = 12

		Plat count ($x10^3/\mu l$)	wbc count ($x10^3/\mu l$)	Hb (g/dL)	Eos counts ($x10^9/l$)
(i)	$\bar{X}$	91.2	5.5	14.3	0.35
	±	±	±	±	±
	SD	31.9	2.5	1.4	0.3
(ii)	$\bar{X}$	198.2	6.5	13.4	0.30
	±	±	±	±	±
	SD	66.6	1.3	1.9	0.1

TABLE 3.2:

Mean platelet, wbc, eosinophil counts and haemoglobin levels in *S. mansoni* patients before treatment (i) and three weeks after treatment (ii). Apart from platelet counts which showed significant rise after treatment ($P<0.01$, $n = 7$) all other parameters remained unchanged.

3.1.3. Induction of thrombocytopaenia.

(i). *Effect of anti-platelet sera (APS) on platelets.*

Fig. 3.1 shows the effect of APS on platelet levels. Platelet depletion was observed as early as one hour after injection. By the second day, all the mice had become thrombocytopaenic compared with the normal controls that were not given the APS. The mice recovered from this state within two days and remained normal upto day fourteen.

(ii). *Effect of thrombocytopaenia on the longevity of mice.*

Fig. 3.2 shows the effect of thrombocytopaenia on the survival of mice. Platelet counts were done starting one hour, affter injection then two days, seven days, nine days snd fourteen days later. All the four mice given multiple doses of APS lived upto the sixth day while two of them died on the eighth day. The mice most probably died due to the thrombocytopaenic state. The remaining two mice lived upto day twelve when no more APS was given.

Three days after the last APS injection the platelet levels of the two mice began to rise.

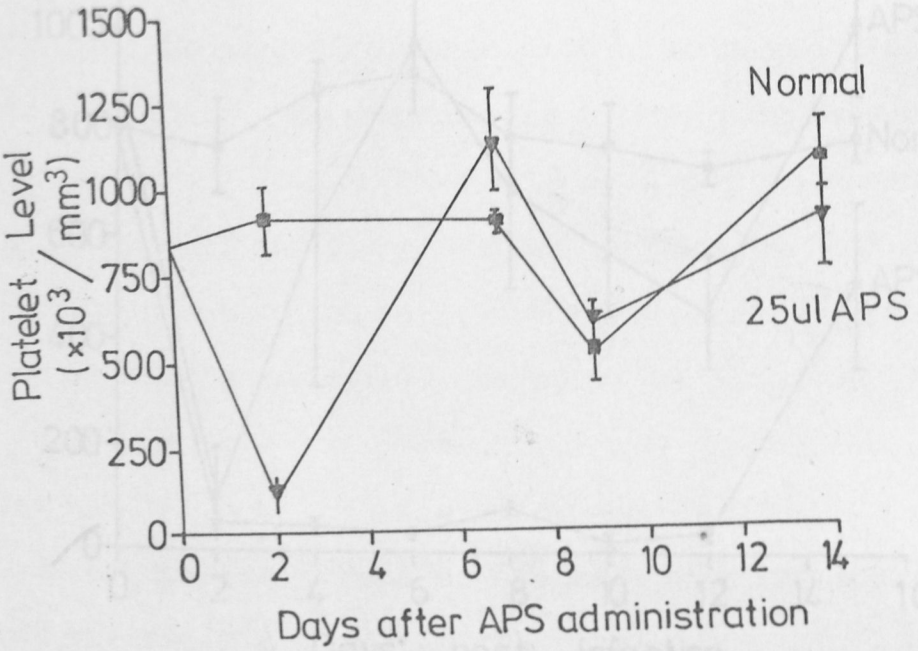


Figure 3.1:

Mean platelet counts in mice treated with APS. The counts reverted to normal levels within two days.

■ = Control mice

▼ = APS treated mice

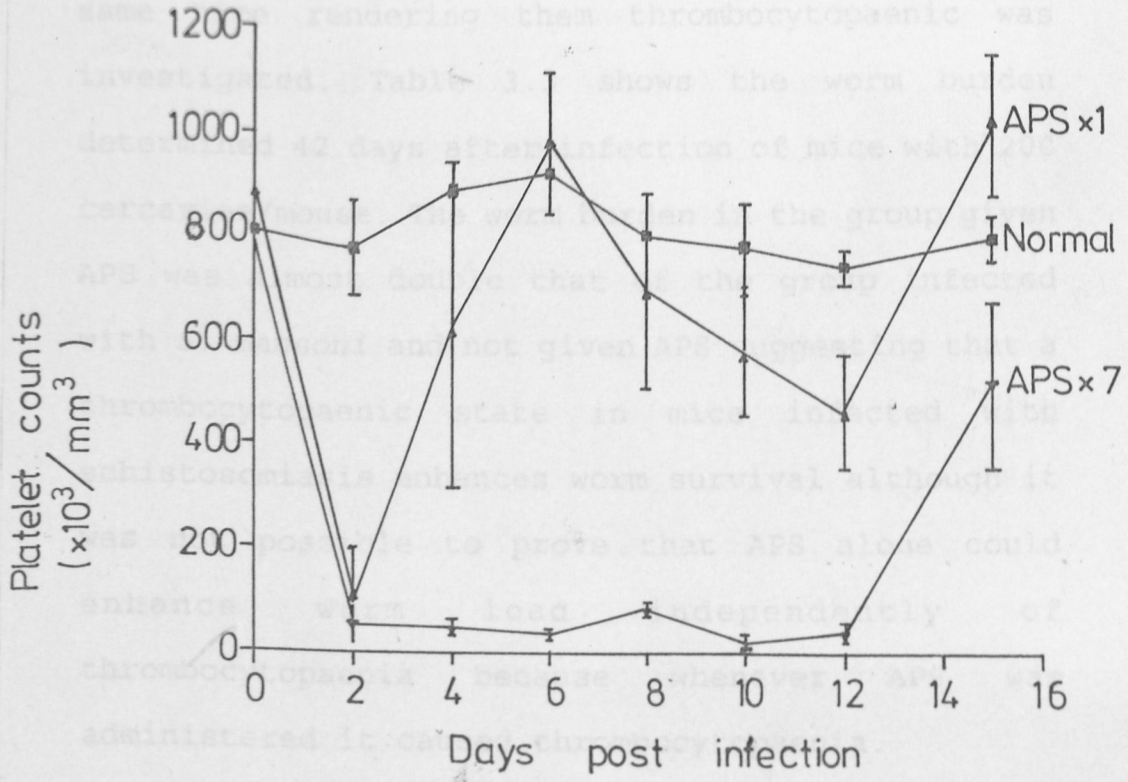


Figure 3.2:

Thrombocytopaenic effects of single or multiple anti - platelet serum treatment of two groups of uninfected mice.

Group (APS x 1) - given a single treatment of APS, Group (APS x 7) - given multiple treatments of APS, Group (Normal) - Untreated controls

(iii). ***Thrombocytopaenia and infection with S. mansoni.***

The effect of infecting mice with *S. mansoni* at the same time rendering them thrombocytopaenic was investigated. Table 3.3 shows the worm burden determined 42 days after infection of mice with 200 cercariae/mouse. The worm burden in the group given APS was almost double that of the group infected with *S. mansoni* and not given APS suggesting that a thrombocytopaenic state in mice infected with schistosomiasis enhances worm survival although it was not possible to prove that APS alone could enhance worm load independently of thrombocytopaenia because whenever APS was administered it caused thrombocytopaenia.

The liver egg counts were correspondingly high in the APS treated group. Fig. 3.3 shows the platelet levels in the two groups. Platelet counts dropped immediately after treatment with APS and remained low upto day six. There was a drop in platelet counts on the second day in the control mice, a

phenomenon that was noted in most experiments but there was no clear explanation for this.

(iv). *Effect of thrombocytopaenia at different stages of infection with S. mansoni.*

The mice that were given APS upto day three post infection with 200 cercariae/mouse had a double worm load compared with controls that were not treated with APS at all and those that were treated from the fourth day post infection. This suggested that worm survival was enhanced when thrombocytopaenia was induced at the skin stage of infection (Table 3.4).

(v). *Platelets and invading schistosomes.*

Platelet depletion in early stages of infection was investigated. Mice were infected with 3,000 cercariae/mouse and platelet counts done. Table 3.5 shows that there was more platelet depletion at the skin stage of infection than at the lung stage.

Group	APS Treated	Worm burden		Egg count	
		$\bar{X}$	SD	$\bar{X}$	SD
A	-	53	± 4	23,733	± 3973
B	+	93	± 8	46,880	± 9943

TABLE 3.3:

Worm burdens and liver egg counts of two groups of mice infected with 200 cercariae of *S. mansoni*.

Group A mice not treated with Anti-platelet serum (APS) at the time of infection. Group B mice treated with APS at the time of infection.

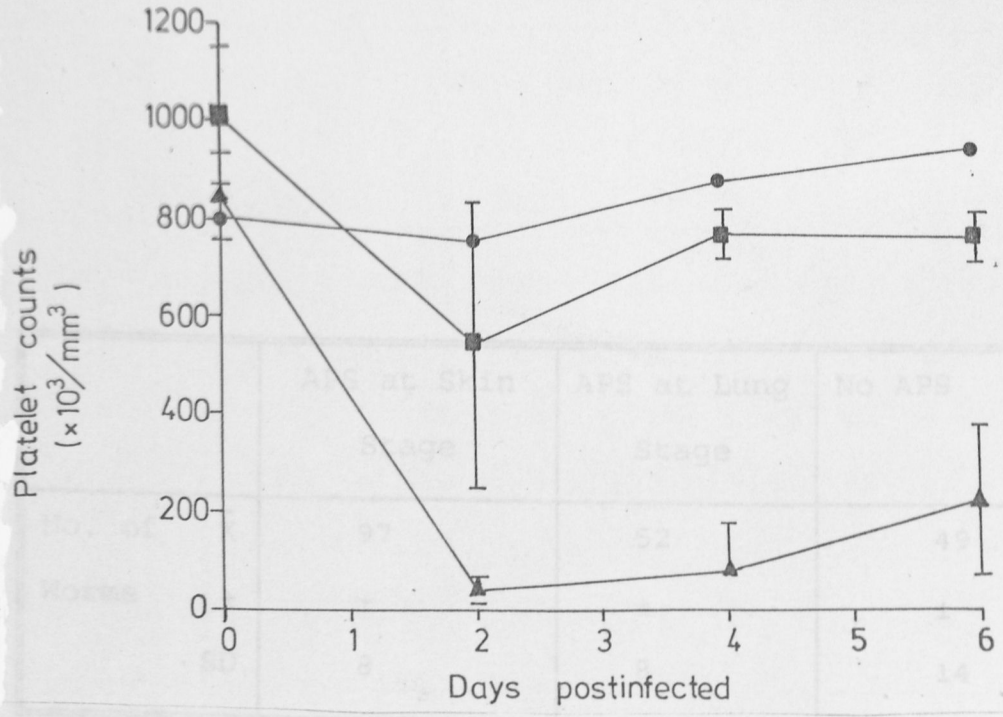


Figure 3.3:

Mean platelet counts in two groups of mice infected with 200 cercariae of *S. mansoni* and treated with APS.

▲ = Infected with 200 cercariae and treated with APS

■ = Infected with 200 cercariae but not treated with APS

● = Uninfected, untreated controls.

		APS at Skin Stage	APS at Lung Stage	No APS
No. of Worms	$\bar{X}$ ± SD	97 ± 8	52 ± 8	49 ± 14

TABLE 3.4:

Worm burden of two groups of mice infected with 200 cercariae *S. mansoni* and treated with APS at the skin stage and lung stage.

Group 1 mice treated with APS at the skin stage, Group 2 mice treated with APS at the lung stage, Group 3 mice not infected and not treated with APS

	Days post infection							
	0	1	2	3	4	5	6	7
NM $\bar{X}$	935	998	802	908	1010	1034	903	835
± SD	91	49	32	57	18	40	18	34
IM $\bar{X}$	906	575	618	708	825	816	749	666
± SD	78	23	94	95	73	130	37	22

TABLE 3.5:

Platelet counts at the skin stage of *S. mansoni* infection in mice.

Mice were infected with 3,000 cerariae/mouse and platelet counts monitored. The count was lowest on the first and second days post infection. NM = Normal Mice, IM = Infected Mice, $P<0.05$ on day 1, n = 12.

3.2. EFFECT OF PRAZIQUANTEL ON PLATELET LEVELS IN MICE INFECTED WITH *SCHISTOSOMA mansoni*.

3.2.1. Platelet levels and infection with *Schistosoma mansoni* in mice.

Platelet counts began declining at five weeks post infection with 300 cercariae/mouse *S. mansoni*. Fig. 3.4 shows the results of the experiment. A total of eight mice were infected while another eight were used as the uninfected controls. At six weeks post infection all the infected mice were thrombocytopaenic. At seven weeks after infection four of the eight infected mice were treated with 1,000 mg/kg body weight praziquantel given orally in a suspension. Following treatment, the mean level of platelet counts for this group showed a dramatic rise while the untreated group remained thrombocytopaenic till the end of the experimental period.

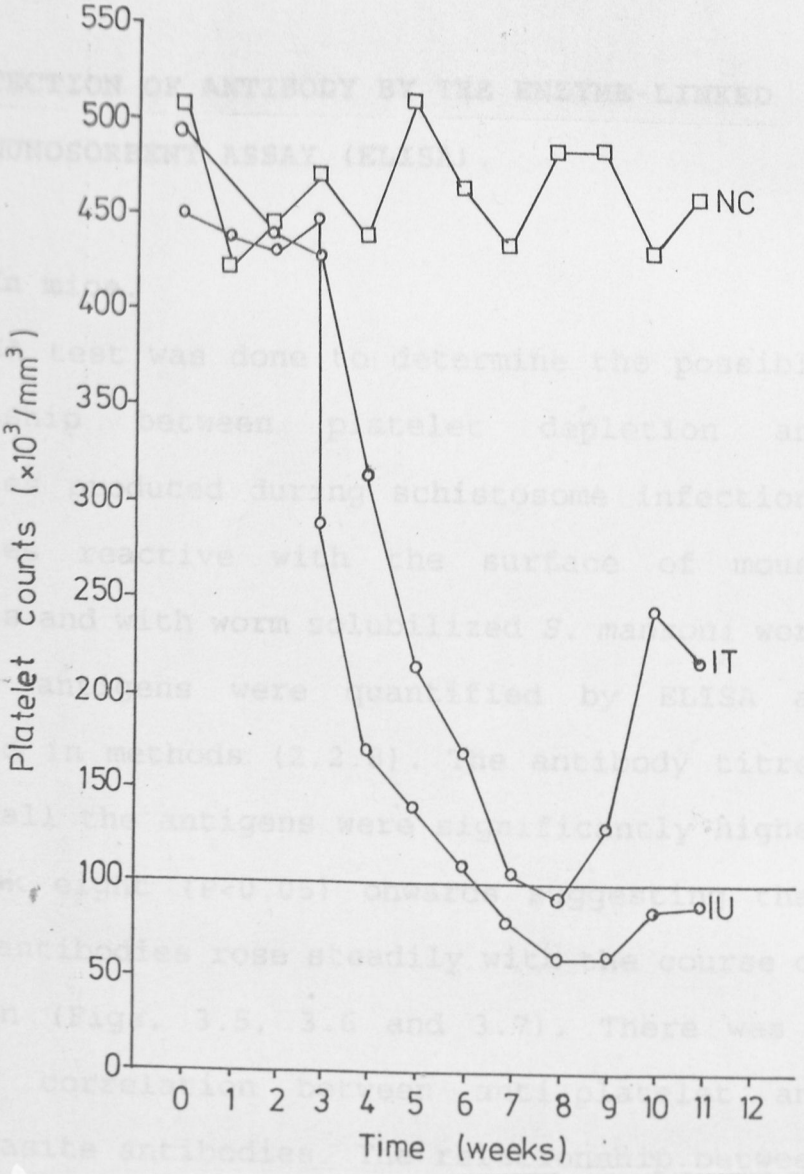


Figure 3.4:

Mean Platelet counts in three groups of mice namely Group (TI) infected then treated after six weeks, Group (IU) infected and not treated and Group (NC) which is the non infected control.

3.3. DETECTION OF ANTIBODY BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

3.3.1. In mice.

The ELISA test was done to determine the possible relationship between platelet depletion and antibodies produced during schistosome infection. Antibodies reactive with the surface of mouse platelets and with worm solubilized *S. mansoni* worm and egg antigens were quantified by ELISA as described in methods (2.2.8). The antibody titres against all the antigens were significantly higher from week eight ($P<0.05$) onwards suggesting that all the antibodies rose steadily with the course of infection (Figs. 3.5, 3.6 and 3.7). There was a positive correlation between anti-platelet and anti-parasite antibodies. The relationship between anti-egg and anti-platelet antibodies was positive but not significant ($R = 0.32$, Fig. 3.8), however that between anti-worm and anti-platelet antibodies was significant ($R = 41$, $P<0.05$, Fig. 3.9). These results suggest that thrombocytopaenia in mice may

be due to autoimmune reactivity against platelet antigens and that these may resemble parasite antigens.

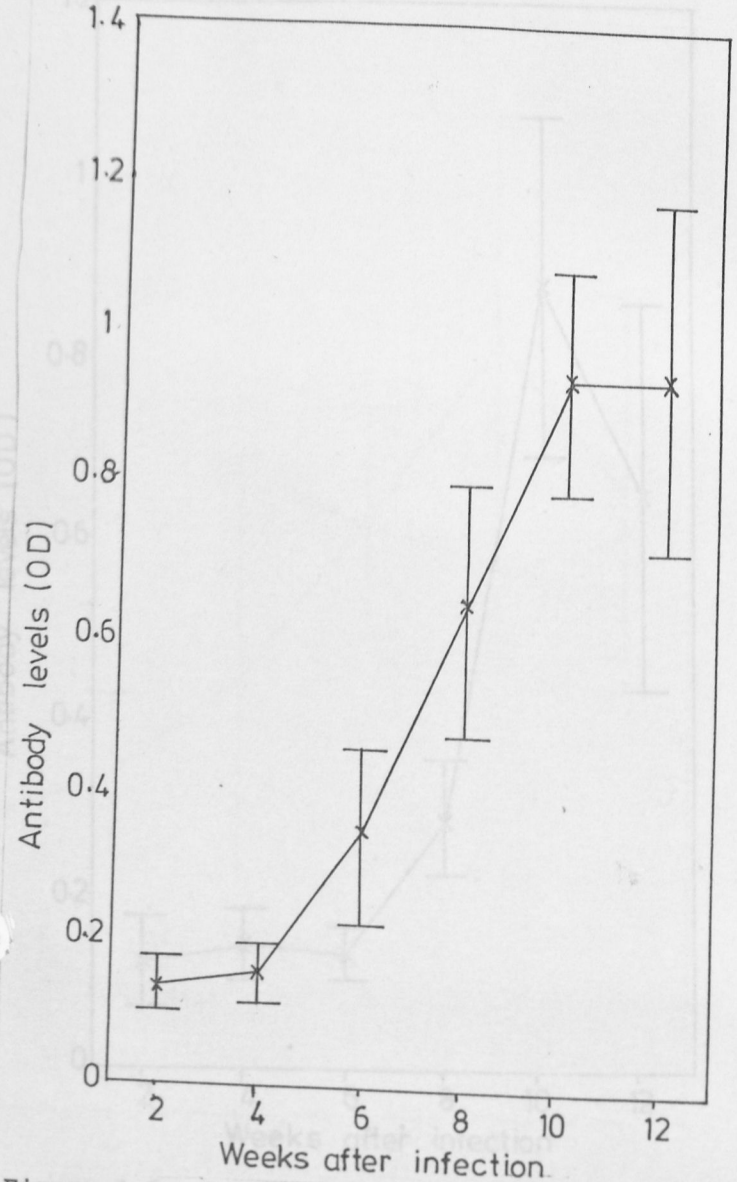


Figure 3.5:

Mean levels of anti-worm antibody detected by ELISA in mice during the first twelve weeks of *S. mansoni* infection.

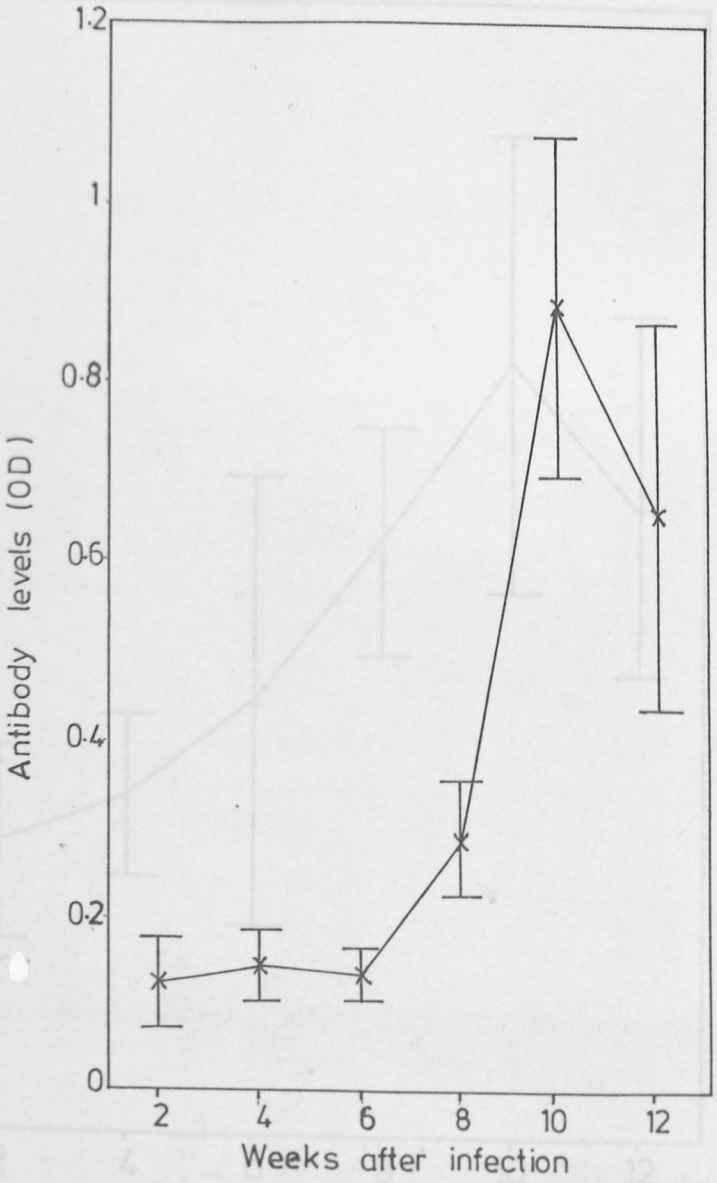


Figure 3.6:

Mean levels of anti-egg antibody detected by ELISA in mice during the first twelve weeks of *S.mansoni* infection.

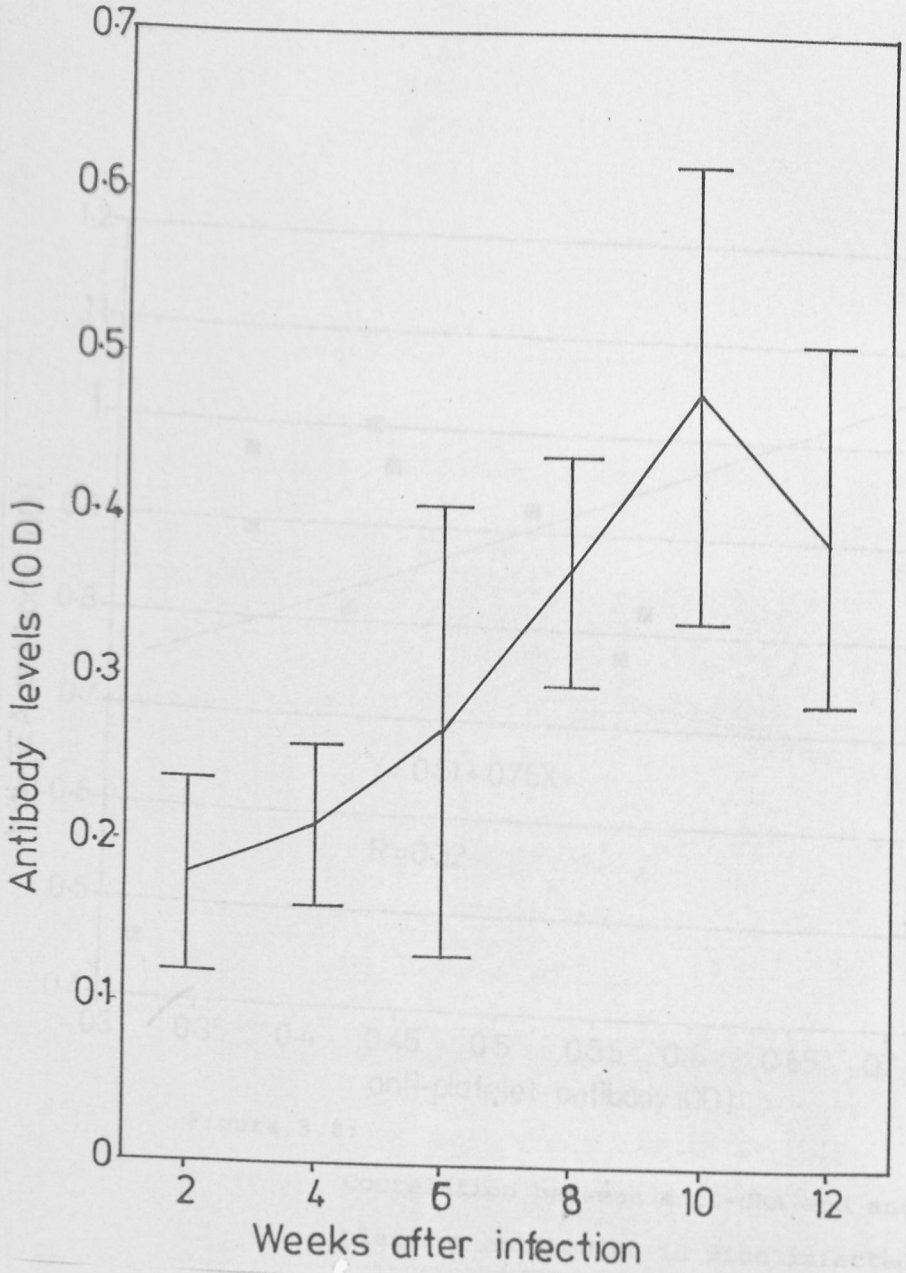


Figure 3.7:

Mean levels of anti-platelet antibody detected by ELISA in mice during the first twelve weeks of *S. mansoni* infection.

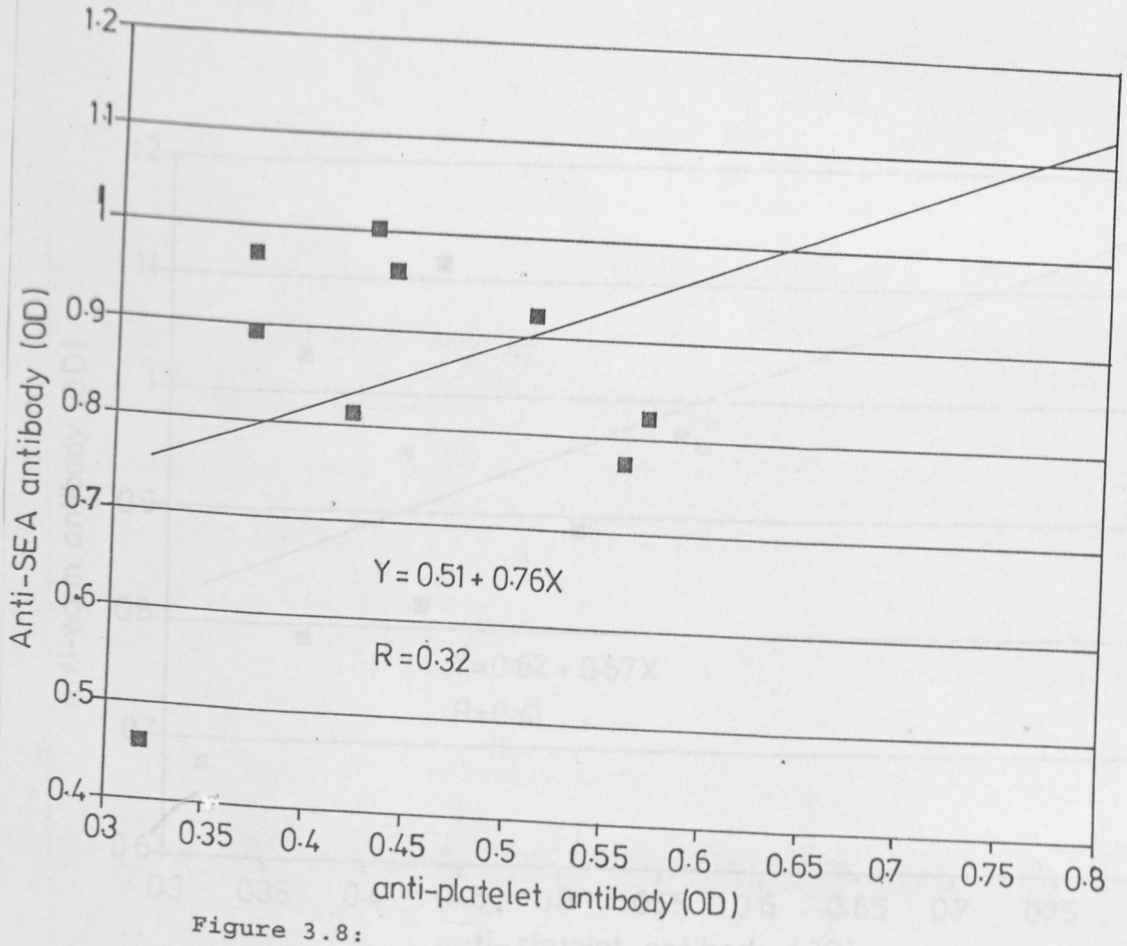


Figure 3.8:

Correlation between anti-SEA and anti-platelet antibodies in mice infected with *S. mansoni* at 15 weeks post infection. The points represent optical densities for 10 individual mice sera obtained 15 weeks post infection, and assayed against two antigens (i) soluble egg antigen (SEA) and (ii) platelet antigen.

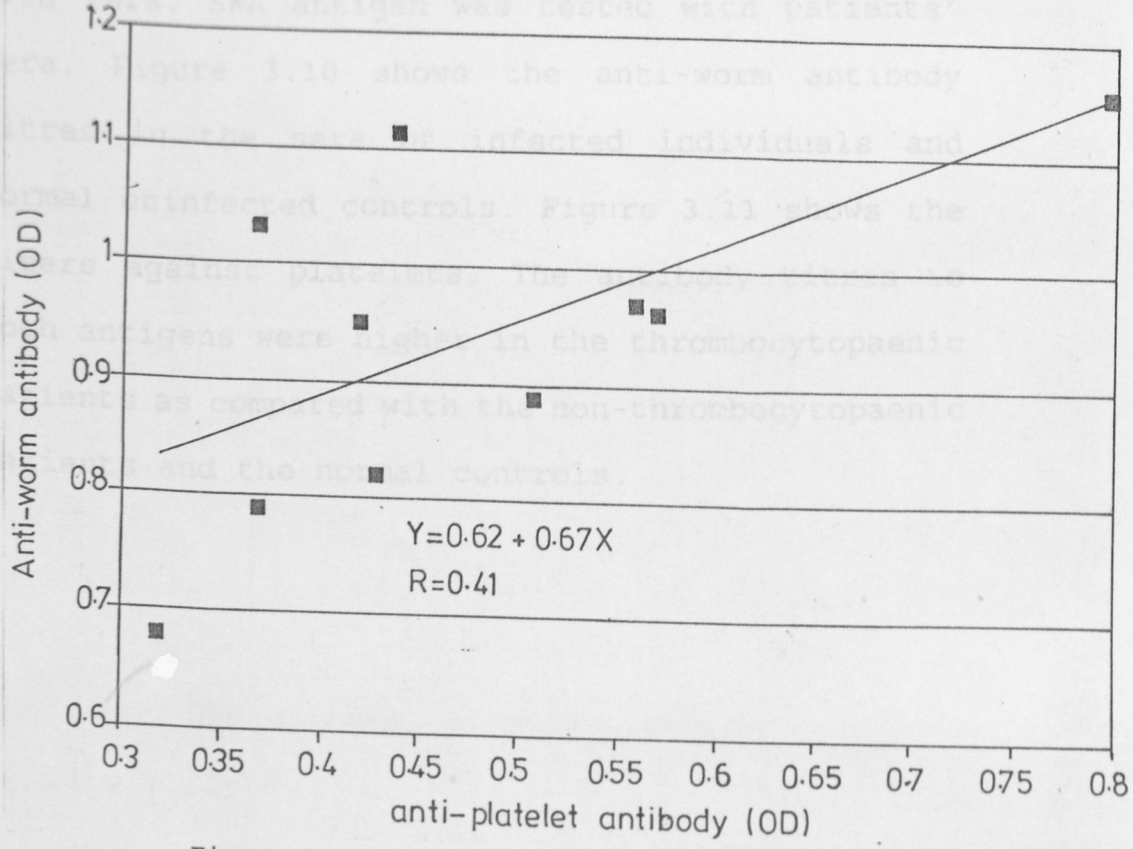


Figure 3.9:

Correlation between anti-worm and anti-platelet antibodies in mice infected with *S. mansoni* at 15 weeks post infection. The points represent optical densities for 10 individual mice sera obtained 15 weeks post infection and assayed against two antigens (i) worm antigen and (ii) platelet antigen.

3.3.2. In human subjects.

Human sera were similarly tested by ELISA as the mice sera. SWA antigen was tested with patients' sera. Figure 3.10 shows the anti-worm antibody titres in the sera of infected individuals and normal uninfected controls. Figure 3.11 shows the titers against platelets. The antibody titres to both antigens were higher in the thrombocytopaenic patients as compared with the non-thrombocytopaenic patients and the normal controls.

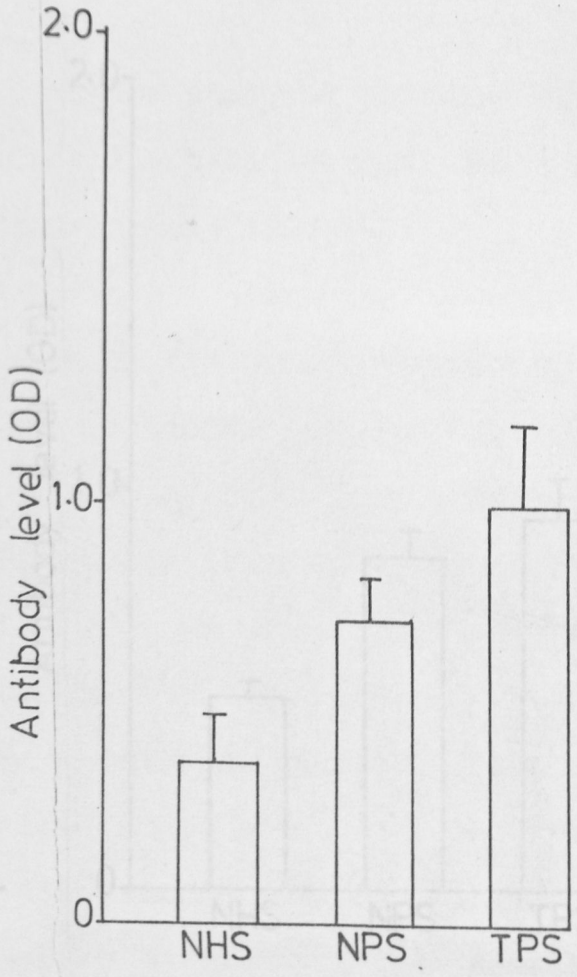


Figure 3.10:

Mean levels of Anti-SWA antibodies in two groups of patients infected with *S. mansoni*. Group 1 = Thrombocytopaenic patients (TPS) Group 2 = Non-thrombocytopaenic patients (NPS) NHS = Normal human sera

$P < 0.01$, $n = 7$

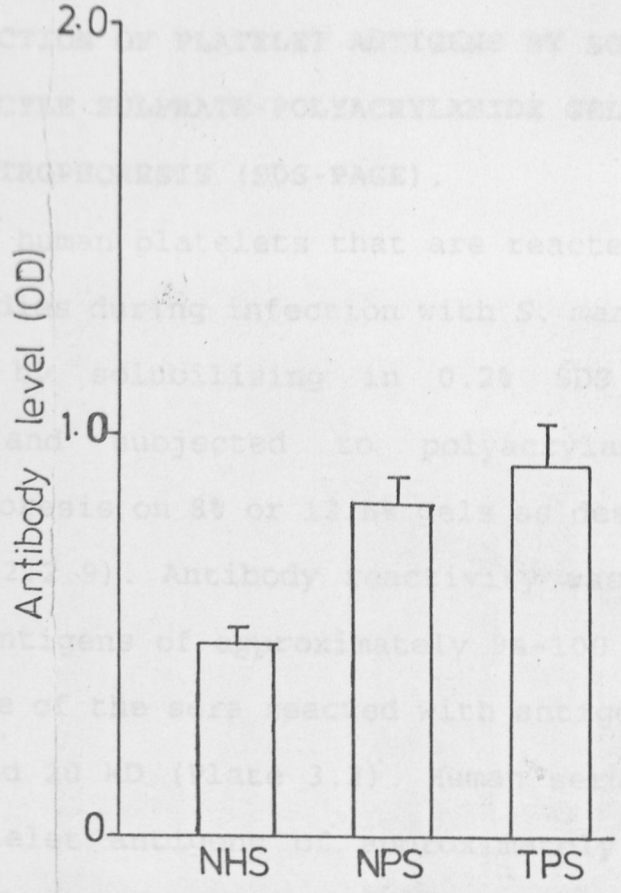


Figure 3.11:

Mean levels of Anti-platelet antibodies in two groups of patients infected with *S. mansoni*.

Group 1 = Thrombocytopaenic patients (TPS), Group 2 = Non-thrombocytopaenic patients (NPS), NHS = Normal human sera. $P<0.01$, $n = 7$

3.4. DETECTION OF PLATELET ANTIGENS BY SODIUM DODECYLE SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

Mouse and human platelets that are reacted against by antibodies during infection with *S. mansoni* were detected by solubilising in 0.2% SDS for two minutes and subjected to polyacrylamide gel electrophoresis on 8% or 12.5% gels as described in methods (2.2.9). Antibody reactivity was detected against antigens of approximately 94-100 kD (Plate 3.1). Some of the sera reacted with antigens of 67, 40, 33 and 20 kD (Plate 3.2). Human serum reacted with platelet antigens of approximately 110, 94, 67, and 50 kD (Plate 3.3). A comparison of the platelet and parasite antigens revealed that human serum reacted with both antigens of approximately 50 and 25 kD (Plate 3.4). In addition the serum reacted with a platelet antigen of 38 kD, with worm antigens of 70, 18 and 10 kD and with egg antigens of 30, 26, 18 and 6 kD.

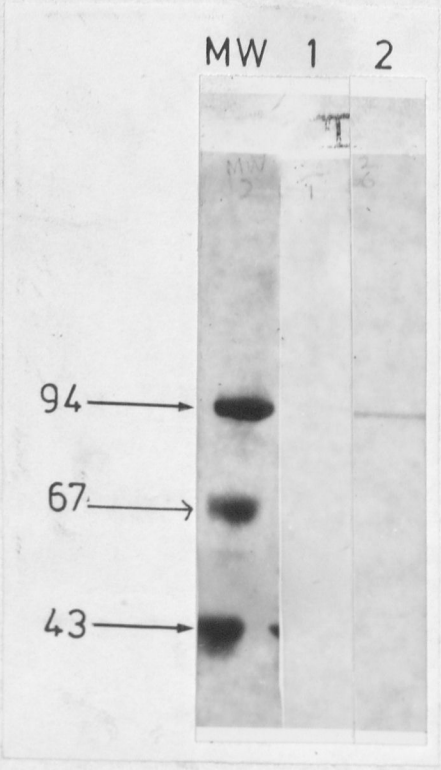


Plate 3.1:

Immunoblot analysis of mouse platelet antigens recognized by pooled sera from *S. mansoni* chronically infected mice

1 = normal mouse serum

2 = pooled chronic infection sera

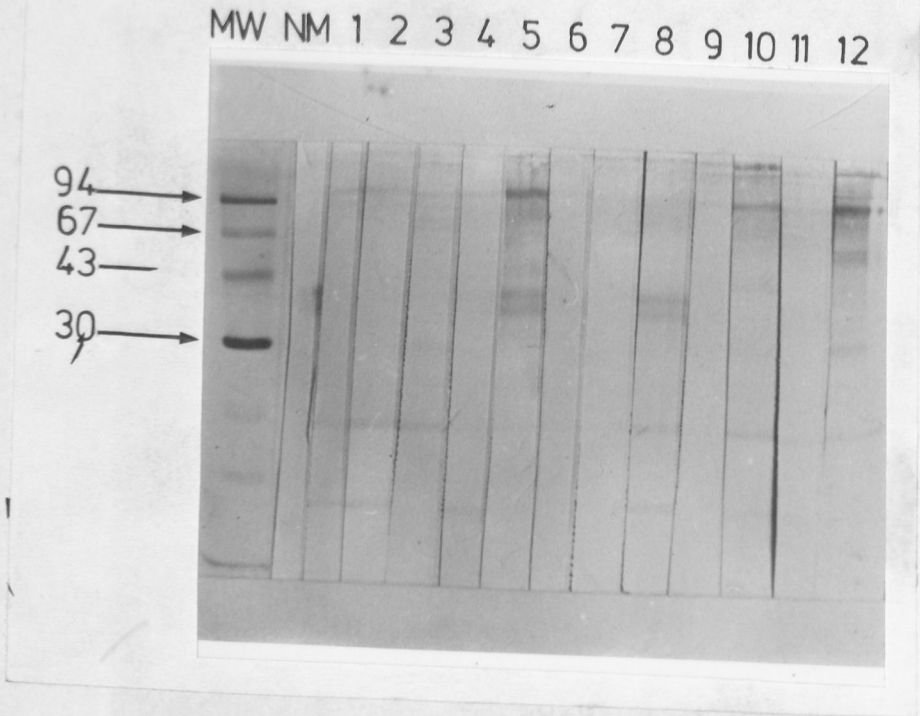


Plate 3.2:

Immunoblot analysis of mouse platelet antigens recognized by sera from individual mice chronically infected with *S. mansoni*

MW = molecular weight markers

NM = normal mouse serum

1 - 12 individual mouse sera

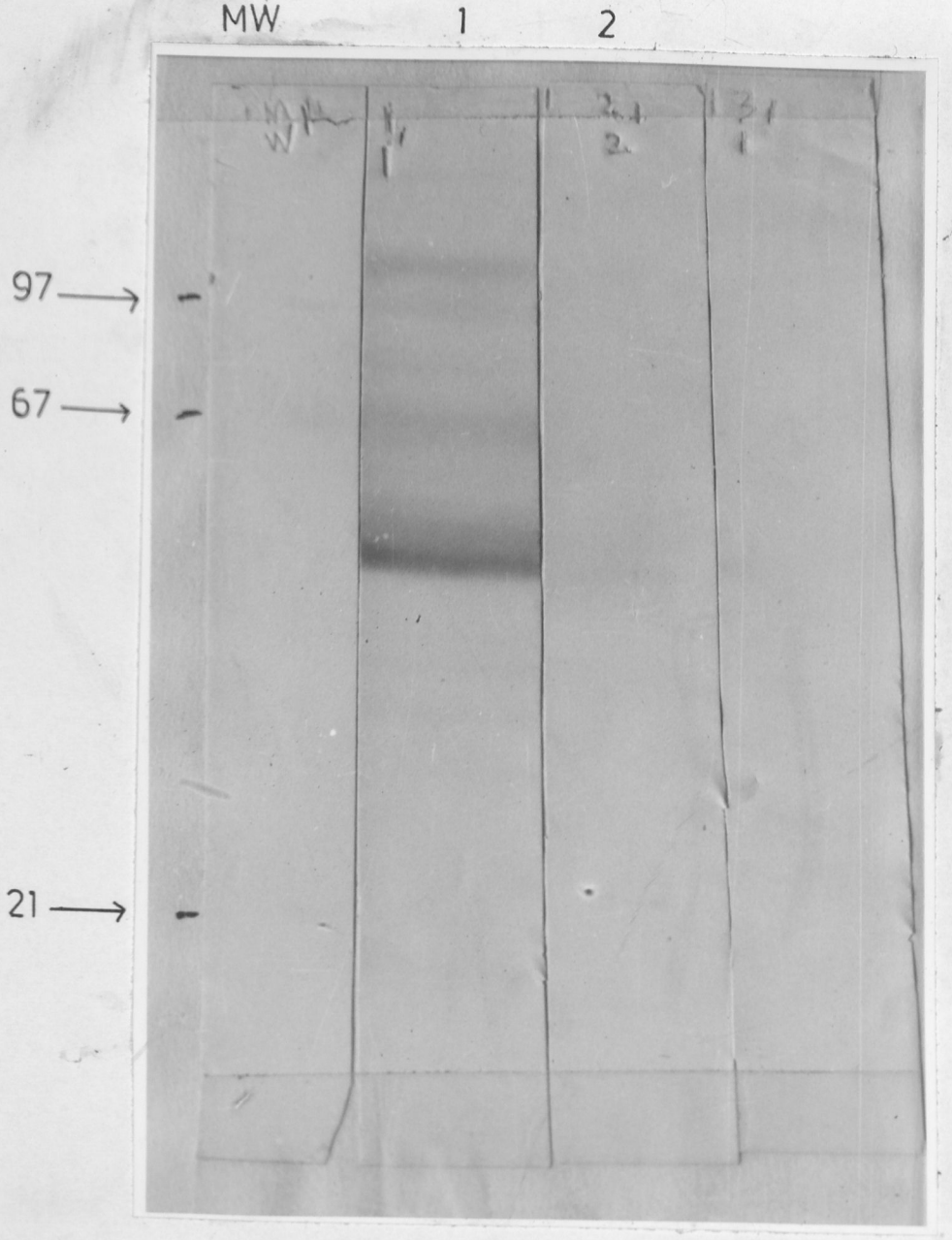


Plate 3.3:

Immunoblot analysis of human platelet antigens recognized by human serum from a *S. mansoni* infected individual

1 = patient serum

2 = normal human serum

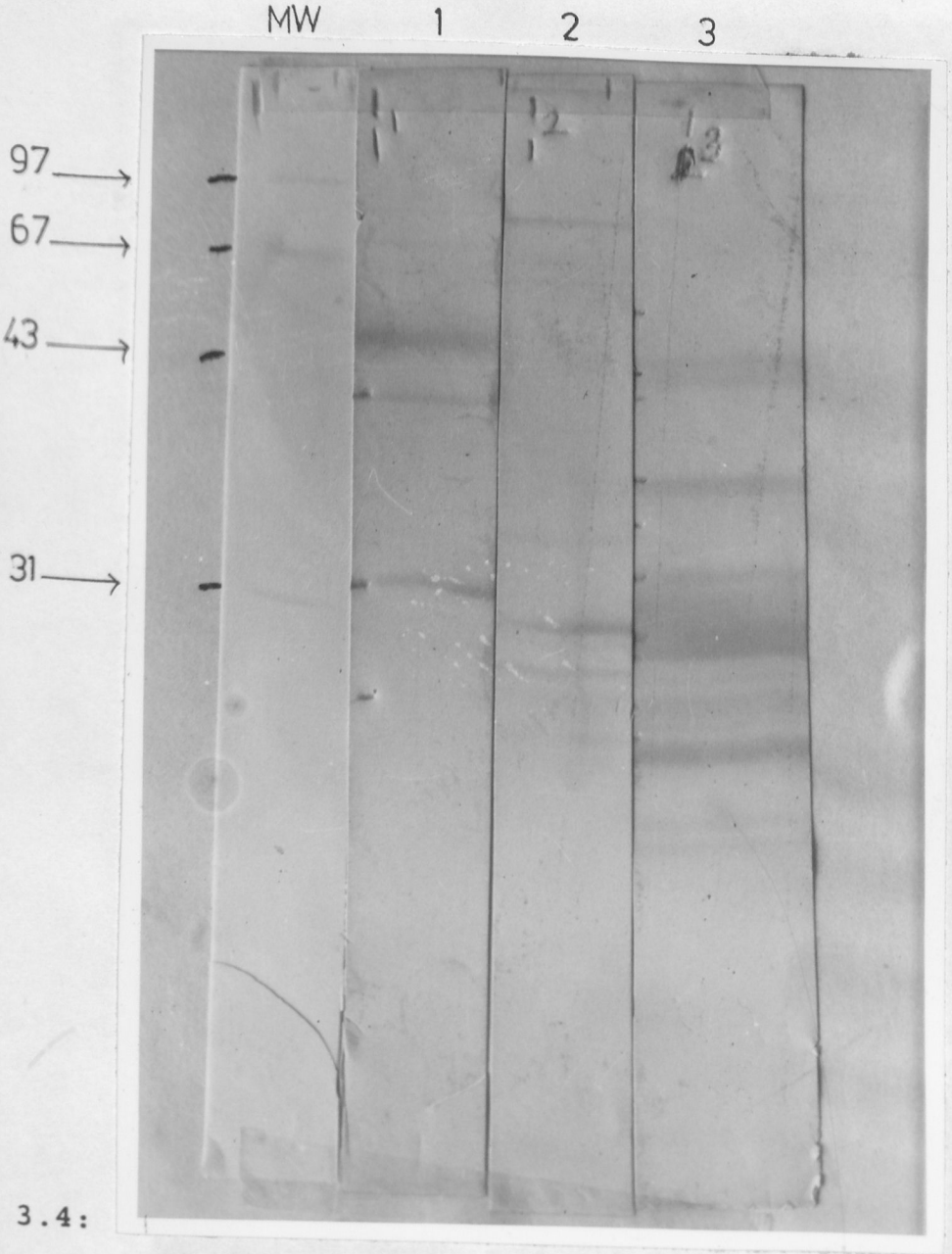


Plate 3.4:

A comparison of platelet and parasite antigens in immunoblot analysis with *S. mansoni* patient serum.

1 = platelet antigen

2 = worm antigen

3 = Egg antigen

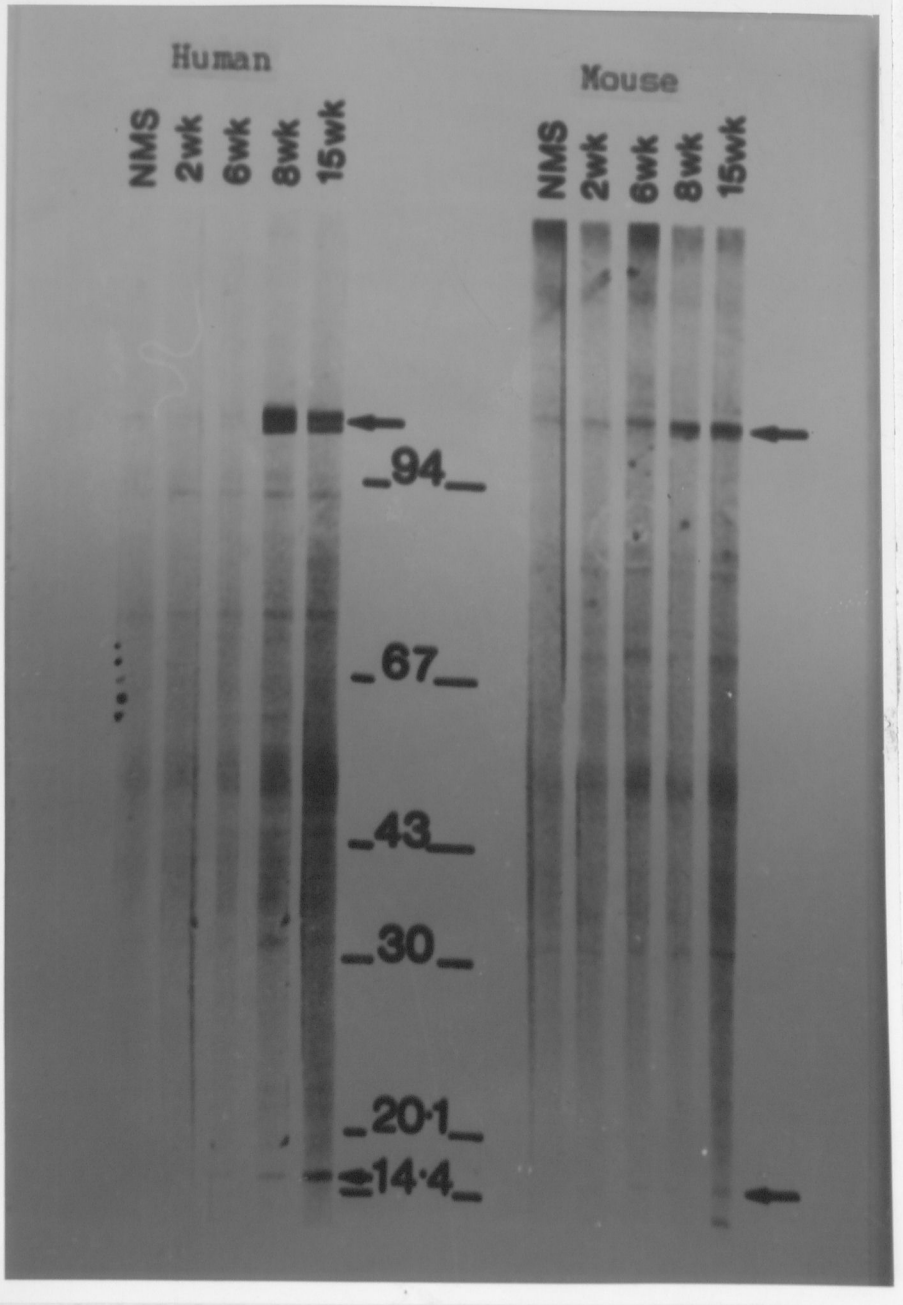


Plate 3.5: Immunoblot analysis of mouse and human platelet antigens recognized by sera from *S. mansoni*-infected mice - time course. *(With permission from Ngaiza's thesis, University of London, 1988)*

3.5. PARASITE / PLATELET INTERACTIONS.

3.5.1. Incubation of schistosomulae with platelets.

Schistosomulae were incubated with freshly washed platelets and platelet attack on the schistosomulae observed at various time intervals. Platelets began to attack the schistosomulae within 30 minutes of incubation and after one hour about 50% of the schistosomulae had platelets on their surfaces. Plate 3.6 shows a schistosomulum with platelets attached to its surface 2 hours after incubation. Plate 3.7 shows a schistosomulum without platelets on its surface.

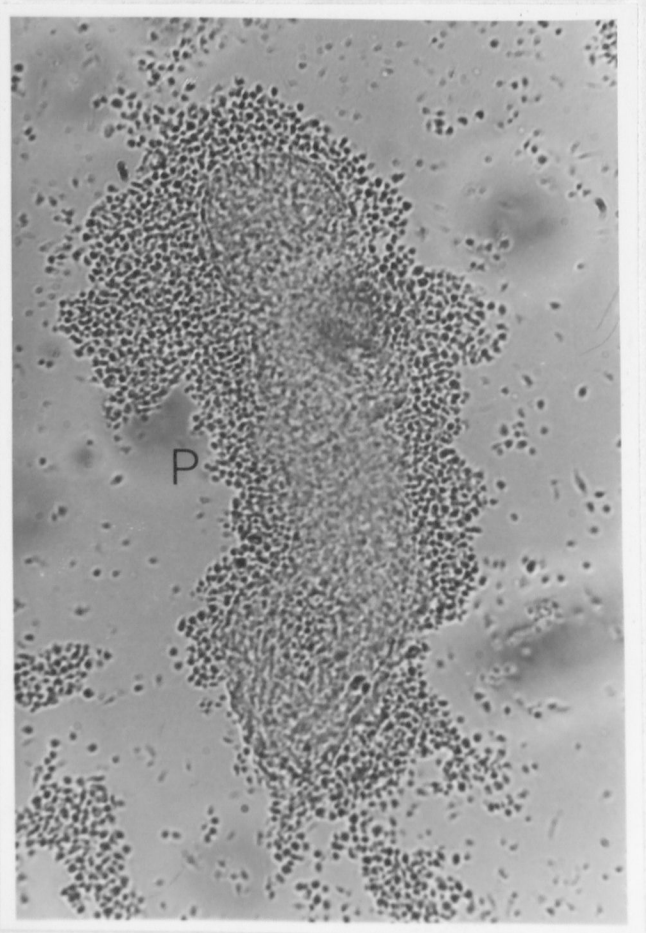


Plate 3.6: Schistosomulum with platelets on the surface.

X160, P = Platelets

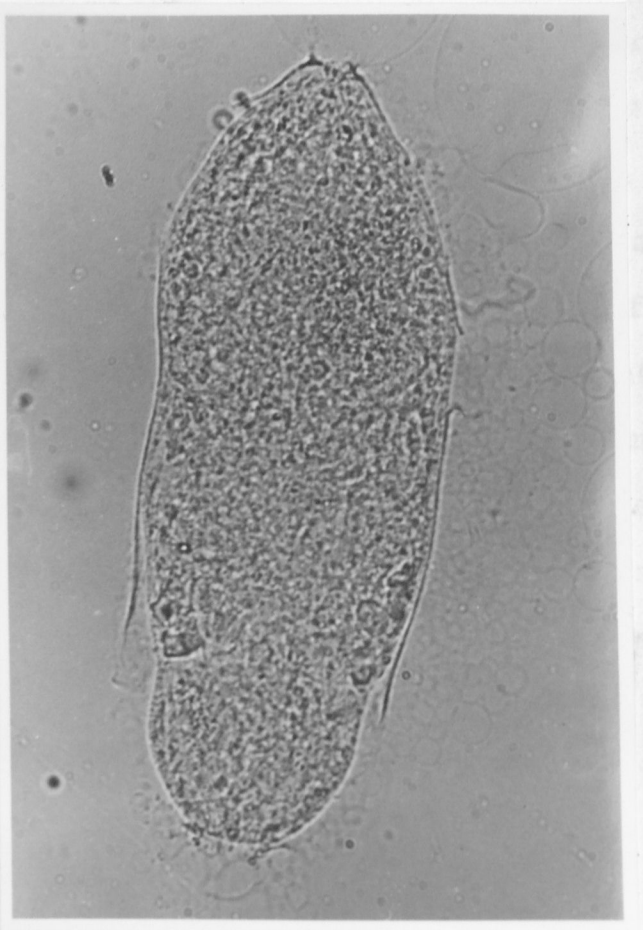


Plate 3.7:

Schistosomulum with no platelets on the surface.

X160

3.5.2. Scanning electron microscopy of schistosomulae with platelets on the surface.

The schistosmulae from the above incubation (3.5.1) were prepared for Scanning Electron Microscopy. Plate 3.8 shows a platelet aggregate. Plate 3.9 shows platelet aggregates covering the entire body of the schistosomulum while plate 3.10 shows platelets aggregated at the posterior end of the worm. Plate 3.11 shows platelets aggregating at the point where the schistosomulum detached from the cercaria. Plate 3.12 & 3.13 show platelets interacting with the worm surface. Protruberances from the platelet surface were seen to interact with the tubercles on the worm surface.

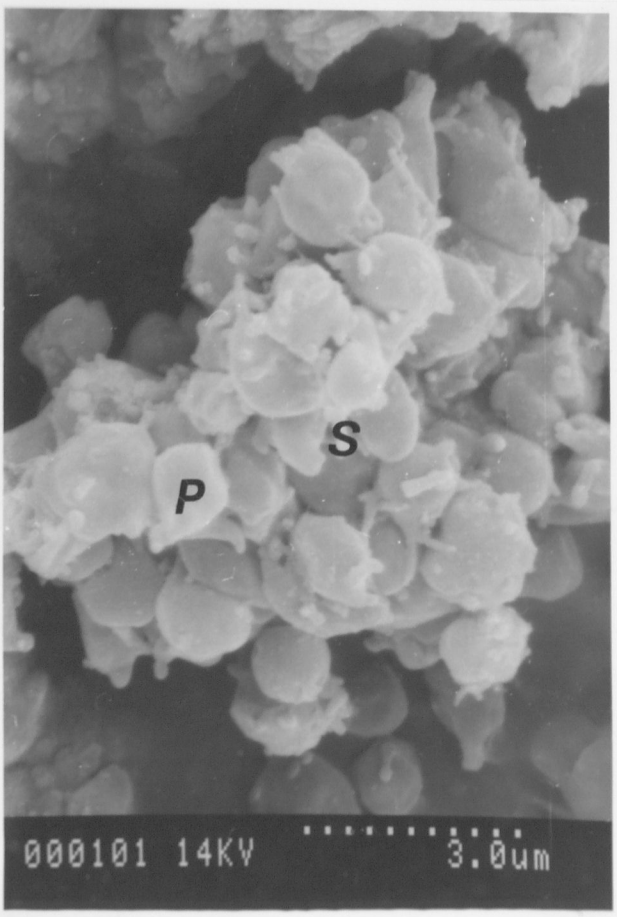


Plate 3.8: Scanning electron micrograph of a platelet aggregate.

X11,000

P = platelets

S = pseudopods

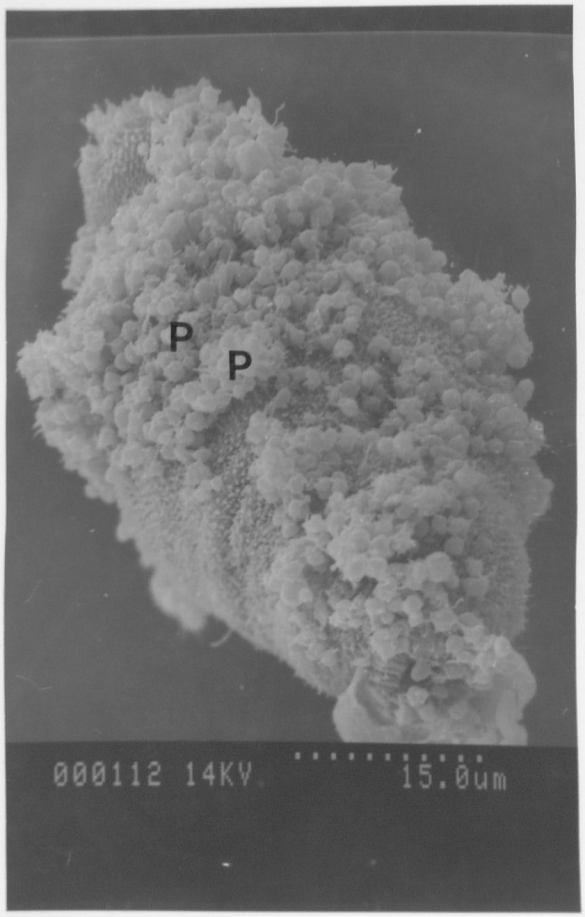


Plate 3.9:

Scanning electron micrograph of schistosomulum with platelets covering the whole surface.

X8,000

p = platelets

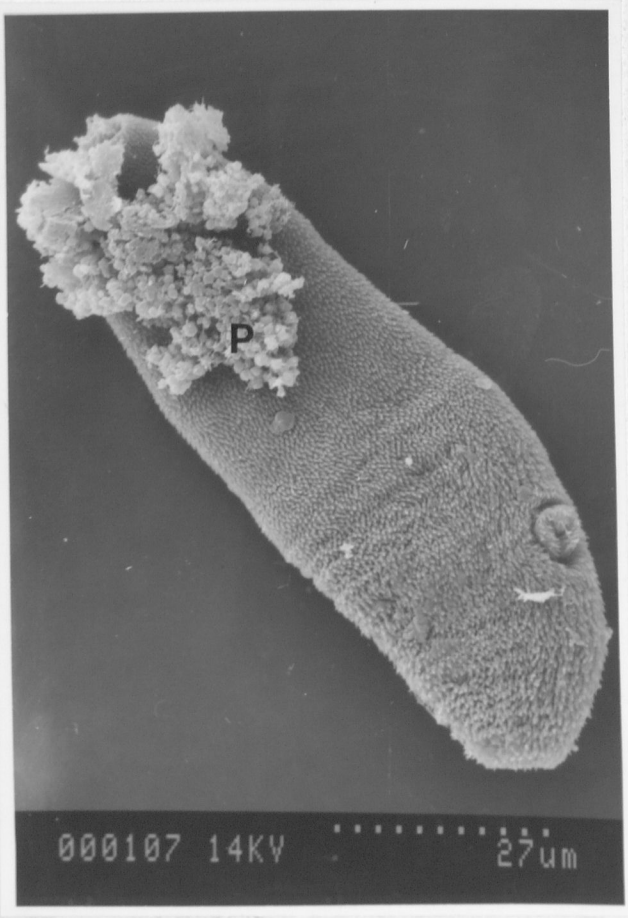


Plate 3.10:

Scanning electron micrograph of schistosomulum with platelets aggregated at the posterior end.

X4,000

P = platelets

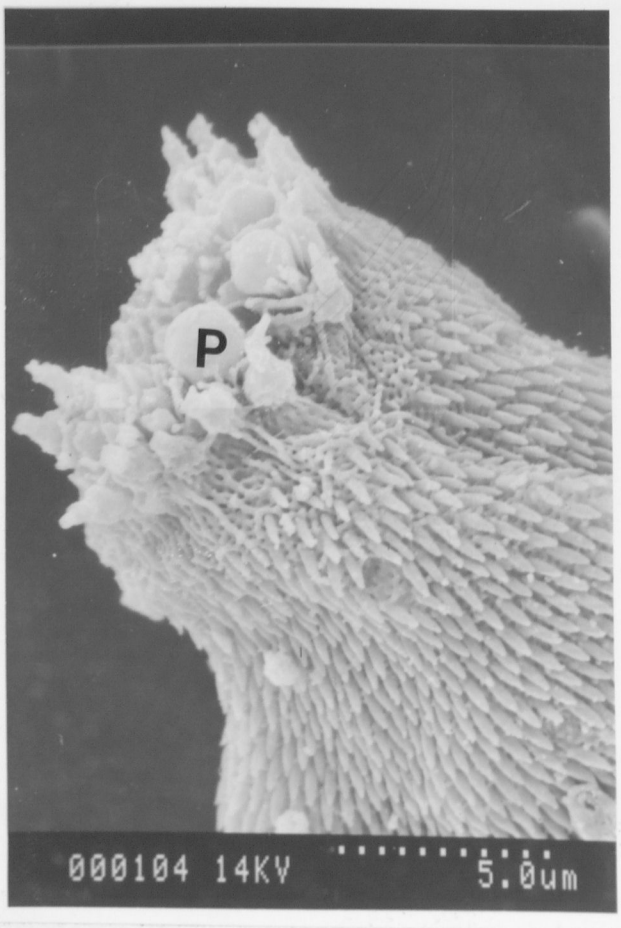


Plate 3.11: Scanning electron micrograph of schistosomulum with platelets at the point of detachment from cercaria.
X9,000
P = platelets

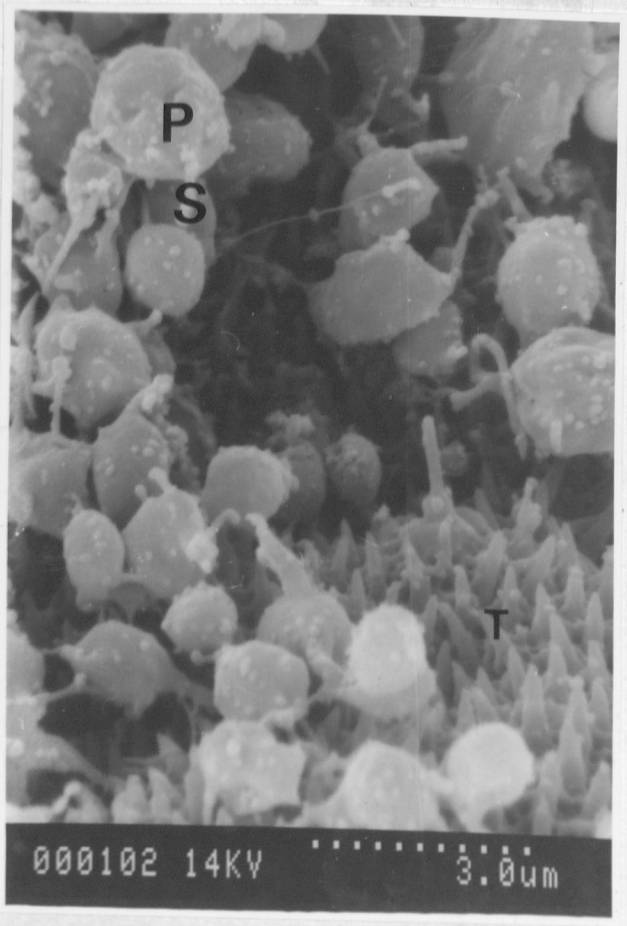


Plate 3.12: **Scanning electron micrograph of schistosomulum with platelets showing protruberances interacting with the worm surface.**

X11,000

P = platelets

S = pseudopods

t = tubercles on the worm surfaces

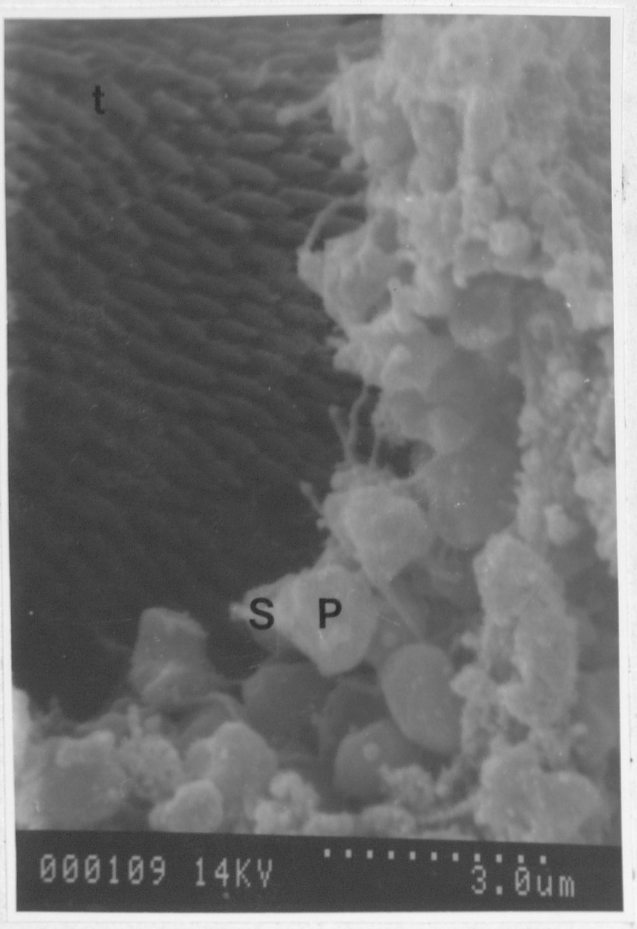


Plate 3.13: **Scanning electron micrograph of schistosomulum with platelets showing protruberances interacting with the worm surface.**

P = platelets

S = pseudopods

t = tubercles on the surface of the worm

3.5.3. Schistosomulae killing assay.

To determine whether the above interaction of schistosmulae with platelets led to the death of the worms, schistosomulae were cultured with platelets. The degree of adherence of platelets onto schistosomulae was recorded daily and the cytotoxicity scored. The percentage death of the schistosomulae was also recorded. Table 6 shows the results of this experiment. Cytotoxicity of 25% was observed on the first day, 40% on the second day and by the third day the cytotoxicity was more than 50%.

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	alive	dead	% dead
Day 1	66	23	25%
Day 2	53	37	41%
Day 3	45	47	51% (P<0.05, n=4 replicates)

TABLE 3.6:

Degree of platelet cytotoxicity against schistosomulae.

CHAPTER FOUR

DISCUSION.

This study was carried out to investigate the interactions between blood platelets, the immune system and schistosomes. The results show that a schistosome infection in mice and human subjects may cause platelet depletion and that this depletion may be immune-dependent. The host-parasite interactions involved may provide clues as to how the parasite evades the host immunity and other defence mechanisms to cause severe disease.

4.1 PLATELET DEPLETION AND PARASITOLOGICAL FINDINGS.

4.1.1. In mice.

Mice were infected with *S. mansoni* to investigate thrombocytopaenia. The results obtained in this study have shown that there is platelet depletion in mice infected with schistosomiasis (3.1.1, Table

3.1). Previously Ngaiza and Doenhoff (1987) reported thrombocytopaenia that was thymus-dependent in mice infected with *S. mansoni*. In this study a thrombocytopaenic state was induced in mice and the mice were able to live for upto twelve days when they were killed. (3.1.3, Fig. 3.2). In Great Britain the local statute does not allow for animals to be subjected to long suffering and so the mice could not be observed further. It was therefore possible to induce thrombocytopaenia in mice and maintain them in that state and observe the effect it had on infection. When the mice were infected with schistosomiasis worm survival was enhanced (Table 3.3). There were correspondingly high liver egg counts in the infected mice compared with the normal infected ones. This suggests that infection with *S. mansoni* in a thrombocytopaenic host can be more severe than in a nonthrombocytopaenic one. It was also observed that inducing thrombocytopaenia at the skin stage of infection results in a bigger worm load compared with inducing it later in infection. This may

suggest that platelets may be activated by invading parasites at the skin stage and probably this may be a cause of platelet depletion in early infection.

4.1.2. In patients.

The results obtained from the human subjects indicate that there is thrombocytopaenia in individuals infected with *S. mansoni* (Table 3.2). A platelet count of less than 100 X $10^3/mm^3$ was indicative of thrombocytopaenia in this study because the mean platelet count among healthy adult kenyans is 211 X $10^3/mm^3$ with 95% values falling between 114 X 10^3 and 300 X 10^3 (Mukiibi *et. al.*, 1981). The observed thrombocytopaenia was not related to the age nor sex of the individuals. There was no correlation between egg output and thrombocytopaenia. The haemoglobin levels of the individuals remained within normal ranges of 14-18g/dL for males and 12-16g/dL for females. This may suggest that when the individuals do not develop severe morbidity from the infection then a

thrombocytopaenic state may not be a serious condition in them. These patients came from an area in Machakos where most individuals with the infection do not present with severe morbidity. Individuals infected with *S. mansoni* from Kibwezi area in the same district have been shown to present with severe disease (Mbugua, personal communications) compared with those involved in this study who only presented with a mild form of the disease.

The individuals from Kibwezi with severe hepatosplenomegaly have been shown to present with thrombocytopaenia and anaemia which persist for more than one year after treatment with praziquantel (Rashid, personal communications). Anaemia that was associated with schistosomiasis in mice infected with *S. mansoni* has been reported before (Mahmoud and Woodruff, 1972). The liver and spleen are key organs in the final differentiation of haemopoietic cells including megakaryocytes from which thrombocytes are formed (Roit *et. al.*, 1987).

In their disease state the organs may affect haemopoiesis leading to reduced numbers of circulating platelets.

Other workers have shown that persistent pathology can cause abnormalities in platelet function and numbers (Omran *et. al.*, 1978; 1988). Therefore the pathology associated with schistosomiasis in Kenyan schistosomiasis patients may affect the platelet numbers similarly. A thrombocytopaenic state in the host may enhance the infection with *S. mansoni* as was shown in this study and this may lead to severe disease in some infected individuals and animal hosts. It has been shown that thrombocytopaenia is also associated with severe disease in other infections like septicaemia (Kelton *et. al.*, 1979) and severe *Plasmodium falciparum* malaria (Dennis *et. al.*, 1967 and Borochovitz *et. al.*, 1970). Similarlry thrombocytopaenia can be a threatening condition in individuals infected with schistosomiasis although the majority of

schistosomiasis patients are already in a compromised state.

The human subjects in this sudy did not present with severe disease and therefore their platelet counts reverted back to normal three weeks after treatment suggesting that treatment with praziquantel in individals who do not present with severe disease or those wo have not developed severe morbidity may restore the platelet levels to normal. This effect of praziquantel was investigated in mice and is discussed in the next section.

4.2. EFFECT OF PRAZIQUANTEL ON PLATELET LEVELS IN MICE INFECTED WITH *Schistosoma mansoni*.

Mice infected with *S. mansoni* and treated with praziquantel recovered from a thrombocytopaenic state as shown in Fig. 3.4. This finding may suggest that recovery from a thrombocytopaenic state was due to loss of infection after treatment. Praziquantel is the drug of choice in the treatment

of schistosomiasis (Jordan *et. al.*, 1993). The efficacy of this drug has been reported to be dependent on the immune status of the host whereby the death of the parasite is partly due to antibody attack on antigens which are exposed on the damaged parts of the adult worm tegument (Doenhoff *et. al.*, 1991; Fallon *et. al.*, 1992). The immune attack mounted against the parasite during this infection may have facilitated the action of praziquantel when the mice were treated with the drug. The anti-parasite and anti-platelet antibodies produced during this infection are discussed in the following section.

4.3. DETECTION OF ANTIBODIES BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

4.3.1. In mice.

The ELISA test was done to determine the possible relationship between platelet depletion and antibodies produced during a schistosome infection. The antibody titres against all the antigens were significantly higher from the eighth week of

infection as shown in Figs. 3.5, 3.6 and 3.7. Figs. 3.8 and 3.9 show a possible correlation between anti-platelet antibodies and anti-parasite antibodies suggesting that the antibodies are not only produced at the same time but that they may also be similar.

These results suggest that thrombocytopaenia in mice may be due to autoimmune reactivity against platelet antigens and that these antigens may resemble parasite antigens. The rise in Platelet-associated IgG (PAIgG) levels began at about five week post infection which is the same time that the production of anti-egg antibodies begins (Dunne *et. al.*, 1981) suggesting further that egg and platelet antigens may be related. The immune-dependent platelet depletion associated with *S. mansoni* that was reported by Ngaiza and Doenhoff (1987) was also associated with high anti-platelet IgG levels (Wambayi, 1987). The results in this study are in agreement with this observation.

Thrombocytopaenia has been reported in other infections where the anti-platelet or PAIgG levels have been found to be in inverse relation with the platelet counts linking the IgG with the platelet depletion (Kelton *et. al.*, 1979). The association of immunoglobulins with platelet surfaces is a common feature associated with immunological destruction of platelets in microbial infections (Kelton *et. al.*, 1979; Kelton and Gibbons, 1982; Kelton, 1984). The clearance of platelets from the circulation in septicaemia is mediated by immune mechanisms (Kelton *et.. al.*, 1979). This observation is supported by the presence of immune complexes. Schistosomiasis is a chronic infection characterised by immune complexes and the mechanisms of platelet depletion may be similar. The platelet Fc receptor has been shown to be the mechanism through which the antigen-antibody complex-induced platelet injury occurs (Israels *et. al.*, 1973). It may therefore be possible that in this study the platelet injury and destruction occured by these mechanisms.

4.3.2. In patients

Sera from human subjects was tested in the same way as the mice sera. The antibody titres showed that there was a high response in patients with thrombocytopaenia compared with the nonthrombocytopaenic ones and the normal controls (Figs. 3.10 & 3.11). The response to worm antigen was as strong as that to the platelet antigens. This may suggest that platelet antigens may be exposed in the presence of adult worms. These results agree with the above obseravation in mice that platelet antigens are exposed during the period of egg laying. Therefore parasite antigens may be instrumental in antibody dependent platelet depletion.

4.4. DETECTION OF PLATELET ANTIGENS BY SODIUM DODECYLE SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

When sera from chronic infections were reacted with mouse platelets antibody reactivity was detected against a wide range of antigens of between 30 and

100 kD (Plates 3.1 & 3.2). In a previous study sera from older infections recognised antigens of 110-115 kD which are well conserved in both mice and humans (Plate 3.5) (Ngaiza, 1988). In another development Ngaiza (1988) showed that binding of antibodies (IgG) from chronic infection was also specific in that they recognised epitopes which were undetected by immune sera from normal infection as well as vaccinated serum IgG. This specificity may be egg specific where it raises an immune response after onset of patency and may be largely responsible for the production of anti-platelet autoantibodies.

Human serum reacted with platelet antigens of approximately 110, 97, 67, and 50 kD (Plate 3.3). A comparison of the platelet and parasite antigens revealed that human serum reacted with both platelet and egg antigens of approximately 43 and 33 kD (Plate 3.4) suggesting that schistosome eggs and platelets may share these epitopes. In addition the serum reacted with a platelet antigen of 38 kD,

worm antigens of 70, 28 and 21 kD, and egg antigens of 35, 14 and 6 kD. These results suggest that parasite and platelet antigens of a wide range are reacted against by schistosomiasis patient sera and sera from infected mice.

It has been postulated that egg antigens may resemble platelet antigens. A cathodally migrating antigen K_3 that was associated with the production of antibodies which inhibit antibody-dependent cell cytotoxicity (ADCC) in *S. mansoni* infections was strongly precipitated on reacting soluble egg antigen extract (SEA) with pooled chronic infection sera (Dunne *et. al.*, 1987). It may be suggested that through a similar mechanism, K_3 is also responsible for the production of platelet function modulatory autoantibodies. It has been shown that K_3 may resemble mouse serum albumin (67 kD) and may therefore bear important evolutionary significance in the protection of the parasite from the host defence mechanisms (Riley, personal communication). The 110-120 kD antigens detected by Ngaiza are

known platelet antigens which may be important in immunological recognition of platelets during infections with *S. mansoni*.

The antigens bearing the lower molecular weight may be similar to some parasite antigens particularly egg and miracidial antigens. It has been shown that there are proteases of molecular weight ranging between 22 and 50 kD in eggs of *S. mansoni* which can degrade fibrinogen in SDS-PAGE (Doenhoff *et. al.*, 1990). Both IgG and IgM antibodies from infected human sera have been shown to immunoprecipitate antigens of between 30-40 kD in labeled detergent extracts of schistosomulum surface (Khalife *et. al.*, 1986). An antigen p40 has been described which is a major protein antigen in eggs of *S. mansoni* (Trzyna and Cordingley, 1993). A 40 kD platelet Fc receptor, Fc γRII which is associated with GPIIb-IIIa complex has been reported by Berndt *et. al.*, (1993). Fc-receptor-mediated platelet activation has been shown to be a cause of thrombocytopaenia associated with sepsis

(Berndt *et. al.*, 1993). Probably the thrombocytopaenia observed in this study may have been caused by a similar mechanism.

4.5. PARASITE / PLATELET INTERACTIONS.

Schistosomulae were incubated with freshly prepared platelets and the platelets were seen to attack the shistosomulae within 30 minutes of incubation (Plate 3.6). The results in this experiment demonstrate the ability of platelets to kill schistosomulae in a non-antibody mediated cytotoxicity assay (Table 3.6). It has been shown before that platelets killed schistosomulae in the presence of IgE via an Fc receptor on the platelets showing that platelets have an immunological function in schistosomiasis (Joseph *et. al.*, 1983). The results in this study have shown that even in the absence of antibody the platelets can still be effective in killing schistosomulae.

Platelets have been shown to procure more protection against challenge infection than known

protective white cells during a passive transfer of purified cell preparations from immune donor rats to naive recipients (Kruger and Wolmarans, 1990). It has also been shown that there is another mechanism of platelet activation other than the antibody-mediated one. Platelet activation dependent on a lymphokine (IFN-Γ) induced platelets from normal rats into cytotoxic effector cells for parasite larvae (Pancre *et. al.*, 1987; 1990). There are other known killing mechanisms of schistosomulae *in vitro* involving both cellular and humoral factors. Neutrophils, eosinophils, Monocytes and Macrophages exhibit cytotoxic properties against schistosomulae in association with antibodies of various isotypes or with Complement (Capron *et. al.*, 1982; Kimani *et. al.*, 1991). The results in this study have further shown the effectiveness of platelets in killing schistosomulae in the absence of antibody.

Electron micrographs of the worms incubated with platelets revealed protruberances from the platelet surface indicating platelet activation (Plates 3.12 & 3.13). These protruberances were seen to interact with tubercles on the worm surface. Such interactions have been reported by other workers where platelets with dendritic structures were seen to adhere to the surface of adult worms (Kruger and Wolmarans, 1990). Platelets are known to aggregate following activation and release substances following such aggregation which could be lethal to foreign bodies in the circulation. Probably these substances were released by platelets in this experiment and such substances may have caused the death of the schistosomulae.

The release of lethal substances has been demonstrated in other infections. In an infection with *Toxoplasma gondii* platelets were activated and they released thromboxane B_2 (TXB_2) (Yong *et. al.*, 1991) and oxygenated products of both arachdonic acid: linoleic acid, 12 - hydroxyheptadecatrienoic

acid (12 - HHT) and 12 - hydroxyeicosatetraenoic acid (12 - HETE) (Henderson *et. al.*, 1992) which may have been important in the cytolytic processes that killed the organisms. Probably these substances were also released when platelets were incubated with schistosomulae in this study. The same may happen when cercariae penetrate the skin of the host and platelets try to attack them immediately. Probably this could lead to the observed thrombocytopaenia during early infection. However, thrombi do not form around schistosomulae and adult worms in the circulation suggesting that the parasites have developed deffensive mechanisms against this form of host defence.

4.6. CONCLUSIONS.

1. Thrombocytopaenia is a prominent feature of chronic Schistosomiasis *mansoni* in both humans as well as mice with platelet levels falling significantly below normal values as the infection progresses.

2. Worm survival is significantly enhanced in a thrombocytopaenic host during infection with schistosomiasis.
3. Platelet depletion in infected mice commences during the fifth week of infection.
4. Anti-platelet IgG is inversely related to platelet levels in the course of infection with *S. mansoni*.
5. There is a positive correlation between anti-platelet and anti-parasite antibodies during infection with *S. mansoni*.
6. Anti-schistosome antibodies react to a wide range of platelet antigens (30-100 kD) while antigens of approximately 33 and 43 kD may be similar to both platelets and schistosomes.
7. Platelets can attack and kill *S. mansoni* schistosomulae in the absence of specific anti-*S. mansoni* antibody.

4.7. FUTURE RECOMMENDATIONS.

1. Thrombocytopaenia is a serious condition in infection and there is need to explore this condition further in individuals with schistosomiasis. Platelet function tests and clotting factors among other parameters need to be studied.
2. Further analysis of the antibodies involved in platelet destruction is necessary in order to establish the actual mechanisms that cause thrombocytopaenia.
3. Immunological probing of the membrane glycoproteins (mainly Gp Ib and Gp IIb-IIIa) with anti-platelet antibodies raised in both bisexual and unisexual schistosome infections would be useful in identifying immunogenic regions in the amino acid sequence of these proteins. Monoclonal antibodies will also identify shared antigens between platelets and schistosomes.

4. An analysis of toxins produced by platelets would be appropriate in order to identify those that may be involved in platelet killing. Long chain fatty acids can be analysed by chromatographic techniques.

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APPENDICES

APPENDIX 1: NORMAL HAEMATOLOGICAL VALUES.

Human

Red cell count	male	5.5 - 1.0 x 10^{12}/l
	female	4.8 - 1.0 x 10^{12}/l
Haemoglobin	male	15. 5 - 2.5g/dl
	female	14.0. - 2.5g/dl
Haematocrit	male	0.47 - 0.07 (1/1)
	female	0.42 - 0.05 (1/1)
Mean cell volume		85 - 8 fl
Mean cell haemoglobin		29.5 - 2.5pg
Mean cell haemoglobulin		

concentration	33.0 - 2g/dl
Mean cell diameter (dry films)	6.7 - 7.7 μm
Reticulocytes	0.2-2.0%(10-100 x10^9/l
Total leucocyte count	7.5 - 3.5 x10^9/l

Differential leucocyte count:

Neutrophils	2.0-7.5 x10^9/l(40-75%)
Lymphocytes	1.5-4.0 x10^9/l(20-45%)
Monocytes	0.2-0.8 x 10^9/l(2-10%)
Eosinophils	0.04-0.4 x 10^9/l(1-6%)
Basophils	<0.01-0.1 x 10^9/l(0-1%)
Platelet count	150-400 x 10^9/l

From Sanderson and Phillips (1981).

Normal haematological values - mouse (4 week old)

Red cell count	male	6.99 x 10^{12}/l
	female	7.29 x 10^{12}/l
Haemoglobin		13.5g/dl

Haematocrit		0.390
Mean cell volume	male	56 fl
	female	53 fl
Mean cell haemoglobulin	male	19.0pg
	female	18.5pg
Mean cell haemoglobulin concentration	male	34.1g/dl
	female	34.5g/dl
Reticulocytes	male	7.6%
	female	7.2%
Total leucocyte count	male	6.7 x 10^9/l
	female	6.4 x 10^9/l
Differential leucocyte count:		
Neutrophils	male	0.6 x 10^9/l
	female	0.8 x 10^9/l
Lymphocytes	male	6.0 x 10^9/l
	female	5.6 x 10^9/l
Monocytes	male	0.1 x 10^9/l
	female	0.0 x 10^9/l
Eosinophils & Basophils		0.0 x 10^9/l

Platelets	male	1633 x 10^9/l
	female	1681 x 10^9/l

From Sanderson and Phillips (1981).

APPENDIX 2: BUFFERS FOR ELISA.

PBS buffer (pH 7.6)

NaCl 42.5g

Na_2HPO_4 ..... 6.4g

$NaH_2PO_4.2H_2O$ 0.78g

Dist. water 5L

Coating buffer (pH 9.6)

Na_2CO_3 (anhydrous) 1.59g

$NaHCO_3$ 2.93g

Dist. water 1L

Incubation buffer

PBS (pH 7.6) 1L

Tween 20 ..... 0.5ml

Washing buffer

NaCl 45g

Tween 20 ..... 2.5ml

Dist. water 5L

Substrate buffer (pH 5)

Citric acid $(CH_2.COOH)_2.H_2O$ 5.25g

Na_2HPO_4 ..... 7.1g

Dist. water 1L

Substrate - stock Orthophenylene diamine (OPD)

OPD 100mg

Methanol 10ml

(store in a cool place)

Working OPD

Substrate buffer 50ml

OPD 0.5ml

H_2O_2 (6%) 25μl

(reaction occurs after 15-30 minutes)

Azino bis 3-ethylbenz-thiazoline-6 sulphonic acid (ABTS)

Substrate buffer 5ml

Substrate 150μl

H_2O_2 ..... 2.5μl

APPENDIX 3: REAGENTS FOR SODIUM DODECYLE SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Resolving gel (8% acrylamide)

Resolving buffer 2.5ml

Acrylamide (protogel) 2.6ml

Dist. water 4.9ml

SDS (10% stock) 100µl

Ammonium persulphate (50mg/ml stock) 100µl

Tetramethly-ethylenediamine (TEMED) 10µl

12.5% gel

Resolving buffer 7.5ml

Acrylamide 12.5ml

Dist. water 9.65ml

SDS (10%) 600µl

APS 50µl

TEMED 10µl

(both recipes make one gel)

Stacking gel (3% acrylamide)

Stacking buffer 2.5ml

Acrylamide 0.5ml

Dist. water 2.0ml

SDS (10% stock) 50µl

Ammonium persulphate 75µl

TEMED 10µl

Phenol red 2.5µl

Loading buffer

50% Glycerol 2.5ml

2% SDS 0.1g

5% 2 mercaptoethanol 0.25ml

All in 0.315M Tris-Cl pH 6.8 + a hint of

bromophenol blue 2.25ml

Ponceau Stain

2% Ponceau (3-hydroxy-4-[2-sulfo-phenylazo)

phenylazo]-2, 7-naphthalene disulfonic acid) in

(i) 30% trichloroaceic acid

(ii) 30% sulfosalicyclic acid

BUFFERS

PAGE BUFFERS

Resolving buffer

3M Tris-Cl pH 8.8 ..... 36.4g

Dist. water ..... 100ml, Filter 0.22µl

Stacking buffer.

0.25M Tris-Cl pH 6.8 ..... 3.0g

Dist. water ..... 100ml, filter.

Lower reservoir buffer X10 stock

50mM Tris-Cl pH 8.8 ..... 30.028g

Dist. water 500ml

Run buffer X5 stock (1L)

0.25M Tris 15.2g

1.92M Glycine 72.0g

1% SDS 5.0g

pH adjusted to 8.3-8.5 with Glycine

IMMUNOBLOTTING BUFFERS

Electroblotting buffer pH 7.0 (5L)

25mM Tris 15.13g

190mM Glycine 71.30g

20% Methanol 1L

Transblotting solution (TBS) (5L)

20mM Tris 12.1g

0.9% NaCl 45.0g

pH adjusted to 7,2 with 1M HCl

Tween transblotting solution (TTBS) (5L)

20mM Tris 12.1g

0.9% NaCl 45.0g

0.1% Tween 20 ..... 5.0ml

pH adjusted to 7.2 with 1M HCl

APPENDIX 4: BIO-RAD PROTEIN ASSAY (1-30 ug).

(As used in this thesis).

1. Dye reagent is prepared by adding 1 volume of dye to 4 volumes of sterile filtered distilled water.

   1ml of dye is needed for each of the 5 standards. Another 1ml is prepared for each sample (dilute dye does not keep for more than two weeks).

2. A standard curve is prepared by making dilutions from the stock (1.39mg/ml) in clean dry test tubes as follows.

Standard	protein (μg/ml) from graph	Stock volume in 50μl Dist. water	Protein total in 20μl (μg)
A		10ul in 50μl	5.7
B		20ul in 50μl	11.4
C		30ul in 50μl	17.04
D		40ul in 50μl	22.7
E		50μl	28.4

These values only applied for the system used in this thesis.

3. 20μl of sample or standard is added to 1ml of the diluted dye reagent.
4. These are vortexed without frothing.
5. They are left for 5 minutes to 1 hour for the colour to develope and stabilise.

6. The samples are poured in unused cuvettes and measured at 595nm.
7. The standard curve is prepared by blotting absorbance against protein total, then the protein concentration of the samples determined from the graph.