# THE EFFECT OF IMMUNIZING SWISS MICE WITH SOLUBLE PROTEINS FROM THE INTERMEDIATE HOST BIOMPHALARIA PFEIFFERI AND CHALLENGING THEM WITH SCHISTOSOMA MANSONI

BY

KOBIA EUNICE NKIROTE

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2011

## DECLARATION

I, Kobia Eunice Nkirote do declare that this thesis is my original work and has not been submitted for a degree in any other University.

Kobia Eunice Nkirote

University of Nairobi,

Signature.....Date.....19/4/2011.....

## SUPERVISORS

This thesis has been submitted for examination with our approval as university supervisors

1. Prof. H. Ochanda
   School Of Biological Sciences,
   University Of Nairobi.

   Signature.....Date..19th April, 2011.....

2. Dr Dorcas S. Yole
   Senior Research Scientist, Department of Tropical and Infectious Diseases
   Institute of Primate Research, National Museums of Kenya

   Signature.....Date.....20/4/2011.....

## DEDICATION

This thesis is dedicated to my parents Tabitha Ncece and Stanley Ataya both of whom God has called to live within his heavenly kingdom. I sincerely thank them for giving life to me and all their teaching that enabled me to value education since childhood.

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| | |
|---|---|
| **0-3 hr** | 0-3 hr protein release |
| **ADCC** | Antibody-dependant cell-mediated cytotoxicity |
| **BSA** | Bovine serum albumen |
| **DTH** | Delayed type hypersensitivity |
| **ELISA** | Enzyme linked immunosorbent assays |
| **IFN-y** | Interferon gamma |
| **Ig** | Immunoglobulin |
| **IgE** | Immunoglobulin E |
| **IgG** | Immunoglobulin G |
| **IL-5** | Interleukin 5 |
| **IPR** | Institute of Primate Research |
| **KEMRI** | Kenya medical research institute |
| **LN** | Lymph node |
| **PBS** | Phosphate buffered saline |
| **RT** | Room Temperature |
| **SEA** | Soluble Egg Antigen |
| **SP** | Spleen |
| **SBgA** | Soluble Biomphalaria glabrata Antigen |
| **SWAP** | Schistosome worm antigen preparation |
| **Th -1** | Helper T-Lymphocyte subset 1 |
| **Th-2** | Helper T lymphocyte subset 2 |
| **T-test** | Student t- test |
| **WHO** | World Health Organisation |

## ABSTRACT

Schistosomiasis, also known as bilharziasis, is second only to malaria in public health importance. It is estimated that 200 million people worldwide are infected and that 20 000 deaths are associated with the severe consequences of infection. Chemotherapy remains the cornerstone of intervention but rapid re-infection demands frequent re-treatment and emphasizes the need for a more long-term approach. The existence of at least partially protective immunity in exposed humans would make vaccination a logical complement to drug therapy. The administration of radiation-attenuated cercariae to laboratory animals provided protection against experimental *S. mansoni* infection by blocking the migration of the parasite out of the lung. Great attention has been paid to the use of antigens from schistosomules, with disappointing protection results so far. It is hoped that better success can be achieved using cocktails of recombinant antigens. Common antigens between different species of *Schistosoma* and their intermediate hosts have been reported. This study was done to investigate the effect of immunizing Swiss mice with soluble proteins from *S mansoni* vector *Biomphalaria pfeifferi* and challenging them with *S mansoni*. The study was done using mice as the models for human schistosomiasis. The mice were divided in four groups, three of which comprised of eighteen mice each and one group (non-infected control) of two mice. The study involved immunization of mice and then challenging them with cercariae of *S. mansoni*. On day 0 (zero) of the experiment DG (mice immunized with antigens from digestive gland of intermediate host) and RT (mice immunized with antigens from the rest of the tissues of intermediate host) groups were immunized with 50 µg of snail antigens in 100 µl of complete Freunds adjuvant intra-peritoneally. In week two and four DG and RT groups of mice were boosted with 50 µg of the snail antigens in 100 µl of incomplete Freunds adjuvant. One week after final vaccination, mice in DG, RT and IC (infected control) groups were challenged with cercariae of *S. mansoni*. In week two and four, six mice from each of the three groups were sampled for cells from the lymph nodes and spleen for cell culture and blood for ELISA. In NC (naïve control) group the two mice were sampled for serum only. In Week six, mice from the three groups were: sampled for blood; perfusion was carried out to recover adult worms; their livers tissues were examined for gross pathology and tissues were preserved for histopathology. The results from this study showed that mice immunized with antigens prepared

from an intermediate host are better protected than non-vaccinated mice. The mean worm burden was higher in IC group than both RT and DG group of mice. The results also reveal that RT candidate vaccine offered a better protection than DG candidate vaccine. Worm reduction in RT was significantly higher (0.05) at 60.5% than that of DG that was at 43.3%. In cellular responses, RT significantly stimulated higher production of interferon gamma as compared to IC at all time points. RT interleukin-5 responses were significantly higher than the DG and IC for both 0-3 hr protein release (0-3 hr) and (Schistosome worm antigen preparation) SWAP antigens for both spleen and lymph node. RT produced higher Immunoglobin G (IgG) responses than DG and IC, meaning it killed more worms using antibody dependant cell-mediated cytotoxicity (ADCC). In RT, all the mice had slightly inflammed livers. DG mice had slightly inflamed livers except one female member who had an inflamed liver. In IC mice, all the sampled animals had inflammed liver tissues. However, none of the mice in all the groups had granulomas most probably due to delayed pathology development in Swiss mice. In conclusion therefore, although the two vaccine candidates were protective, RT was a better vaccine candidate in terms of higher worm reduction, higher cellular and humoral protective responses and least pathology.

## CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

### 1.0 General Introduction

Schistosomiasis is a parasitic disease caused by trematode of the genus *Schistosoma*. It is estimated that 200 million people worldwide are infected with the snail-transmitted, water-borne parasitic helminth, and that 200 000 deaths per year are due to schistosomiasis in sub-Saharan Africa (WHO, 2009). Chemotherapy remains the cornerstone of intervention but rapid re-infection demands frequent re-treatment and emphasizes the need for a more long-term approach. The existence of at least partially protective immunity in exposed humans would make vaccination a logical complement to drug therapy (Bergquist & Colley, 1998).

It has become clear that host immune responses are absolutely central to strategies for controlling both infection and pathology (Capron, 1992). Parasite egg productions, granuloma formation, disease severity, resistance to re-infection and drug efficacy have all been shown to depend on immunological factors (McCarthy & Nutman, 1996). Therefore vaccine strategies represent an essential component of the control of this chronic debilitating disease where the deposition of millions of eggs in tissues is the main cause of pathology.

The optimism towards the development of Schistosomiasis vaccine stems from the findings that continual exposure to Schistosomiasis elicits partial immunity and that complete, sterilizing immunity is not required since the parasite does not replicate within the human host. The evidence from field studies suggests that partial resistance due to infection probably develop in most people (James *et al.*, 2001).

Great attention has been paid to the use of antigens from schistosomules, with disappointing protection results so far. Somewhat better results have been obtained with antigens that are shared between schistosomules and schistosomes, such as the 63 kD parasite myosin, the 97 kD paramyosin, the 28 kD triose phosphate isomerase (TPI), a 23 kD integral membrane protein (Sm23), and the 26 and 28 kD glutathione-S-transferases (GSTs).

Snails have been found to have shared proteins with the *S. mansoni* parasite. Common antigens between different species of *Schistosoma* and their intermediate hosts have been reported (Chacón *et al., 2000*). This study demonstrated that sera from schistosome-infected persons reacted against soluble crude *Biomphalaria glabrata* antigen (S*Bg*A) by ELISA (100% of

sensitivity) and that sera from mice immunized with S*Bg*A recognized several homologous snail molecules by Western-blot (Chacón *et al., 2000*).

The present study was done to investigate the effect of immunizing Swiss mice with soluble proteins from the intermediate host *Biomphalaria pfeifferi* and challenging them with *S. mansoni.*

### 1.1 Geographical distribution of *S. mansoni*

Figure 1.1 shows the geographical distribution of *S.* mansoni . The parasite *S. mansoni* is found in many countries in Africa, South America (Brazil, Surinam and Venezuela), the Caribbean (including Puerto Rico, St Lucia, Guadeloupe, Martinique, Dominican Republic, Antigua and Montserat) and in parts of the Middle East. These are areas with either slow moving streams or large-scale water projects infested with the vector snails (WHO, 1987).

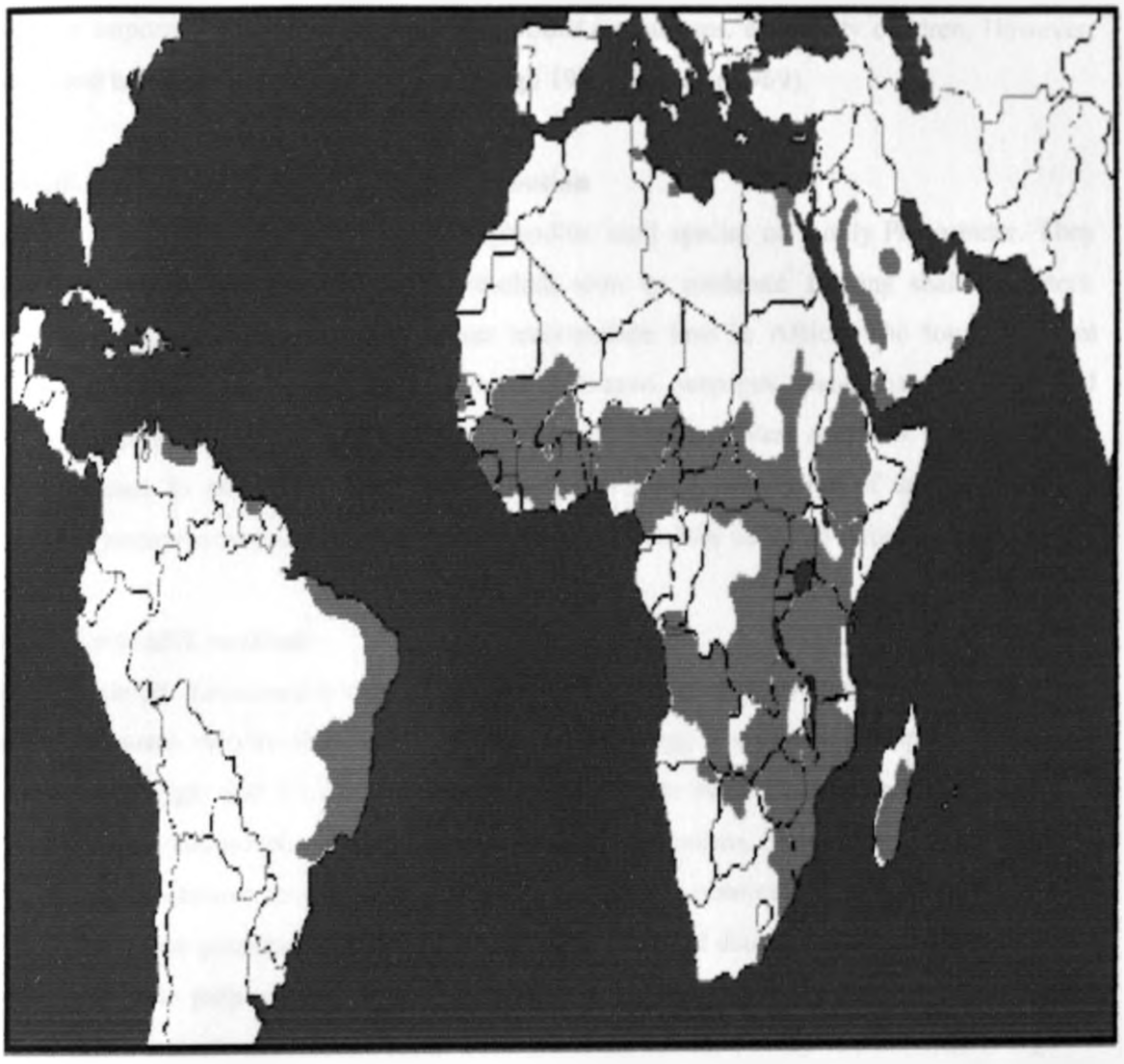

**Figure 1.1 Geographical Distribution of *Schistosoma mansoni*** (www.striepen.uga.edu/med para/ Schistosome.ppt)

### 1.2 Definitive hosts of S.mansoni

The most important definitive hosts of *S. mansoni* are humans, especially children. However, rodents and baboons are rare hosts (Cheesbrough 1987; Chandler 1969).

### 1.3 Snail hosts and their geographical distribution

*S. mansoni* is transmitted by aquatic hermaphroditic snail species of family Planorbidae. They are found among vegetation in lightly shaded, slow to moderate flowing shallow waters. *Biomphalaria* genus is the most important intermediate host in Africa. The four important species of *Biomphalaria* are: *B. pfeifferi* found in streams, seepages, water channels, dams and swimming pools, *B. sudanica* exists in swamps in both East and West Africa, *B. choanomphala* species, occurs in lakes and forms the main vector along the shores of lake Victoria, *B. Alexandria* occurs in moderate flowing shallow water in the Nile valley in Africa (WHO, 1993).

### 1.4 Life Cycle of *S. mansoni*

The life cycle of *S.mansoni* is shown in figure 1.2. After the eggs are passed out in the faeces and into the water, the ripe miracidium hatches out of the egg. The hatching happens in response to temperature, light and dilution of faeces with water. The miracidium searches for a suitable freshwater snail (*Biomphalaria glabrata*, *Biomphalaria straminea*, *Biomphalaria tenagophila* or *Biomphalaria sudanica*) to act as an intermediate host and penetrates it (Gatlix *et al.*, 2009). Following this, the parasite develops via mother-sporocyst and daughter-sporocyst generation to the cercaria. The purpose of the growth in the snail is the numerical multiplication of the parasite. From a single miracidium result a few thousand cercaria, every one of which is capable of infecting man. (Gatlix *et al.*, 2009).

The cercaria emerge from the snail during daylight and they propel themselves in water with the aid of their bifurcated tail, actively seeking out their final host. When they recognise human skin, they penetrate it within a very short time. This occurs in three stages, an initial attachment to the skin, followed by the cercaria creeping over the skin searching for a suitable penetration site, often a hair follicle, and finally penetration of the skin into the epidermis using proteolytic secretions from the cercarial post-acetabular, then pre-acetabular glands. On penetration, the head of the cercaria transforms into an endoparasitic larva, the schistosomule. Each

schistosomule spends a few days in the skin and then enters the circulation starting at the dermal lymphatics and venules. Here they feed on blood, regurgitating the haem as hemozoin. (Olivera *et al.*, 2009). The schistosomule migrates to the lungs (5–7 days post-penetration) and then moves via circulation through the left side of the heart to the hepatoportal circulation (>15 days) where, if it meets a partner of the opposite sex, it develops into a sexually mature adult and the pair migrate to the mesenteric veins. Such pairings are monogamous (Beltran & Boissier, 2008). Male schistosomes undergo normal maturation and morphological development in the presence or absence of a female, although behavioural, physiological and antigenic differences between males from single-sex, as opposed to bisex, infections have been reported. On the other hand, female schistosomes do not mature without a male. Females' schistosomes from single-sex infections are underdeveloped and exhibit an immature reproductive system. Although the maturation of the female worm seems to be dependent on the presence of the mature male, the stimuli for female growth and for reproductive development seem to be independent from each other (Beltran & Boissier, 2008).

The adult female worm resides within the adult male worm's gynaecophoric canal, which is a modification of the ventral surface of the male forming a groove. The paired worms move against the flow of blood to their final niche in the mesenteric circulation where they begin egg production (>32 days). The *S. mansoni* parasites are found predominantly in the small inferior mesenteric blood vessels surrounding the large intestine and caecal region of the host. Each female lays approximately 300 eggs a day (one egg every 4.8 minutes), which are deposited on the endothelial lining of the venous capillary walls (Loverde &Chen, 1991). Most of the body mass of female schistosomes is devoted to the reproductive system. The female converts the equivalent of almost her own body dry weight into eggs each day. The eggs move into the lumen of the host's intestines and are released into the environment with the faeces (Loverde &Chen, 1991). Other eggs are carried by circulation and are deposited in tissues.

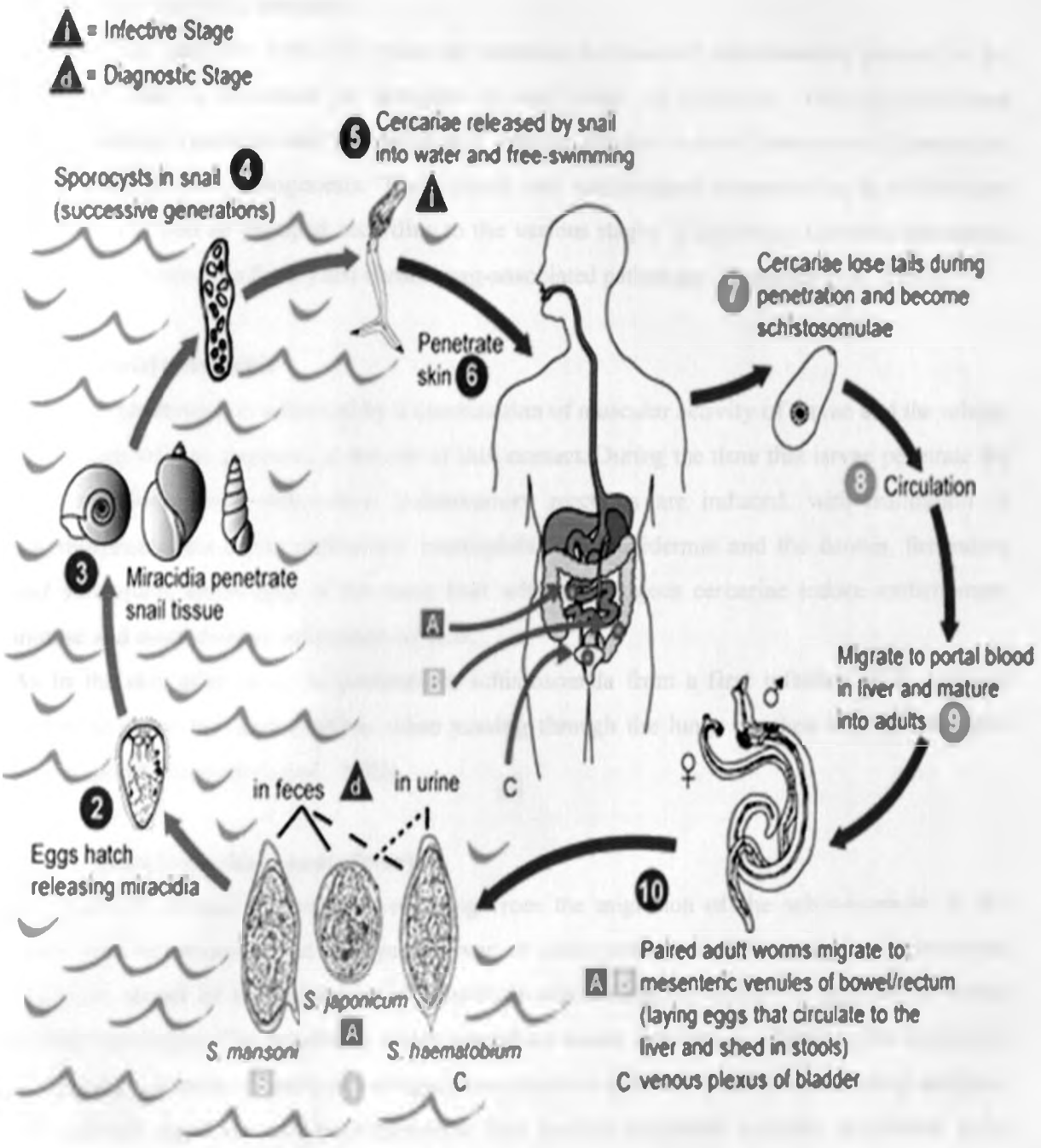


Figure 1.2: Life cycle of human *Schistosoma* species (CDC, 2009)

### 1.5 Pathogenesis of *S. mansoni*

Multicellular parasites with life cycles as complex as those of schistosomes, present to the definitive host a multitude of antigens at each stage of infection. The egg associated granulomatous reactions and the direct and indirect injuries to host tissues are of paramount importance in the pathogenesis. The clinical and pathological responses to a schistosome infection can best be grouped according to the various stages of infection: Cercarial dermatitis, acute form (Katayama fever) and chronic egg-associated pathology. Stadecker *et al.*, 1992).

### 1.6 Cercarial dermatitis

Cercarial penetration is achieved by a combination of muscular activity of larvae and the release of a variety of lytic enzymes at the site of skin contact. During the time that larvae penetrate the skin, relatively mild oedematous inflammatory reactions are induced, with infiltration of polymorphonuclear cells, particularly neutrophils in the epidermis and the dermis. Secondary and subsequent challenges of the same host with homologous cercariae induce earlier, more intense and more durable infiltration of cells.

As in the skin after cercarial penetration, schistosomula from a first infection of *S. mansoni* appear to cause few perturbations when passing through the lungs on their way to the portal blood system (Stadecker *et al.*, 1992).

### 1.7 Acute form (Katayama fever)

The transient allergic pneumonitis resulting from the migration of the schistosomules in the lungs may be observed but Katayama fever or acute toxaemic schistosomiasis is the most important sequel of the migration of schistosomulae and adult worms as well as the initial deposition of eggs. The condition, which resembles serum sickness, is related to the deposition of the large amounts of antibody-antigen complexes as a result of excess circulating antigens. The clinical signs are non-pathognomonic but include persistent pyrexia, abdominal pain, diarrhea and myalgia. Pathological lesions of katayama fever include lymphoadenopathy, hepatomegaly and characteristic peripheral eosinophilia (Stadecker *et al.*, 1992)

## 1.8 Chronic egg-associated pathology

The eggs of all schistosome species have the common property of inflammatory granulomas or pseudotubercles at the site of their embolization. Considerable evidence indicates that the inflammatory reaction induced by *S. mansoni* eggs is immunological in nature and induced by antigenic egg products. It has the characteristics more typical of a cell-mediated immune response than that of an immediate (antibody-mediated) hypersensitivity reaction and that it is subject to amnestic recall. *S. mansoni* eggs embryonate and the miracidia mature within about six days after leaving the female worm, and during this time a minimal host response is evoked. Release of antigen factors by secretory glands in mature miracidia inside the egg is considered to be necessary for granuloma induction, and it is these antigens that diffuse out through micropores or fenestration in the eggshell (Stadecker *et al.*, 1992).

In unsensitized mice, the early granuloma consists predominantly of large and small mononuclear cells, but as the reaction proceed, the proportion of small mononuclear cells decreases, while the proportion of total numbers of eosinophils increases to constitute 50% of the granuloma by day 32. Large granulomas composed of macrophages, lymphocytes and eosinophils, result as a consequence of the host's T cells sensitization to antigens emanating from the eggs but new granulomas formed around the continuously arriving eggs appear reduced in size, a phenomenon that has been termed as immunomodulation. The scarring process may lead to portal hypertension, portal systematic shunting, haemorrhage and death (Stadecker *et al.*, 1992).

Hepatic granulomas are initiated endovascularly and they are presinusoidal, mostly in inlet venules or terminal portal venules. Following initial endothelial proliferation, the blood vessels rapidly become occluded with eventual obliteration of the lumen and deplacement of hepatocytes by the developing granuloma. Portal hypertension and collateral blood vessel formation is generally a pre-requisite for development of pathology in the lungs in chronic human Schistosomiasis mansoni and japonicum. Direct passage of eggs to the lungs induces granulomatous inflammation and arteritis develops particularly in instances where massive egg embolization occurs over a short period. The intestine is another most pathologically involved tissue in *S. mansoni* infections. Intestinal polyps are more common in African schistosomiasis mansoni and can contribute to protein loss (Stadecker *et al.*, 1992).

## 1.9 Immunological responses in murine schistosomiasis

### 1.9.1 Humoral immune response

The involvement of antibodies in protective immunity was demonstrated earlier on by passively transferring resistance to naïve mice using serum of vaccinated mice (Perlowagra, 1964). The protective capacity is restricted to sera obtained from multiply vaccinated mice (Mangold, 1986). The IgG isotype, particularly IgG1, seems to be protective and may be synergistically enhanced by the presence of IgM (Jwo & Loverde, 1989). Parasite specific-antibodies are detected as early as two weeks after vaccination. Their levels peak at week 5 to 6 and then gradually decline. Antibodies are still detectable 15 weeks after vaccination. Titers are enhanced by repeated vaccinations with irradiated cercariae, or after challenge infection with non-attenuated cercariae showing a typical amnestic response. Although the presence of antibodies is essential, there is no consistent association between antibody titers and level of resistance. However, overall levels of schistosome specific antibodies are greater in mice vaccinated with 15 or 20 krad irradiated cercariae. Therefore, antibody specificity rather than quantity seem to be relevant to protective immunity (Lewis *et al.*, 1987).

### 1.9.2 Cellular immune responses

Proliferation response of lymphocytes to schistosomal antigens peak during the first two weeks after vaccination and wane after the fourth in mice (Pemberton *et al.*, 1991). Lymphocytes derived from draining lymph nodes respond considerably more strongly than those derived from spleen (Constant *et al.*, 1990; Pemberton *et al.*, 1991). The relevance of regional rather than systematic stimulation is supported by the observation that attenuated parasites release significant amount of antigenic material during the passage through the skin, lymph nodes and lungs. As a result the time and the site of lymphocyte priming coincide closely with the parasites migration, that is, proliferation is observed first in skin and later in lungs (Constant *et al.*, 1990; Pemberton *et al.*, 1991), since greater amounts of antigens are released over an extended period in axillary's and inguinal lymph nodes as compared to mice infected with non-attenuated cercariae, (Mountford *et al.*, 1988), the cells of lymph nodes, as well as mediastinal nodes, proliferate more strongly. In contrast, no proliferation is observed in cells from brachial, periaortic or mesenteric nodes (Constant *et al.*, 1990).

The kinetics of interferon gamma production in vaccinated mice coincides with the migratory pattern of the immunizing parasites. Interferon gamma is initially produced by lymphocytes obtained from axillary and inguinal lymph nodes five days after vaccination, peaking two weeks later. Therefore, secretion of interferon gamma correlates with level of resistance (Pemberton *et al.*, 1991). Th1 cell responses appear to participate in the development of inflammatory foci. After vaccination, large numbers of T cells infiltrate the lungs (Coulson & Wilson, 1993). When attenuated challenge schistosomules arrive in the lungs of vaccinated mice 4 days after skin penetration, they become surrounded by focal inflammatory infiltration, rich in mononuclear cells (Wynn *et al.*, 1994: Crabtree & Wilson, 1986). Moreover, alveolar macrophages are activated after challenge, suggesting that they interact with T lymphocytes (Menson *et al.*, 1989). Taken together, these factors imply that challenge parasites stimulate delayed type hypersentisivity (DTH) responses in the pulmonary tissues (Crabtree *et al.*, 1986). Schistosome reactive DTH-mediating lymphocytes are present in the circulation between days 10 and 17 post vaccination (Ratcliffe & Wilson, 1991) and will be among the mononuclear cells that will infiltrate the pulmonary tissues. This may explain the decline in circulating reactivity as a result of the recruitment of DTH- mediating cells in the lungs (Ratcliffe & Wilson, 1991). Hence, it appears that there are two basic requirements for development of protective immunity in the mouse. Firstly, a population of CD4+ T cells must be generated which responds to the antigens of lung schistosomula by initiating DTH responses. Secondly, these cells must be recruited to the lungs in advance of the challenging parasites (Ratcliffe & Wilson, 1992).

### 1.10 Control of schistosomiasis

Control of schistosomiasis is undertaken in various ways. These include: chemotherapy, sanitation, health education and vector control.

### 1.11 Chemotherapy of Schistosomiasis

In areas of high endemicity, control involves different forms of population-based chemotherapy, aimed at reducing prevalence, intensity and severity of infection to a level where it is not a major public health problem (WHO, 1985). Praziquantel, oxamniquine and metrifonate are currently the most widespread drug in use of treatment of schistosomiasis. Praziquantel is effective in all forms of schistisomiasis while oxamniqiune is effective only on *S. mansoni*. Metrifonate is effective *on S. haematobium* only. The set back to chemotherapy is high tendancy of re – infection, toxicity and resistance.

### 1.12 Sanitation and health educationp

The provision of safe and adequate water supplies and sanitation facilities contributes significantly to the reduction of prevalence and severity of schistosomiasis. Health education is aimed at improving water use practices preventing indiscriminate urination and defecation, which contaminate water bodies with eggs in faeces and urine (WHO, 1985).

### 1.13 Vector control

#### 1.13.1 Chemical control of the vectors

Snails being the intermediate host play an integral role in transmission. Snail control by use of molluscides has been in use since the 1950s. The use of molluscides has always been considered to be a major supportive procedure in intergrated control approach. Niclosamide is still the molluscide of choice, being highly active at all stages of snail life cycle and also on schistosome larvae. However, Niclosomide is toxic to fish and is costly. Moreover, it does not prevent recolonization of sites by the remaining snails, which could lead to selection of molluscide resistant populations (Larden & Dissous, 1998).

#### 1.13.2 Environmental management

As mentioned by several authors, no single control strategy has proven effective on its own in the control of schistosomiasis (Thomas 1987; Madsen & Christensen 1992; World Health Organization 1998; Sturrock 2001). An integrated approach, involving the application of several measures simultaneously, is needed to control schistosomiasis. In an intervention study in Central Morocco the authors compared three different environmental snail control measures that removed snails, egg masses, aquatic plants and silt (Laamrani & Boelee, 2002). Intensified cleaning of hydraulic structures led to a short-term reduction in density of *B. truncatus* but the intervention could not be sustained by the farmers in this area and snail populations rapidly re-colonized the habitats (Laamrani *et al.*, 2000). In the Rahad irrigation scheme in the Sudan, (Meyer-Lassen, 1992) observed the disappearance of *B. truncatus* after the removal of aquatic plants. In contrast, (Hilali *et al.*, 1985) reported less impressive results from the Gezira irrigation scheme, where mechanical weed removal alone did not lead to a significant reduction of the snail population, because of rapid re-colonization of the canals by weeds.

### 1.14 Biological control of the intermediate hosts

There are different biological control agents used to control the snail population. Predators such as small fish are successfully used in limiting the snail populations in islands such as Saint Lucia and Puerto Rico (Pontier, *et al.,* 1992). Snail pathogens such as *Echinostomatidae*, a dominant trematode, with the ability to sterilize snails are used to control snail population in China, Japan, and Brazil (WHO, 2002). Introduction of snail competitors to displace target snail populations (Madsen, 1990) or to compete for parasites by possible decoy effect (Combes, *et al.*, 1987) *Marisa cornuarieties* and *Melanoides tuberailata* have been successful in displacing *Biamphalaria glabrata* and *Biomphalaria stramineae* in Martinique Island. A major setback however is the potential of colonizing snails becoming susceptible to local schistosomes (Sturrock, 1995).

### 1.15 Vaccines against Schistosomiasis

Research findings reveal that continual exposure to Schistosomiasis elicits partial immunity and that complete, sterilizing immunity is not required since the parasite does not replicate within the human host. The evidence from field studies suggests that partial resistance due to infection probably develop in most people (James *et al.*, 2001). The intesinty of infection is generally lower in adults (Butterworth *et al.*, 1996), and following treatment with Praziquantel, adults also display some degree of resistance exhibited as lower intensities upon re- infection (Colley *et al.*, 1986). Therefore, it is possible that immunization may enhance the naturally occurring protective mechanisms noted in infected people, although it does not have to, because it may result in the stimulation of distinct immune modes of action. In this regard, the initial target group of immunization may be infants and children who have never been infected. Children normally are not heavily exposed to the threat of infection until they are toddler age: hence, this target period is the first two years of life.

The administration of radiation-attenuated cercariae to laboratory animals provided protection against experimental *S. mansoni* infection by blocking the migration of the parasite out of the lung. IFN-γ and Th1 cellular immune responses appear to play a key role in this process (WHO, 2009).

Most of the research has been done by utilizing antigens from schistosomules, with disappointing protection results so far. Somewhat better results have been obtained with antigens that are

shared between schistosomules and schistosomes, such as the 63 kD parasite myosin, the 97 kD paramyosin, the 28 kD triose phosphate isomerase (TPI), a 23 kD integral membrane protein (Sm23), and the 26 and 28 kD glutathione-S-transferases (GSTs). In recent Phase I and II clinical trials, the 28 kD *S. haematobium* GST (Sh28GST) developed by Institut Pasteur de Lille (France), was safe and showed good immunogenicity in human volunteers in France, Niger and Senegal (WHO 2009).

The Schistosomiasis Vaccine Development Programme (SVDP), based in Egypt and supported by United States Aid for International Development (USAID), has focused on two *S. mansoni* antigens: paramyosin and a synthetic peptide construct containing multiple antigen epitopes (MAP) from the triose phosphate isomerase (Bachem Company, Los Angeles, USA). Another candidate vaccine, which is developed by Fiocruz (Rio de Janeiro, Brazil), is based on the use of Sm14, a 14 kD fatty acid-binding *S. mansoni* protein with cross-reactivity with *Fasciola hepatica*. In mice, Sm14 provided a 67% protection against challenge with *S. mansoni* cercariae and full protection against *F. hepatica* metacercariae (WHO 2009).

None of the above candidate vaccines has, however, been able so far to provide more than a partial reduction in challenge-derived worm burdens relative to non-immunized controls. It is hoped that better success can be achieved using cocktails of recombinant antigens.

Another approach to vaccination against schistosomiasis has been to target the fecundity of the female schistosome in order to diminish egg excretion. Success with this approach has been reported in mice and large animal reservoir hosts, including pigs and water buffaloes, using *S. japonicum* 26 kD GST and paramyosin.

### 1.16 Common antigens between *Biomphalaria* snails and *Schistosoma* parasites

Snails have been found to have shared proteins with the *S mansoni* parasite. Common antigens between different species of *Schistosoma* and their intermediate hosts have been reported (Chacón *et al., 2000*). This study demonstrated that sera from schistosome-infected persons reacted against soluble crude *Biomphalaria glabrata* antigen (S*Bg*A) by ELISA (100% of sensitivity) and that sera from mice immunized with S*Bg*A recognized several homologous snail molecules by Western-blot (Chacón *et al., 2000*).

Immunoelectrophoretic studies on common antigenicities between the Puerto Rican strain of *Schistosoma mansoni* and *Biomphalaria* snails was carried out by using rabbits sera immunized with the Puerto Rican strain of *Schistosoma mansoni* adult worms or eggs and antigens of several adult *Biomphalaria* snails and vice versa. *S. mansoni* adult worm extracts produced 8 bands both with extracts of *B. glabrata* pigmentation and *B. glabrata pigmentado*. There were *3* to 4 bands with those of *B. glabrata* albino and 1 to 2 bands with those of *B. straminea* in the above study. On the other hand, *S. mansoni* egg extracts produced 5 bands with extracts of *B. glabrata* pigmentation, 4 bands with those of *B. glabrata pigmentado*, 2 bands with those of *B. glabrata* albino and 1 band with those of *B. straminea*. In the experimental infection of adult *Biomphalaria* snails with five *S. mansoni* miracidia, the infection rate in *B. glabrata* pigmentation was 78.8%, and 71.2% in *B. glabrata* pigmentado, whereas the infection rate in *B. glabrata* albino was 10.3%, and *B. straminea* was not susceptible to *S. mansoni*. The infectivity of each snail corresponded with the number of bands representing common antigenicities between host and parasite. Crude antigens of *Biomphalaria* snails were fractionated by Sephadex G-100 column, and each antigen fraction was tested with anti-*S. mansoni* adult worm and egg sera by immunoelectrophoresis. The common antigenicities between fractionated antigens of Biomphalaria snails and of anti-*S. mansoni* adult worm or egg sera mostly existed in the first fraction 1 with Mr > 45 kDa.( Iwanaga , 1994 ).

In a study of structural homology of tropomyosins from the human trematode *S.mansoni* and its intermediate host *B. glabrata,* characterization of protein determinants shared by *S.mansoni* and its intermediate host *B.glabrata* were carried out. Parasite (Sm39) and mollusc (Bg 39) cross-reactive proteins were identified and shown to induce in rabbit and mouse, antibodies specific for invertebrate determinants. Ultra structural studies demonstrated that antibodies to Sm39 specifically bound to muscular structures of parasite and mollusc. Molecular cloning and sequencing indicated that Bg39 corresponded to a muscular isoform of tropomyosin. The mollusc sequence showed a 51-65% homology with seven different muscular tropomyosins from vertebrate and invertebrate species. The highest score of homology was observed with *S. mansoni* tropomyosin, suggesting that cross-reactive determinants could be specific for the trematode and its intermediate host. In miracidia, Sm39 epitopes were also shown to be contained in the vesicles present in epidermal ridges and cellular bodies. Such vesicles are involved in the formation of a protective tegument around sporocysts, suggesting a possible role

of cross-reactive tropomyosins in miracidia and/or sporocyst-snail interactions (Dissous *et al.*, 1990).

### 1.17 Use of shared antigens in vaccine development

Studies on Antigenic Community between *S. mansoni* and *B.glabrata*: on the Search of Candidate Antigens for Vaccines (Chacón *et al.* 2000) confirmed the presence of common antigens between *S. mansoni* and its vector, *B. glabrata*. Cross-reactive antigens may be important as possible candidates for vaccine and diagnosis of schistosomiasis. Sera from outbred mice immunized with a soluble *B. glabrata* antigen (SBgA) of non-infected *B. glabrata* snails recognized molecules of SBgA. In order to determine which epitopes of SBgA were glycoproteins, the antigen was treated with sodium metaperiodate and compared with non-treated antigen. Molecules of 140, 60 and 24 kDa in the SBgA appear to be glycoproteins. Possible protective effects of the SBgA were evaluated by immunizing outbred mice in two different experiments using Freund's adjuvant. In the first one (12 mice/group), they obtained a significant level of protection (46%) in the total worm load, with a high variability in worm recovery. In the second experiment (22 mice/group), no significant protection was observed, neither in worm load nor in egg production per female (Chacón *et al.*, 2000)

### 1.18 Justification and significance of the study

Schistosomiasis is second to malaria in public health importance of parasitic infections. It is estimated that 600 million people in 74 countries are exposed to the risk of (infection with) schistosomiasis (WHO, 1996).

Chemotherapy remains the cornerstone of intervention but rapid re-infection demands frequent re-treatment and emphasizes the need for a more long-term approach. The existence of at least partially protective immunity in exposed humans would make vaccination a logical complement to drug therapy.

One of the main concepts that have emerged during recent years is that the potential control of these major parasitic diseases will rely on multiple and intergrated strategies, among which the modern tools of immuno-intervention, will play a significant role.

A number of vaccines utilizing well-explored parasite antigens with reported consistent high protection have been shown in various experiments and laboratories. They include paramyosin, a myosin-like molecule implicated in the irradiated cercariae model and Sm14, a protein characterized by its ability to bind fatty acids. None of the above candidate vaccines has, however, been able so far to provide more than a partial reduction in challenge-derived worm burdens relative to non-immunized controls (WHO, 1996). This study therefore aimed at investigating the effect of immunizing Swiss mice with soluble proteins from colony bred *B. pfeifferi* snails and challenging them with *S. mansoni* worms to generate information that would be useful in the control of schistosomiasis through vaccination.

### 1.19 Problem statement

Snails have been found to have shared proteins with the *S. mansoni* parasite. Cross-reactive antigens may be important as possible candidates for vaccine and diagnosis of schistosomiasis. The snail is larger than the parasite and would be a larger source of vaccine as compared to the parasite if a vaccine would be found from it.

### 1.20 Null hypothesis

Mice immunized with soluble proteins from digestive gland ( DG) and the rest of the tissues( RT) of an intermediate host are not protected against *S. mansoni.*

### 1.21 General objective

To determine the effect of immunizing Swiss mice with soluble proteins from the intermediate host *B. pfeifferi* and challenging them with *S. mansoni.*

### 1.22 Specific objectives

1 To determine worm recovery in mice immunized with soluble proteins from the digestive gland and the rest of the snail tissue from colony bred intermediate host *B. pfeifferi*

2 To determine humoral and cellular immune responses in mice immunized with soluble proteins from the digestive gland and the rest of the snail tissue from colony bred intermediate host *B. pfeifferi*

3 To examine gross pathology of liver tissues from mice immunized with soluble proteins from the digestive gland and the rest of the snail tissue from colony bred intermediate host *B. pfeifferi*

4 To examine histopathology in liver tissues from mice immunized with soluble proteins from the digestive gland and the rest of the snail tissue from colony bred intermediate host *B. pfeifferi*

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Experimental design

The study was done using mice as the models for human schistosomiasis. The mice were in four groups, two experimental groups DG (Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged); RT (Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged) and two controls IC (Infected control) and NC (Naïve). The first three groups comprised of eighteen mice each and the last one group (NC) of two mice. The study involved vaccination of mice and then challenging them with cercariae of *S. mansoni*. On day 0 (zero) of the experiment DG and RT groups were vaccinated with 50 µg of snail antigens in 100 µl of complete Freunds adjuvant intra-peritoneally. In week two and four DG and RT groups of mice were boosted with 50 µg of the snail antigens in 100 µl of incomplete Freunds adjuvant. One week after final vaccination, mice in DG, RT and IC groups were challenged with cercariae of *S. mansoni*. In week two and four after challenge, six mice from each of the three groups were sampled for cells from the lymph nodes and spleen for cell culture and blood for ELISA. In NC group the two mice were sampled for serum only. In Week six post challenge, mice from the three groups were: sampled for blood; perfusion was carried out to recover adult worms; their livers were examined for gross pathology and tissues were preserved for histopathology. The experimental design is shown in table 2.1.

**Table 2:1 Experimental design for immunization, challenge and sampling for serum and cells**

| EXPERIMENTAL GROUPS | n | TIME IN WEEKS | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | -2 | -4 | 0 | 2 | 4 | 6 |
| DG | 18 | IM | Bo | Bo | Ch | S | S | P/SERUM |
| RT | 18 | IM | Bo | Bo | Ch | S | S | P/SERUM |
| IC | 18 | - | - | - | Ch | S | S | P/SERUM |
| NC | 2 | - | - | - | - | SERUM ONLY | - | - |

**Key**

- DG- Mice immunized with antigens from snail digestive glands
- RT- Mice immunized with antigens from rest of snail tissue
- IC- infected control
- Naïve-Non-infected control
- N-Total number of mice per group
- IM-Immunization
- Bo-Boost
- Ch-Challenge
- S-Sampling for serum and cells P-Perfusion, gross pathology and histopathology
- - No activity

## 2.2 Definitive host – Swiss Mice

Swiss mice (*Mus musculus*) are permissive definitive hosts of *S. mansoni*. Six weeks old mice were used in this study. They were obtained at Kenya Medical Research Institute (KEMRI) and maintained at the Animal Resources Department at the Institute of Primate Research (I.P.R). Mice were fed with nutrient pellets (Laboratory Cho from Unga Feeds ® CO.) and supplemented with carrots and kale leaves. Water was supplied ad *libitum*.

## 2.3 Intermediate host – Snails

*Biomphalaria pfeifferi* snails originally collected from streams in Kakuyuni, Kangudo (Machakos District) and housed at Institute of Primate Research for over five years were used in this experiment.

## 2.4 Laboratory maintenance of infected snails

Snails were maintained in snail tanks, which contained snail water and sterilized sand from Kakuyuni, Kangundo, where the snails were initially collected from. During the entire period, the room temperature (RT) was maintained at 22-25$^0$C. The snails were kept in Chlorine free water from IPR well (Snail water) which were changed once per week. The tanks were aerated by Daphnea which feed on decaying organic matter. The snails were fed on dried lettuce.

## 2.5 Harvest of *S. mansoni* eggs from baboon faeces and infection of snails

Feacal samples from Olive baboons (*Papio anubis*) with chronic *S. mansoni* infection were collected. Feacal samples were thoroughly mixed with PBS (x1) in a plastic jar. The slurry was poured on a standard test sieve (Arthur Thomas Co. USA) of 600µm placed on another sieve of 250 µm; the latter had a collecting tray placed under it. Water was poured on the feacal sample. After sieving, the feacal debris was discarded, while the feacal suspension was poured into urine glass jars. The suspension was placed in the dark and left to sediment for at least 30 minutes. The supernatant was poured out and the pellet was re-suspended in water and then allowed to sediment in the dark. This procedure was repeated three times until the supernatant became clear. The clear supernatant was poured out. The sediment was placed on Petri dish containing water. This Petri dish was placed under a 100 Watts lamp for at least 30 minutes to allow

hatching of free swimming larvae (miracidia) from the eggs. Procedure is as used by (Kanyugo *et al* 2009)

Snails were placed individually in wells of a 24 microwell plate (Nunclon, Denmark), and 3-5 miracidia were dispensed into each well to infect the snails. After 30 minutes, snails were transferred to snail tanks. Four weeks post infection (pi), snail tank were covered with black clothe to prevent light from stimulating trickle shedding of cercariae. Five weeks PI, the snails were carefully removed using forceps from tanks and placed in 100 ml beakers containing snail water (Non-chlorinated water from IPR wells). They were exposed to 100 Watt lamp shaded with glass for 1 hr to shed cercariae. Cercariae suspensions were pooled together. Three 50 µl aliquots of the cercariae suspension were counted under the dissecting microscope. Volume containing 150 cercariae for challenge was worked out.

### 2.6 Preparation of soluble proteins from the S. mansoni intermediate host Biomphalaria pfeifferi

A hundred colony bred *B. pfeifferi* snails were dissected under the dissecting microscope to extract the digestive gland and the rest of the snail tissues. The digestive gland and the rest of the snail tissue were placed in separate nunc tubes containing a small volume of PBS and placed on ice. The tissues were crashed in a glass mortar and pestle to obtain a homogenate which was centrifuged in a microfuge for 1 hr at 14,000/rpm at $4^{0}$C to obtain the soluble protein. The concentration of the protein was assayed using Bradford method (1976). This method utilized bovine serum albumin, BSA, (Biorad Co.) as a standard protein. The optical densities at 595nm, of serial dilution of BSA were read using ELISA reader. Protein concentration was adjusted to 1mg/ml before being used in *in vitro* assays. The protein was aliquoted and sterized by exposure of UV light (10 minutes, 5cm from a 30 watt ultra violet OSRAM bulb). The aliquots were stored at -$20^{0}$C (Mutua, 1988).

### 2.7 Immunization of mice

At day 0 (zero) of the experiment DG group was vaccinated with 50 µg of snail antigens extracted from the snails digestive gland in a 100 µl of complete Freunds adjuvant intra-peritoneally while the RT groups were vaccinated with 50 µg of snail antigens extracted from the rest of the snail tissues in a 100 µl of complete Freunds adjuvant intra-peritoneally. In week two

and four DG and RT groups of mice were boosted with 50 µg of their specific snail antigens in 100 µl of incomplete Freunds adjuvant.

### 2.8 Challenge of mice

A week after administration of final immunization, general anaesthesia was administered to the mice to produce loss of consciousness, analgesia and suppression of reflex of activity, and muscle relaxation. A mixture of Rompun and Ketamine 20:1 was used to provide a combined effect of anesthesia [10mls of Ketamine (Agrar, Holland) were mixed with 0.5 mls of Rompun]. Anesthesia at 0.02ml/30mg-mouse body weight was injected intraperitonealy. The anaesthetized mice were shaved on the stomach area. Cotton wool dipped in water was used to moisten the shaven area to allow easy penetration of the cercariae. A 1-cm diameter metal ring was placed on the shaven area of each mouse. A suspension containing 150 live cercariae were dispensed in the metal ring using a micropipette. A period of 30 minutes was allowed for cercariae to penetrate into the mice (Smithers and Terry, 1965).

### 2.9 Collection of blood samples

#### 2.9.1 Heart puncture

At specified time points week 2 for NC group, week 2, 4 and 6 for DG, RT, and IC mice were anaesthetized by intraperitoneal injection of 0.02ml of Ketamine/ xylaxine mixture as described above. A small incision was made at the centre of the abdominal skin and the skin torn around the waist of the mouse. The skin was then peeled upwards to expose the thoracic region. A small cut was made through the ribs on the right side of the mouse just above the diaphragm. The cut was made up to the sternum. The same was done to the left side and the ribs trimmed carefully to prevent puncture of the lungs or the heart. Another cut was made on either sides of the sternum taking care not to puncture the interthoracic arteries. The left ventricle was located and a 1 ml syringe inserted. Blood was sucked in small jerks in order to create a vacuum and prevent collapsing of the heart due to sucking of large amounts of blood. After draining all the blood from the left ventricle, the same process was used to suck blood from the right ventricle. The whole volume of blood collected was dispensed into microfuge (eppendorf) tube and left on the bench for 2 hrs to clot. Thereafter, the clotted blood was processed into serum and stored at -20$^{0}$C as described below (Yole, 1993).

### 2.9.2 Serum Preparation

The collected blood was spun at high speed of 700xgor 700g/700xG for 10 minutes in a microfuge .The clear supernatant (serum) was transferred to another clean epperdorf tube and stored at -20$^{0}$ C until use for antibody ELISA later.

## 2.10 Sampling procedure for lymph nodes and spleen

The skin of the mouse bleed in 2.9.1 above was peeled downwards to the groin region. Auxillary lymph nodes that are located near the arm pit, slightly above the blood vessel and inguinal lymph nodes that are located at the lower part of the abdominal skin, at the junction of three blood vessels on either side of the abdominal skin, were removed and placed in a Petri dish containing incomplete media (RPMI/1640, Gentamycin, L-Glutamine,Beta-mecarptoethanol, sodium bicarbonate). In addition, the abdominal cavity was opened and the spleen was removed using sterile forceps and the spleen placed on Petri dish containing incomplete media (Yole, 1993).

### 2.10.1 Preparation of Lymph Node Cells

Both inguinal and auxiliary lymph nodes were picked using a pair of sterile forceps and placed in a sterile Petri dish containing incomplete media. They were transferred to a sterile petri dish containing sterile medium in a sterile culture hood. The lymph nodes were teased using a pair of sharp sterile forceps and the suspension dispensed into a 15 ml tube. The cells were washed two times in incomplete media by centrifugation at 450xgor 450g/450xG, for 10 minutes at room temperature, supernatant discarded, and pellet resuspended and fresh sterile incomplete media was added. This washing process was repeated once. After the final wash, supernatant was discarded and cells resuspended in 4 ml of complete medium (incomplete medium fortified with 10% foetal serum). The supernatant was discarded and the pellet resuspended in 1 ml of the complete media, RPMI10 [(RPMI 1640 {Gibco BRL, Life Technologies Ltd. Scotland}, containing 10% fetal calf serum (Gibco BRL, Germany), Gentamyicine 2mM (Sigma Co.) and 1% β-mercaptoethanol (Ladzins, 1982)].

Lymphocyte viability was determined by the Trypan blue exclusion test. (The plasma membrane of viable cells does not permit the entry of non – electrolyte dye substances such as Trypan blue. This phenomenon is used to distinguish dead lymphocytes from living ones). Lymphocyte suspension was mixed with Trypan blue at 1:10 ratio and incubated at RT for at

least 5 minutes to facilitate dye uptake. After incubation, the lymphocyte count was done using a haemocytometer. Lymphocyte tryphan blue suspension was charged on to the haemocytometer chamber and counting was done under light microscope at ×40. Cells from culture were made up to 3 x$10^6$ cells/ml in the complete media (Yole, 1993).

### 2.10.2 Preparation of Spleen Cells

Spleen from each mouse was transferred to sterilized wire gauze, in a Petri dish containing sterile incomplete media in sterile culture hood and a 10ml syringe piston was used to squash the spleen. A Pasteur pipette was used to disperse the cells. The cells were then dispensed in 15ml tube and topped up with incomlete media. The cell suspension was centrifuged at 450xgor 450g/450xG, for 10 minutes at room temperature, supernatant discarded, and pellet resuspended and fresh sterile incomplete media was added. This washing process was repeated once. After the final wash, supernatant was discarded and cells resuspended in 4 ml of complete medium (incomplete medium fortified with 10% foetal serum).Lymphocyte count was carried out as described for lymph node cells (Yole, 1993).

## 2.11 Immunological assays

### 2.11.1 Preparation of 0-3 hr release protein

*S. mansoni* cercariae were obtained by shedding infected snails with a patent infection of five weeks. Heads and tails of *S. mansoni* cercariae were separated as described by Ramolho-pinto *et al.*, (1974). The heads were isolated on discontinuous percoll gradient and washed two times in complete media. Two concentrations of Percoll (Sigma Co. Sweden) were prepared; one which was 70% Percoll mixed with 9 ml of RPMI 1640 media). The other set consisted of 45% Percoll (9 ml of Percoll mixed with 11 ml of RPMI 1640). A drop of Hepes (Flow Laboratories, Scotland) was added into each tube to keep the pH constant. The 45% Percoll was layered over the 70% Percoll to make a discontinous gradient. Cercariae suspension was chilled for one hour at $4^0$ C. They settled at the bottom of the beaker. Excess water from the chilled cercariae suspension was sucked out using a Pasteur pipette and the cercariae resuspended. The cercariae suspension was placed in chilled glass tubes and centrifuged for 10 seconds at 450xgor 450g/450xG.

Glucose was made up to 5% in double distilled water. After centrifugation, the supernatant was sucked out of the glass tubes containing cercariae and the 0.5 ml glucose added. The suspension was shaken on a vortex for 90 seconds to separate the heads from the tails of the chilled cercariae. The separated heads and tails of cercariae were dispensed gently on the Percoll gradient using a Pasteur pipette and centrifuged at 450xgor 450g/450xG, for 10 min. The heads formed a band at the top of the interface of the gradient.

The heads were aspirated and washed three times in complete media. The heads were re-suspended in complete media and transferred to the Bijou tubes and incubated at 37 $^{0}$C, in the presence of 5% carbon dioxide for 0-3 hr release protein. After 3 hr of incubation, the schistosomula (head) suspension was centrifuged (10 minutes at 450g, at 37 $^{0}$C). The supernatant was obtained. This contains the proteins released by penetrating schistosomula between 0-3 hr of penetration. Protein concentration was assayed as described below.

The concentration of the protein was assayed using Bradford method (1976). This method utilized bovine serum albumin, BSA, (Biorad Co.) as a standard protein. The optical densities at 595nm, of serial dilution of BSA were read using ELISA reader. The protein was aliquoted and sterized by exposure of UV light (10 minutes, 5cm from a 30 watt ultra violet OSRAM bulb) before use in *in vitro* assays. The aliquots were stored at -20$^{0}$C. Protein concentration was adjusted to 1mg/ml before being used in *in vitro* assays (Yole, 1993).

### 2.11.2 Schistosome worm antigen preparation (SWAP)

Adult *S. mansoni* worms were obtained from infected mice perfused at week five post-infection. The worms were placed in a tube containing PBS, and sonicated (23khz, 16um amplitude) for 10 minutes and the homogenate was centrifuged for 1 hour at 14, 000g, under 4 $^{0}$ C to obtain the soluble protein. The protein estimation was done based on the procedure described for 0-3 hr. The protein concentration was adjusted to 100 µg/ml in PBS before use in vitro assays (Yole, 1993).

### 2.11.3 Culture Supernatant

Flat-bottomed 48-well microtitre plate was used for culture and 6 x 105 cells were dispensed in each well. Duplicate wells were set for each regime. Negative control had only medium and

cells. Positive control had 1µg/ml of Concanavalin A and test wells, 10µg/ml of (SWAP) and 0-3 hr preparation. The total volume of culture medium per well was 400µl. The plates were incubated at 37°C, 5% CO2 for 48hr for Con A and 72 hr for the other set-ups. The supernant was collected from each well and were stored at -20 $^0$C for the assays below.

**2.11.4 Mouse Interferon gamma ELISA**

Nunc-Immulo$^{TM}$ (Maxisorp$^{TM}$ surface ) ELISA plates from MAb Tech was used for assay. They were coated with 50 µl of 5 µg/ml solution of monoclonal anti-IFN antibody. They were incubated overnight at 4°C. The plates were then washed six times and 100 µl/well blocking buffer 0.1% Bovine Serum Albumin (BSA) was added. The plates were incubated for 1 hr at 37$^0$ C and washed six times and 50 µl/well of samples and mouse interferon gamma standards were added and plate incubated for 2 hrs at 37° C. They were washed six times and 50 µg/well of 3 µg/ml rabbit anti-mouse IFN (mAb TRFK 4-biotin) added. The plates were then incubated for 1hr at 37$^O$C. The plates were washed six times and 50 µl/well of Streptavidin-Horseradish peroxide added. They were incubated for 1 hr at 37$^0$C and washed six times and 50 µg/well of the substrate (Sure Blue $^{TM}$ TMB) added. The plates were incubated at 37$^O$C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader (Dynatech MRX) (Yole, 1993)

**2.11.5 Mouse Interleukin - 5 (IL – 5 ELISA)**

Nunc-Immulo$^{TM}$ (Maxisorp$^{TM}$ surface) ELISA plates from MAb Tech was used for assay. They were coated with 50 µl of 5 µg/ml solution of monoclonal anti-IL-5 antibody. They were incubated overnight at 4°C. The plates were then washed six times and 100 µl/well blocking buffer 0.1% Bovine Serum Albumin (BSA) was added. The plates were incubated for 1 hr at 37$^0$ C and washed six times and 50 µl/well of samples and mouse interferon gamma standards were added and plate incubated for 2 hrs at 37° C. They were washed six times and 50 µg/well of 3 µg/ml rabbit anti-mouse IL-5 ( mAb TRFK 4-biotin) added. The plates were then incubated for 1 hr at 37$^O$C. The plates were washed six times and 50 µl/well of Streptavidin-Horseradish peroxidase added. They were incubated for 1 hr at 37$^0$C and washed six times and 50 µg/well of the substrate (Sure Blue $^{TM}$ TMB) added. The plates were incubated at 37$^O$C in the dark for 30

minutes. Optical density was read at 630 nm in an ELISA microplate reader (Dynatech MRX) (Yole, 1993).

### 2.11.6 Enzyme-Linked Immunosorbent Assay (ELISA) for Schistosome Specific IgG

Nunc-Immuno™ plates (MaxiSorp ™ Surface) ELISA plates were coated overnight at 4 °C with 50 µl of soluble, adult worm antigen preparation (SWAP) 10 ug/ml and 0-3 hr antigens, diluted in bicarbonate buffer, pH 9.6. The antigens were then dispensed off on a blotting paper. The plate was washed six times using the washing buffer (0.05 % Tween 20 in PBS). This was followed by blocking of the non-specific binding sites with 100 µl 3% BSA in PBS for 1 hr at 37 °C and washing off unbound BSA six times with washing buffer. Diluted (1:200) serum samples (50 µl) were dispensed into each well in duplicates and incubated for 1 hr at 37 °C, and then washed as above. After washing the unbound serum, 50 µl of 1:2000 peroxidase conjugated goat anti-mouse IgG (SIGMA Goat anti-mouse IgG peroxide) was dispensed into the wells and incubated for 1 hr at 37 °C. The unbound conjugate was washed off as before and 50 µl SureBlue ™ microwell peroxide substrate (1-component) was added. The plates were incubated at 37 °C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader (Yole, 1993).

## 2.12 Parasitological Assays

### 2.12.1 Perfusion

Mice from each group were perfused to recover the adult worms, using a modified method of Smithers and Terry (1965). Each mouse was anaesthetized with a dose of 0.02ml/30gm of Ketamine/Rompun mixture (3:1). A transverse mid vertal cut was made on the skin of the abdomen with a pair of scissors and the skin peeled off. The abdomen was opened without cutting the viscera. The diaphragm was cut carefully to expose the heart and the ribcage trimmed. The hepatic portal vein was incised. Perfusion needle containing perfusion fluid (0.85% Sodium chloride and 1.5% Sodium nitrate) was inserted on the left ventricle of the heart and perfusion carried out until the liver, lower limbs and mesenteries were clear. The perfusate was collected in a 20 cm glass petri-dish and transferred in a urine jar to settle.

### 2.12.2 Adult Worm Recovery

Worms were recovered using the method of the Yole *et al* 1996. The perfusate containing the recovered worms were transferred into urine jar and topped with phosphate buffered saline (PBS). After the worms had settled, the supernatant was sucked out, and the settling procedure repeated three times. When the supernatant was clear, the worms were then placed on Petri dish containing PBS and then counted. Percentage worm recovery and reduction for each group was calculated as shown in the formulae below.

Percentage worm recovery =

$$\frac{\textit{Mean of worms from the experimental group}}{\textit{Mean of worms from the IC}} \times 100$$

Percentage worm reduction

$$\frac{\textit{Mean of worms from the IC - the mean of worms from the experimental group}}{\textit{The mean of worms from the IC}} \times 100$$

## 2.13 Pathological Examination

### 2.13.1 Gross Pathology

Gross pathology examination was done before perfusion. It focused on the general and overt appearance of the liver. The observation that was considered in the liver included inflammation, adhesions and presence of granulomas. Granulomas appear as raised pinheads sized foci distributed over the surface of the liver lobes. 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and ≥ 10 granulomas per lobe was considered severe (Yole et al.,1993).

### 2.13.2 Histopathology

The livers were fixed immediately in 10% buffered formalin for at least two weeks. The fixed tissue samples of liver sections were transferred into tissue cassettes and then immersed into 80%, 95% and 100% ethanol respectively to achieve optimum dehydration. The tissues were

cleared in toluene and then infiltrated in hot paraffin. The tissues then were embedded on tissue embedding paraffin wax (Sherwood Medical Co. USA). The tissue was sectioned serially at 6 microns using a Rotary microtome (Leitz, Germany). The thin tissue sections were mounted on glass slides and stained with Haemotoxylin and Eosin. Observation was done under microscope at × 100 (Kanyugo *et al* 2009).

### 2.14 Statistical analysis

Student t-test was used for comparing mean differences in worm burdens of groups DG, RT and IC. Comparisons of humoral and cellular immune responses among Groups of mice were performed using Student t- test; p values < 0.05 were considered significant.

## CHAPTER 3: RESULTS

### 3.1 Parasitological findings

Mice in DG group were immunized with antigen derived from the digestive glands of the intermediate host then infected. Mice in RT were immunized with antigens derived from the rest of the body tissues of the intermediate host and then infected, while mice in IC group were infected only, i.e. the infected control. The mean number of *S.mansoni* worms that were recovered from Swiss mice in the three groups, percentage worm recovery and percentage worm reduction are shown in Table 3. The mean number of worms for RT group was 3.5±0.495, DG group 4.9 ±0.69 and IC was 8.7 ±0.94. This indicates that RT had a lower mean number of worms than the other vaccinated group, DG and also the infected control.

### 3.2 Percentage worm recovery

Percentage worm recovery for the two experimental groups was calculated using the formula shown below.

Percentage worm recovery =

$$\frac{\textit{Mean of worms from the experimental group}}{\textit{Mean of worms from the IC}} \times 100$$

Adult male and female *S.mansoni* worms were recovered from the DG and RT groups of mice that were challenged with 150 cercariae (Table 3). The percentage worm recovery for RT group was 39.5% while for the DG group was 56.66% when perfused 6 weeks post infection. This indicates that RT had lower percentage worm recovery as compared to DG.

### 3.3 Percentage worm reduction

Percentage worm reduction for the two experimental groups was calculated using the formula shown below.

Percentage worm reduction =

$$\frac{\textit{Mean of worms from the IC - the mean of worms from the experimental group}}{\textit{The mean of worms from the IC}} \times 100$$

Percentage worm reduction for the RT group was 60.5% while for the DG group was 43.3% indicating RT vaccine reduced more worms than DG vaccine (Table 3).

A t-test analysis was done on RT and IC group of mice for comparing mean worm burden. RT had significantly lower worm burden than IC. The difference between DG and IC was also significant with the number of worms recovered from RT being significantly lower than that for DG. This shows that the two vaccines reduced worms significantly, although RT was better than DG.

**Table 3.1: Worm recovery and reduction in mice immunized with soluble proteins from *B. pfeifferi* and challenged with *S. mansoni***

| TREATMENT GROUPS | MEAN NUMBER OF WORMS (mean, S.E) | % WORM RECOVERY | % WORM REDUCTION |
|---|---|---|---|
| RT | 3.5±0.495 | 39.5 | 60.5 |
| DG | 4.9±0.69 | 56.7 | 43.3 |
| IC | 8.7±0.94 | - | - |

**Key: RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then infected; **IC**- Infected control.

## 3.4 Cellular responses

### 3.4.1 IFN-gamma responses in lymph node cells stimulated with 0-3 hr protein release and swap

Figure 3.1(a) show IFN-gamma responses in lymph node cells stimulated with 0-3 hr protein release in RT, DG and IC group of mice. In RT mice, the response was elevated at week 2 with a mean of 379.65 ± 46.54 pg/ml. The response declined significantly at week 4 to a mean of 262.35 ± 29.69 pg/ml ($p< 0.05$). The response in DG group was high in week 2 with a mean of 230.25 ± 8.99 pg/ml. However, the responses decreased at week 4 with a mean of 201.25 ± 28.47 pg/ml. This decline was not statistically significant ($p> 0.05$). In IC mice, the response was elevated at week 2 with a mean of 229.35 ± 14.17 pg/ml. At week 4 however, the response declined with a mean of 175.05 ± 25.63 pg/ml. The decreased response in IC was not statistically significant ($p> 0.05$).

A significant difference was noted between RT and IC ($p< 0.05$) in week 2 and 4; the response was higher in RT than in IC group. There was no significant difference in responses at week 2 and 4($p> 0.05$) between DG and IC Groups. However, the response was slightly higher in DG than in IC group .There was a significant difference found between RT and DG groups of mice at week 2 ($p< 0.05$). At week 4 however, no significant difference ($p> 0.05$) was noted though RT showed higher response than DG.

Figure 3.1(b) shows IFN-gamma responses in lymph node cells stimulated with SWAP in RT, DG and IC groups of mice. In RT mice, the response was elevated at week 2 with a mean of 126.5 ±48.57 pg/ml. The response insignificantly declined at week 4 with a mean of 109.75± 14.56pg/ml ($p> 0.05$). The response in DG group was high in week 2 with a mean of 99.75±35.63 pg/ml. However the response insignificantly decreased at week 4 with a mean of 77.98± 29.10 pg/ml ($p> 0.05$). In IC mice, the response was elevated at week 2 with a mean of 93.5±28.46 pg/ml. At week 4 however, the response declined with a mean of 75.25±21.66 pg/ml. This decline was not significant ($p > 0.05$). No significant difference ($p > 0.05$) was noted between RT and IC in week 2 although responses were slightly higher in RT group than IC. A significant difference ($p < 0.05$) was noted between RT and IC in week 4. The response was higher in RT than in IC group. There was no significant difference ($p > 0.05$) in responses at week 2 and 4 between DG and IC Groups; the response was higher in DG than in IC group

There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at week 2 and 4 with RT showing higher response at all the time points.

A major observation made throughout the sampling points was that RT responses were higher in all cases; elevation from week 2 to 4 for spleen and decrease from week 2 to week 4 for lymph node. This trend was recorded for both RT and DG. 0-3 hr gave higher responses than SWAP.

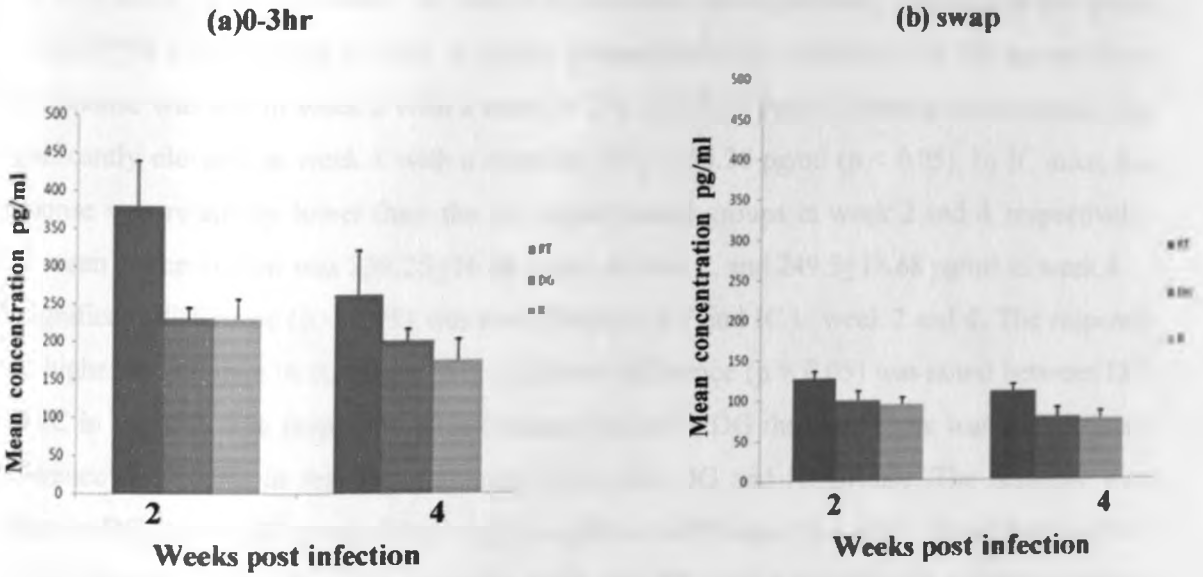


**Figure 3.1: IFN-gamma responses in mice lymph node cells stimulated with 0-3 hr protein release and swap**

**Key: RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control.

### 3.4.2 IFN-gamma responses in spleen cells stimulated with 0-3 hr protein release and swap

Figure 3.2(a) show IFN-gamma responses in spleen cells stimulated with 0-3 hr protein release in RT, DG and IC groups of mice. The response in RT mice was low at week 2 with a mean of 410.75±138.42 pg/ ml. However, at week 4 the response was significantly elevated with a mean of 498.25±98.411 pg /ml($p < 0.05$). A similar phenomenon was observed with DG group where the response was low in week 2 with a mean of 251.25±29.27 pg/ml. However the response was significantly elevated at week 4 with a mean of 367.25±66.28 pg/ml ($p < 0.05$). In IC mice, the response was relatively lower than the two experimental groups in week 2 and 4 respectively. The mean concentration was 230.25±16.68 pg/ml at week 2, and 249.5±18.68 pg/ml in week 4.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2 and 4. The response was higher in RT than in IC group. No significant difference ($p > 0.05$) was noted between DG and IC in week 2. The responses were however higher in DG than IC. There was a significant difference ($p < 0.05$) in responses at week 4 between DG and IC Groups. The response was higher in DG than in IC group. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at all the sampling point with RT having significantly higher responses than DG in the two time points.

Figure 3.2(b) show IFN-gamma responses in spleen cells stimulated with SWAP in RT, DG and IC groups of mice. In RT mice, the response was high at week 2 with a mean of 250.25 +14.77 pg/ml. The response was elevated at week 4 with a mean of 280.35±18.98 pg/ml. The response in DG group was low in week 2 with a mean of 75.25±19.48 pg/ml. However the response was elevated at week 4 with a mean of 110.15±22.73 pg/ml. In IC mice, the response was low at week 2 with a mean of 103.75±13.83 pg/ml. At week 4 however, the response was elevated with a mean of 120.65±31.72 pg/ml. The elevated response in RT and IC was not statistically significant ($p > 0.05$).

A significant difference ($p < 0.05$) was noted between RT and IC in week 2 and 4; the response was higher in RT than in IC group. A significant difference ($p < 0.05$) was noted between DG and IC in week 2 .The responses was higher in IC group than DG. There was no significant difference ($p > 0.05$) in responses at week 4 between DG and IC Groups. However, the response was higher in IC than in DG group. There was a significant difference ($p < 0.05$) found between

RT and DG groups of mice. No significant difference ($p > 0.05$) was noted at week 4 between RT and DG though RT showed a higher response than DG.

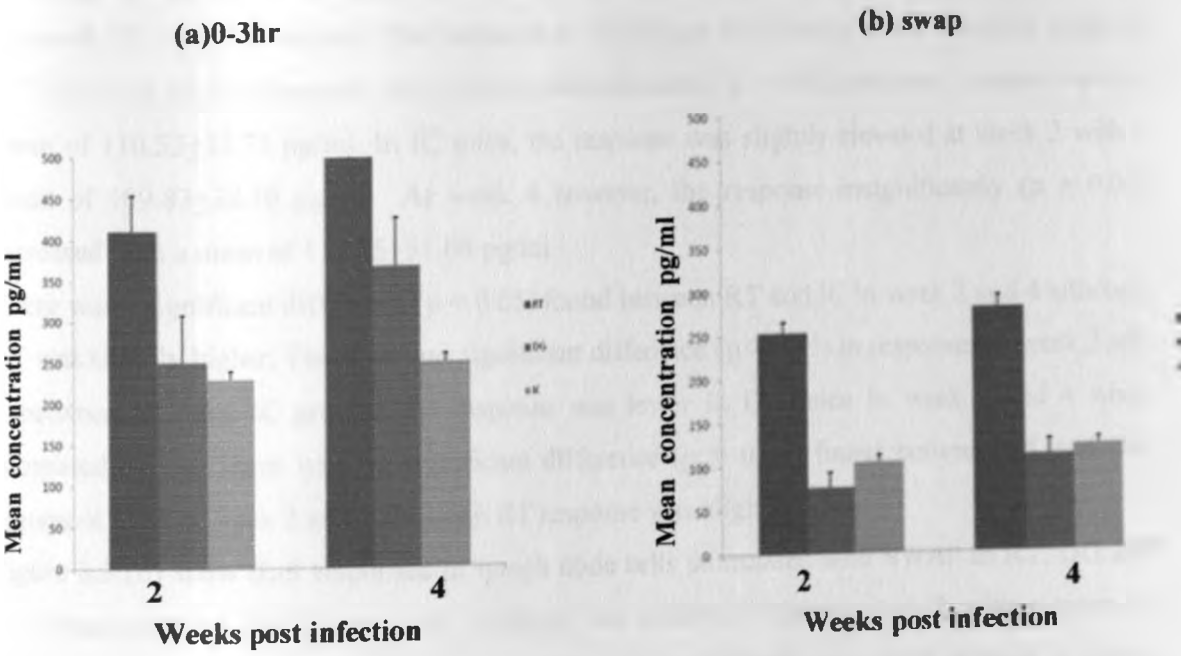


**Figure 3.2: IFN-gamma responses in mice spleen cells stimulated with 0-3 hr protein release and swap**

Key: **RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control.

### 3.4.3 IL-5 responses in lymph node cells stimulated with 0-3 hr protein release and swap

Figure 3.3 (a) shows IL-5 responses in lymph node cells stimulated with 0-3 hr in RT, DG and IC groups of mice. In RT mice, the response was relatively high at week 2 with a mean of 115.08±34.64 pg/ml. The response however declined insignificantly ($p > 0.05$) at week 4 with a mean of 112.75±31.03 pg/ml. The response in DG group was low in week 2 with a mean of 107.83±31.22 pg/ml. However, the response insignificantly ($p > 0.05$) increased at week 4 with a mean of 110.53±33.71 pg/ml. In IC mice, the response was slightly elevated at week 2 with a mean of 109.83±38.10 pg/ml. At week 4 however, the response insignificantly ($p > 0.05$) increased with a mean of 111.85±31.08 pg/ml.

There was a significant difference ($p < 0.05$) found between RT and IC in week 2 and 4 although RT was slightly higher. There was no significant difference ($p > 0.05$) in responses at week 2 and 4 between DG and IC groups; the response was lower in DG mice in week 2 and 4 when compared to IC. There was no significant difference ($p > 0.05$) found between RT and DG groups of mice at week 2 and 4 although RT response was slightly higher.

Figure 3.3 (b) show IL-5 responses in lymph node cells stimulated with SWAP in RT, DG and IC groups of mice. In RT mice, the response was relatively high at week 2 with a mean of 45.6±8.12 pg/ml. The response however declined insignificantly ($p > 0.05$) at week 4 with a mean of 43.18±5.62 pg/ml. The response in DG group was low in week 2 with a mean of 40.57±4.81 pg/ml. However, the responses insignificantly ($p > 0.05$) increased at week 4 with a mean of 44.25 ±5.67 pg/ml. In IC mice, the response was low at week 2 with a mean of 38.15±8.44 pg/ml. At week 4 however, the response was insignificantly ($p > 0.05$) elevated at week 4 with a mean of 42.95±5.84 pg/ml.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2; the response was higher in RT than in IC group. No significant difference ($p > 0.05$) was noted between RT and IC in week 4. The responses were slightly higher in RT group than IC. No significant difference ($p > 0.05$) was noted between DG and IC group at the two sampling points. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at week 2 with RT showing higher response. There was no significant difference ($p > 0.05$) noted between RT and DG in week 4. Cells stimulated with 0-3 hr protein release had higher responses than cells stimulated with SWAP.

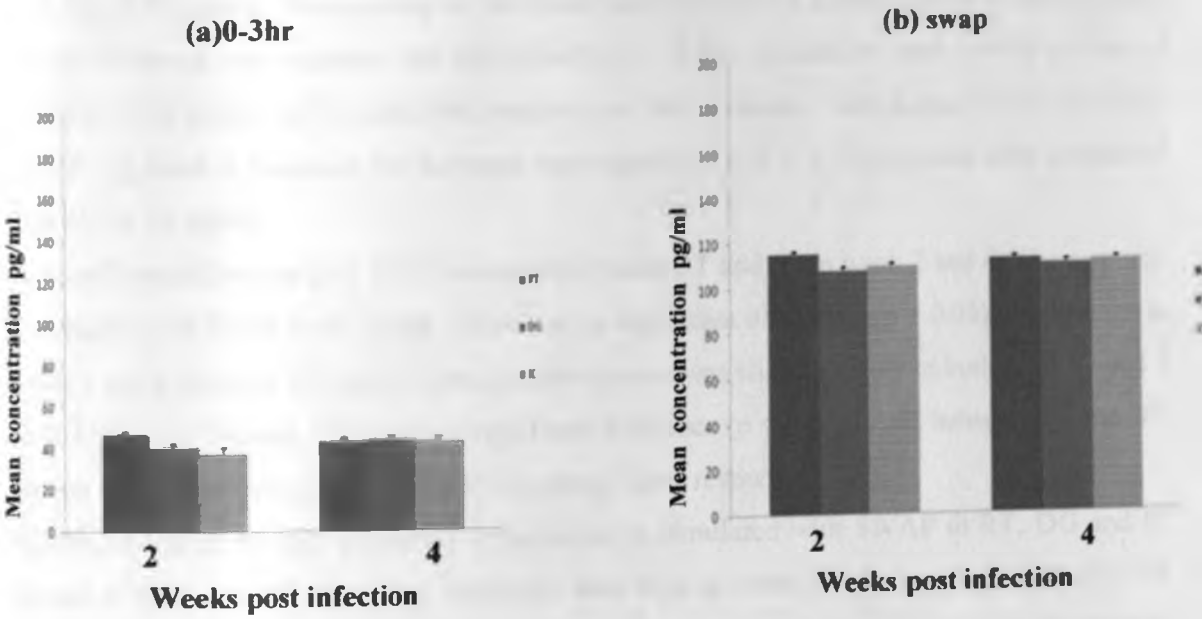


**Figure 3.3: IL-5 responses in mice lymph node cells stimulated with 0-3 hr protein release and swap**

Key: **RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control.

### 3.4.4 IL-5 responses in spleen cells stimulated with 0-3 hr protein release and swap

Figure 3.4 (a) show IL-5 responses in spleen cells stimulated with 0-3 hr protein release in RT, DG and IC groups of mice. In RT mice, the response was elevated at week 2 with a mean of 119.38±39.90 pg/ml. The response significantly ($p < 0.05$) increased at week 4 with a mean of 138.98±51.79 pg/ml. The response in DG group was low in week 2 with a mean of 80.13±16.31 pg/ml. However, the response was significantly ($p < 0.05$) elevated at week 4 with a mean of 107.83±30.19 pg/ml. In IC mice, the response was low at week 2 with a mean of 78.23±13.63 pg/ml. At week 4 however, the response was significantly ($p < 0.05$) elevated with a mean of 106.85±36.22 pg/ml.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2 and 4. The response was higher in RT than in IC group. There was no significant difference ($p > 0.05$) in responses at week 2 and 4 between DG and IC groups; the response was slightly higher at both weeks 2 and 4 in DG than in IC group. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at week 2 and 4 with RT showing higher response.

Figure 3.4 (b) show IL-5 responses in spleen cells stimulated with SWAP in RT, DG and IC groups of mice. In RT mice, the responses were high at week 2 with a mean of 44.35±5.01 pg/ml. The response insignificantly ($p > 0.05$) declined at week 4 with a mean of 43.08±6.84 pg/ml. The response in DG group was low in week 2 with a mean of 34.0 ±2.62pg/ml. The response insignificantly ($p > 0.05$) decreased further at week 4 with a mean of 32.23±1.62 pg/ml. In IC mice, the response was elevated at week 2 with a mean of 33.15±2.46 pg/ml. At week 4, the response insignificantly ($p > 0.05$) declined further with a mean of 31.9 ±2.78 pg/ml.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2 and 4; the response was higher in RT than in IC group. There was no significant difference ($p > 0.05$) in responses at week 2 and 4 between DG and IC Groups. However, the response was slightly lower in IC group than in DG mice at both weeks. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at week 2 and 4 with RT showing higher response.

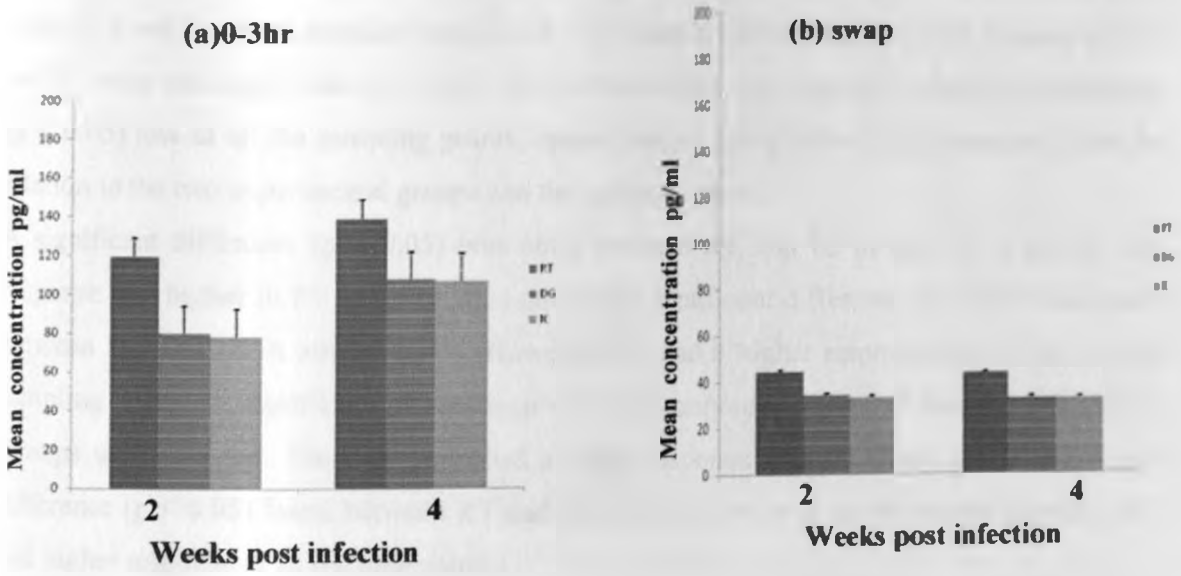


**Figure 3.4: IL-5 responses in mice spleen cells stimulated with SWAP**

**Key: RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control

## 3.5 Humoral responses

### 3.5.1 0-3 hr specific IgG responses

Figure 3.5 shows the 0-3 hr Schistosome specific IgG responses for two experimental groups DG, RT and control IC. In RT mice, the optical density in week 2 was 0.22 while in week 4 it declined to 0.16. A significant elevation was observed in week 6 with optical density being 0.239. In DG and IC mice, 0-3 hr schistosome specific IgG response gradually increased from week 2, 4 and 6 optical densities being 0.11, 0.133 and 0.154 respectively. The increase in DG and IC mice was significant ($p < 0.05$). In the Naïve mice, the response remained significantly ($p < 0.05$) low at all the sampling points, optical density being 0.094 at all sampling points in relation to the two experimental groups and the infected control.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2, 4 and 6. The response was higher in RT at all the time points. No significant difference ($p > 0.05$) was noted between DG and IC in week 2 and 4. However, DG had a higher response than IC at the two sampling points. A significant difference ($p < 0.05$) in responses at week 6 between DG and IC Groups was observed. The DG group had a higher response than IC. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at all the sampling points. RT had higher response at all the time points.

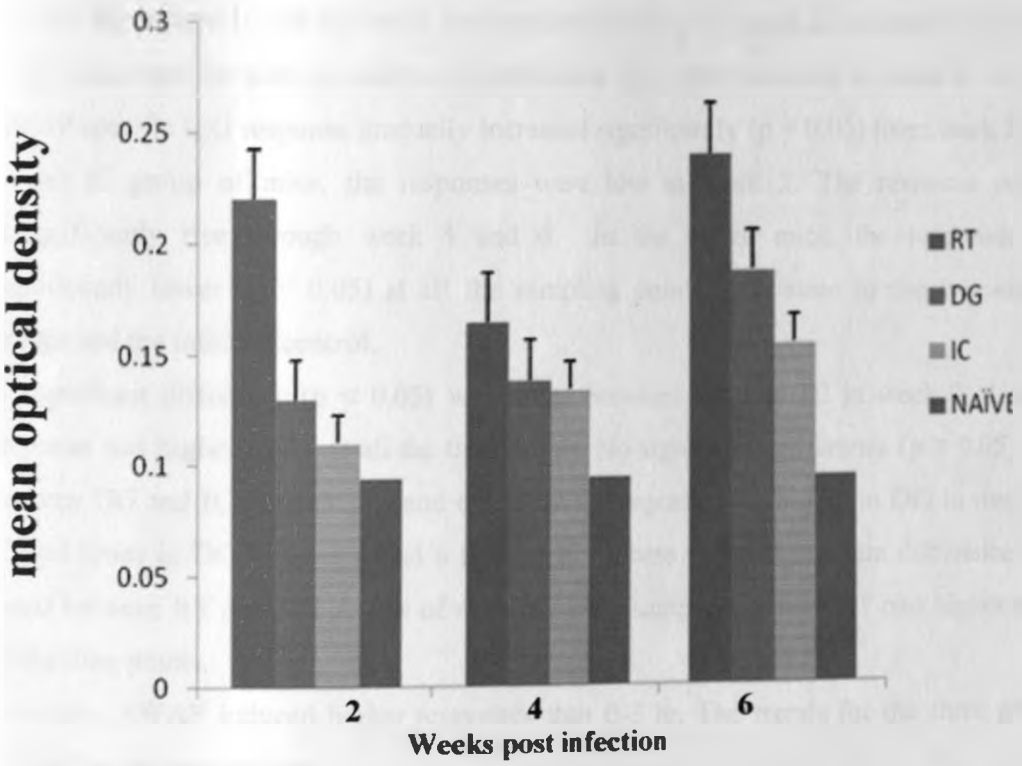


**Figure 3:5 0-3 hr Schistosome specific IgG responses**

Key: **RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control.

### 3.5.2 SWAP schistosome specific IgG responses

Fig. 3.6 shows the SWAP Schistosome specific IgG responses for two experimental groups DG, RT and the control IC. In RT mice, the response was high in week 2, and then insignificantly ($p > 0.05$) declined in week 4, and was significantly ($p < 0.05$) elevated in week 6. In DG mice, SWAP specific IgG response gradually increased significantly ($p < 0.05$) from week 2 to week 6. In the IC group of mice, the responses were low in week 2. The response continued to insignificantly rise through week 4 and 6. In the Naïve mice, the responses remained significantly lower ($p < 0.05$) at all the sampling points in relation to the two experimental groups and the infected control.

A significant difference ($p < 0.05$) was noted between RT and IC in week 2, 4 and 6. The response was higher in RT at all the time points. No significant difference ($p > 0.05$) was noted between DG and IC in week 2, 4 and 6. However, response was higher in DG in week 2 than in IC and lower in DG in week 4 and 6 than in IC. There was a significant difference ($p < 0.05$) found between RT and DG groups of mice at all the sampling points. RT had higher response at all the time points.

Generally, SWAP induced higher responses than 0-3 hr. The trends for the three groups were similar for the two antigens.

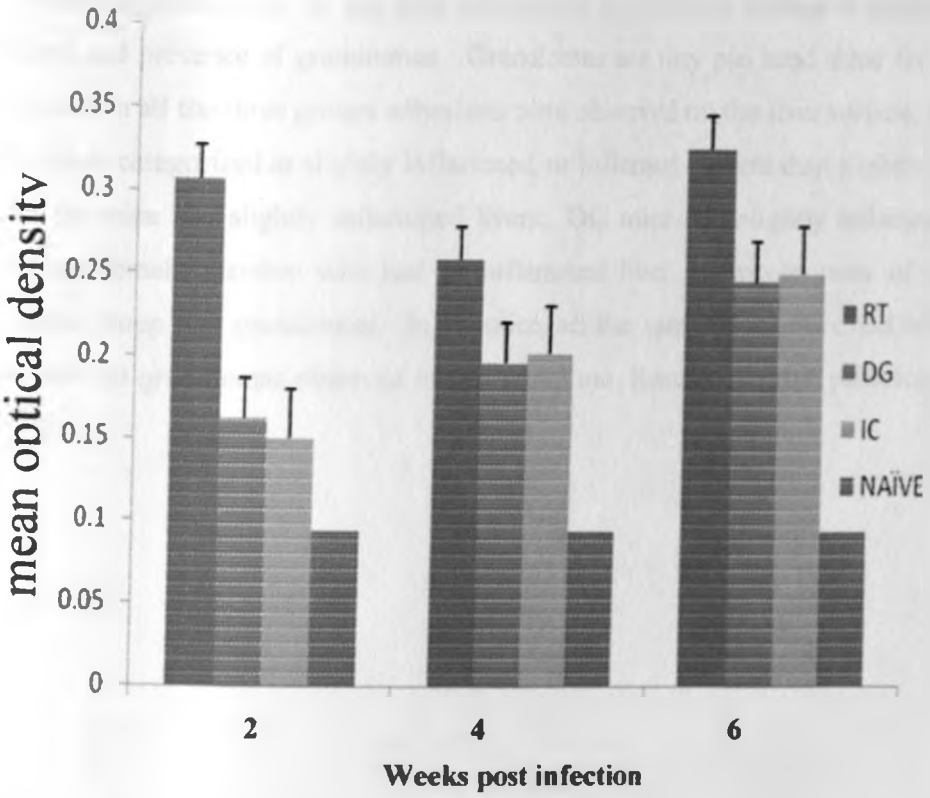


**Figure 3:6 SWAP Schistosome specific IgG responses**

**Key: RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the digestive gland of the intermediate host and then challenged; **IC**- Infected control.

### 3.6 Pathological findings

#### 3.6.1 Gross pathology

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granulomas. Granulomas are tiny pin head sized foci on the surface of the liver. In all the three groups adhesions were observed on the liver surface. Inflammation in the liver was categorized as slightly inflammed, or inflamed (severe than slightly inflammed). In RT, all the mice had slightly inflammed livers. DG mice had slightly inflammed liver tissues except one female member who had an inflammed liver. However, none of the mice in the vaccinated group had granulomas. In IC mice, all the sampled animals had inflammed livers. There were no granulomas observed in this group too. Results of gross pathology are shown on table 3.2

**Table 3.2: Gross pathology of mice immunized with soluble proteins from *Biomphalaria pfeifferi* and challenged with *Schistosoma mansoni***

| SAMPLES | RT | | | | | | DG | | | | | | IC | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | | | F | | | M | | | F | | | M | | | F | | |
| **Number** | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| **Adhesion** | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* | *p* |
| **Inflammation** | *s* | *s* | *s* | *s* | *s* | *s* | *s* | *s* | *s* | *s* | *s* | *infl* | *infl* | *infl* | *infl* | *infl* | *infl* | *infl* |
| **Granulomas** | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* | *n* |

**Key: M**-male mice;**F**-female mice; **p**-Present **S**-Slightly **n**-Non **Infl**-Inflammed, **RT**-Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged; **DG**- Mice immunized with antigens derived from the rest of the body tissues of the intermediate host and then challenged ; **IC**- Infected control.

### 3.6.2 Histopathology

Figure 3.5 shows a photomicrograph of the liver tissues of the sampled mice in the two experimental groups and the control group which did not reveal any damage to the tissues at week six post infection.

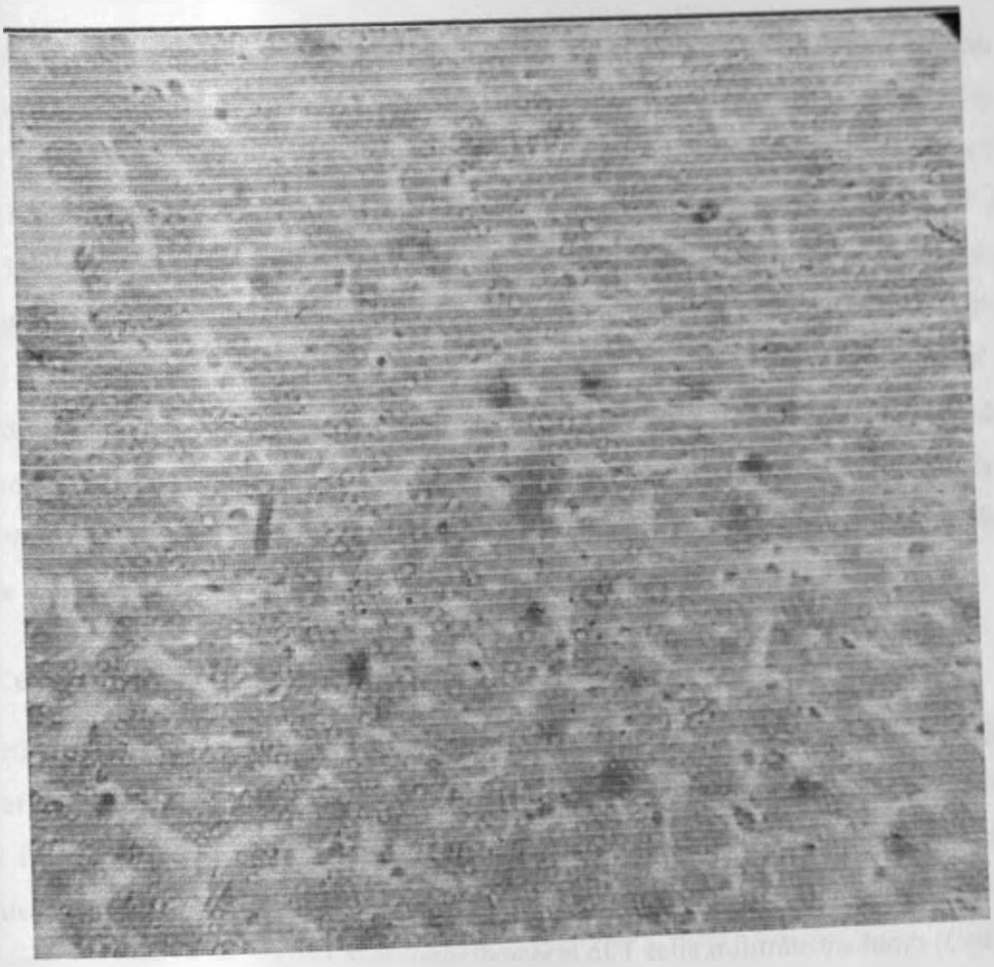

**Figure 3.7: Photomicrograph of liver tissue (H & E, 100x)**

## CHAPTER 4: DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

### 4.1 Parasitological findings

RT had significantly lower worm burden than IC (t-test; $p< 0.05$). The difference between DG and IC was also significant ($p<0.05$) with the number of worms recovered from RT being less than that for DG. The difference between RT and DG was also significant ($p<0.05$) with the worm burden lower in RT mice than DG group. The percentage worm recovery for RT group was 39.5% while for the DG group was 56.7% when perfused 6 weeks post infection. Percentage worm reduction for the RT group was 60.5% while for the DG group was 43.3%. RT mice had higher stimulation of interferon gamma and IL-5 than the DG and IC groups of mice. These indicate that RT was able to invoke both Th1 and Th2 protection in Swiss mice leading to higher worm reduction than DG group. The results also show that the two candidate vaccines reduced worms significantly; however RT was better than DG. These results indicate that RT candidate vaccine was more efficacious than the DG candidate vaccine.

### 4.2 Cellular immunological responses

The kinetics of interferon gamma production in vaccinated mice coincides with the migratory pattern of the immunizing parasites. Interferon gamma is initially produced by lymphocytes obtained from the axillary and inguinal lymph nodes five days after vaccination, peaking two weeks later (Pemberton *et al,* 1991). Th1 cell responses appear to participate in the development of inflammatory foci. After vaccination, large numbers of T cells infiltrate the lungs (Coulson & Wilson, 1993). When attenuated challenge schistosomules arrive in the lungs of vaccinated mice 4 days after skin penetration, they become surrounded by focal inflammatory infiltration, rich in mononuclear cells (Crabtree &Wilson, 1986; Wynn *et al,* 1994). Moreover, alveolar macrophages are activated after challenge, suggesting that they interact with T lymphocytes (Melson *et al,* 1998). Taken together, these factors imply that challenge parasites stimulate delayed type hypersensitivity (DTH) responses in the pulmonary tissues (Crabtree *et al,* 1986). Schistosome reactive DTH-mediating lymphocytes are present in circulation between days 10 and 17 post vaccination (Ratcliffe & Wilson, 1991) and will be among the mononuclear cells that will infiltrate the pulmonary tissues.

### 4.2.1 IFN gamma levels in SP and LN cells stimulated with 0-3 hr and SWAP

RT group of mice had significantly higher ($p< 0.05$) IFN gamma responses than both DG and IC at all time points for both antigens.

In most of the times, DG had higher responses than IC, for both cells and both antigens, but the difference was not always significant.

Therefore RT vaccination had the stronger interferon gamma production, implying better Th1 protection produced by RT, compared to DG (Pearce & sher., 1991).

Lymph node cells had a peak production of IFN gamma at week two post challenge, which is in agreement with Pemberton *et al.*, (1991).

Spleen cells had peak responses at week four. This is expected since antigens take longer time to reach the spleen compared with the draining lymph nodes, which encounter parasite antigens presented by dendritic cells soon after the infection.

0-3 hr antigens gave better responses than SWAP antigens. This is expected since sampling was done at early part of migration of parasites, before most worms had matured to adult worms. 0-3 hr antigens had more shared antigens with these early stages of development.

### 4.2.2 IL-5 levels in SP and LN cells stimulated with 0-3 hr and SWAP

It is universally accepted that in humans, primates and rats, protective immunity to schistosome is associated with Th2 responses {interleukin-4 (IL-4), IL-5 production, IgE, IgG and eusinophils}, in contrast with murine schistosomiasis, where immunity is associated with Th1 responses (IL-1, interferon gamma and IL-12) (Pearce & Sher., 1991). It is of particular interest to note that IL-5 production appears to be associated with lymphocyte proliferation to specific antigens and resistance to re-infection of a human population (Roberts *et al.*, 1993)

In RT group of mice there was significantly higher IL-5(0.05) responses compared to DG and IC expect for just one time point were the difference was not significant (LN cells, SWAP week 4).

These results show that, in addition to IFN gamma, RT was able to stimulate production of IL-5, and hence invoke Th2 protection in Swiss mice. This phenomenon is reported in man, primates and rats, but not in mice (Pearce & Sher., 1991).

Again, like in the case of IFN gamma, cells stimulated with 0-3 hr had higher responses than those stimulated with SWAP implying more shared antigens with 0-3 hr antigens and early stages of development of *S. mansoni* before maturation.

### 4.3 ELISA for Schistosome specific IgG antibodies

IgG antibodies is involved in antibody-dependant cell-mediated cytotoxicity (ADCC) with neutrophils, macrophages and eosinophils which are damaging to the schistosomulae stage (Hagen *et al.*, 1991). Different antibody isotopes may have different effects in *S. mansoni* infection. IgG2 and IgG4 block or compete with IgGE which is associated with resistance to infection. IgG, IgE and IgA are important in protection, acting at several stages to prevent invasion, migration, development and egg production (Hagan *et al.*, 1991).

RT had very strong IgG responses at week 2, which declined at week 4, before rising again at week 6. The strong response at week 2 correlates very well with higher IL-5 responses at the same time point. IL-5 is involved in the Th2 responses, which leads to production of antibodies.

RT had significantly higher IgG responses than both DG and IC at all sampling points.

The IgG responses for DG and IC showed an increasing trend from week 2 to 6. There was no significant difference between IgG responses between DG and IC although DG responses for 0-3hr were consistently slightly higher than those of IC.

The three groups –RT, DG and IC had significantly higher IgG responses than naïve control. This shows that all the groups produced IgG which is involved in ADCC (Hagan *et al,* 1991) However, the strongest response was in RT, and this correlates very well with the greatest worm reduction recorded in this group.

### 4.4 Pathological findings

#### 4.4.1 Gross pathology

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granulomas. Granulomas are tiny pin head sized foci on the surface of the liver. In all the three groups adhesions were observed on the liver surface an indication of an encounter with infection. None of the mice had granuloma. This could be attributed to a slower development in the Swiss mice Model compared to, for example, BALB/C mouse where granulomas were present at week 6 (Kanyugo *et al.*, 2009).

Inflammation in the liver was categorized as slightly or inflamed. All the mice in RT group had slightly inflamed liver tissues. DG had 5 mice with slightly inflamed liver tissues and one mouse

with inflamed liver. All the mice in IC had inflammed liver tissues. This shows that RT had the least pathology, while IC had the worst, with DG lying in between.

### 4.4.2 Histopathology

Histopathology was similar in all the 3 groups. This could be as a result of delay of eggs arriving in the liver, and hence delay in granuloma formation around the eggs.

## CONCLUSION

The results from this study showed that mice immunized with antigens prepared from an intermediate host are better protected than non-vaccinated mice. The mean worm burden was higher in IC group than both RT and DG group of mice. The results also reveal that RT vaccine offered a better protection than DG vaccine. Worm reduction in RT was 60.5% while that of DG was 43.3%. In cellular responses, RT significantly stimulated higher production of interferon gamma as compared to IC at all time points. This also shows that RT induced better protection related to Th1, compared to DG. RT IL-5 responses were significantly higher than the DG and IC for both 0-3 hr and SWAP antigens for both spleen and lymph node; IL-5 is responsible for Th2 protective responses .This means RT had induced better Th2 protective responses. IgG is involved in antibody-dependant cell-mediated cytotoxicity. RT produced higher IgG responses than DG and IC, meaning it killed more worms using antibody-dependant cell-mediated cytotoxicity. This is in agreement with higher worm reduction, higher cellular and humoral protective responses and least pathology. In conclusion therefore, although the two candidate vaccines were protective, RT was a better candidate vaccine in terms of higher worm reduction, higher cellular and humoral protective responses and least pathology.

## RECOMMENDATIONS

1. There is a need to identify which of the tissues of the rest of body (RT) is inducing protection.
2. After identifying the protective tissue, the proteins involved in protection should be characterized.
3. After characterization and purification of the proteins, tests should be carried out in permissive hosts such as primates.

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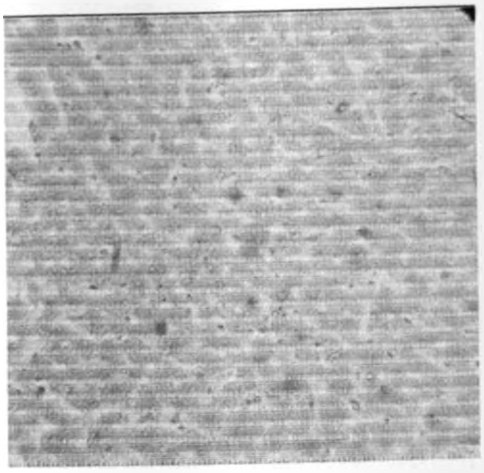
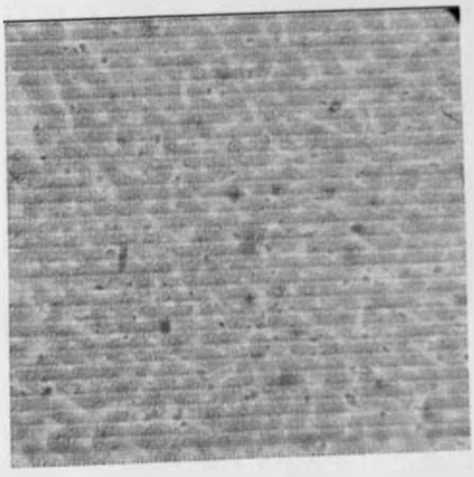
## APPENDIX 1



**Photomicrograph of RT group 6wks PI**



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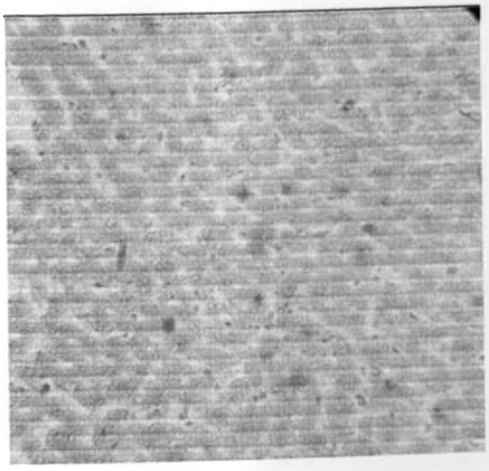

**Photomicrograph of IC group 6wks PI**