PARASITOLOGICAL AND IMMUNOPATHOLOGICAL RESPONSES IN MICE WITH CONCOMITANT *SCHISTOSOMA MANSONI* AND *PLASMODIUM BERGHEI* INFECTIONS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my son Henry.

Henry, may you grow up to carry on this scientific legacy.

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My gratitude goes beyond words to Dr Dorcas Yole, in whom I saw not just a supervisor but much more. I can not fail to mention how much she taught me, and the research discipline she instilled in me. I will live to remember her patient yet firm resolve to see science done as it should be.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADCC	Antibody dependent cell cytotoxicity
BSA	Bovine serum albumin
FCS	Fetal calf serum
g.	Acceleration due to gravity for centrifugation
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-4,-5, -6, -10	Interleukin-4,-5, -6, -10
IPR	Institute of Primate Research
NEG	Negative control
PbC	*Plasmodium berghei* control
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SEA	Soluble egg antigen
SEM	Standard error of means
SmC	*Schistosoma mansoni* control
SmPb	*Schistosoma* mansoni/ Plasmodium *berghei* co-infection.
SWAP	Schistosome worm antigenic preparation
TGF-α	Transforming growth factor- alpha
Th1	Helper T-lymphocytes subset 1
Th2	Helper T-lymphocytes subset 2
TNF-β	Tumour necrosis factor-beta
WHO	World Health Orgarnisation

ABSTRACT

Usually, areas endemic for schistosomiasis have malarial infections and vice versa. In Kenya, areas like Mwea, Lake Victoria region, along the coast and Ukambani, are known for this. On the other hand, not much information is available on the consequences of having the two infections together. At the same time, study of the co-infections in humans may not allow one to know when the infections started. This study is therefore aimed at investigating parasitological, pathological and immunological responses that occur in mice as models for humans, during a schistosomiasis-malaria co-infection.

A group of mice was infected with *S. mansoni* and then divided into three separate groups. At weeks 4, 5, and 6 post-infection, each separate group was super-infected with *P. berghei*. There were also uninfected and single-infected controls of both *S. mansoni* and *P. berghei*. At various times post-infection with *P. berghei,* blood samples were taken for assays of parasitaemia and immunoglobulin G (IgG). Liver tissues were also obtained for histopathology, and perfusion done for establishing the worm burden. The procedures were carried out in the experimental mice and their controls.

It was found out that mice infected by both parasites developed higher malaria parasitemia, and showed higher IgG responses compared to the single-infected mice. It was also noted that the worm load in the *S. mansoni*-only-infected group was significantly higher than in the co-infected mice. The granuloma sizes were also larger in the *S. mansoni*-only-infected groups as compared to the co-infected groups. These results were consistent regardless of whether the super-infection was done at week 4, 5, or 6 post- *S. mansoni* infections, though the difference in worm load at week 4 was not significant. Taken together, the data from this study show that schistosome and malaria infections profoundly affect each other. These findings may have implications in the treatment and control of both infections. The results suggest that co-infections with schistosome and malaria parasites would aggravate malaria severity. This would neeessitate the need for considering schistosome infection in clinical as well as therapeutic management of malaria patients in areas where the two diseases are co-endemic. On the other hand, the results indicate that malaria confers an advantage to the *S. mansoni* infection, since the granuloma sizes in co-infected mice were significantly smaller than in the *S. mansoni*- only infected mice. The worm

burden was also significantly higher in the *S. mansoni*- only infected mice than in the co-infected mice. These findings call for a concerted approach in the control of malaria and schistosomiasis.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Although polyparasitism is a common phenomenon in human populations living in malaria endemic areas, little is known about how co-infections affect the immune responses against malaria and *vice versa*. If made available, such knowledge would be important in the design and optimization of vaccines and chemotherapy (Helena *et al.*, 1998).

Some situations are known in which the association of infection by *S. mansoni* with other pathogens in the same host results in a type of disease, which differs, from the simple summation of the individual effects of each infection (Helena *et al.*, 1998). If the combined responses were determined, this would probably serve as a ground for proper vaccination and treatment.

Concurrent infection with helminth parasites, which are highly prevalent in many areas where malaria is endemic, has recently been recognized as a possible confounding factor modulating immune responses to other pathogens, including malaria parasites (Nacher, 2001).

Malaria is highly endemic in Sub-Saharan Africa, Southeast Asia, and South America, where there is also a high prevalence of helminth parasite infections. For instance, infections with the major human gastro-intestinal nematodes, including *Ascaris lumbricoides, Trichuris trichura,* and the hookworm species *Ancylostoma duodenale* and *Necator americanus*, are widespread in most of these areas. The combined prevalence of infections with gastro-intestinal nematodes can be as high as 90% in some parts of the African continent (Chan *et al.*, 1994; Chan, 1997; De Silva

et al., 2003).

Modulation of immune responses to viral, bacterial, and protozoan pathogens by concurrent helminth infection has been observed in many human epidemiological studies and in laboratory animal models. Patients with helminth infections have been observed to have higher viral load of Human Immunodeficiency Virus (HIV) in plasma, and reduced delayed-type hypersensitivity responses to *Mycobacterium tuberculosis* purified protein derivative or house dust mite antigen (Borkow *et al.*, 2001; Van den *et al.*, 2004).

Human subjects infected with the filarial parasite *Onchocerca volvulus*, have been observed to produce significantly lower levels of anti-tetanus antibody following tetanus vaccination (Cooper *et al.*, 1999).

In laboratory animal studies, mice co-infected with *S. mansoni* and *Leishmania major* showed impaired ability to resolve *L. major* infection (LA Flamme *et al.*, 2002; Yole *et al.*, 2007). Similar impairment of protective immunity by concurrent helminth infection has also been observed in other co-infection models, such as the nematode *Nippostrongylus bransiliensis* and the bacterium *Chlamydophila arbortus* (Buendia *et al.*, 2002), the cestode *Taenia crassiceps* and the protozoan *Trypanosoma cruzi* (Rodriguez *et al.*, 1999), and *S. mansoni* and the recombinant Vaccinia virus.

It has also been reported that mixed *Plasmodium falciparum* and *Plasmodium vivax* infections are more frequent in *A. lumbricoides*-infected patients in Thailand (Nacher, 2001). Epidemiological studies also showed that worm infection in humans alters the development of cerebral malaria (Nacher *et al.*, 2000). Mice co-infected with *S. mansoni* and *Plasmodium chabaudi* develop increased malaria parasitaemia (Helena *et al.*, 1998).

CD4+ T cells play a major role in protective immunity against the blood stage of

malaria. Both TH1 and TH2 subsets of CD4+ T cells are protective. TH1 cells protect by a nitric oxide-dependent mechanism, whereas TH2 cells protect by the enhancement and accelerated production of specific IgG1 antibody. A switch follows the initial CD4+ TH1 cell response to cytokine production, stimulating antibody-dependent mechanisms. The antibody-dependent mechanisms are involved in the final control and clearance of parasitaemia (Taylor *et al.*, 1993).

In experimental *S. mansoni* infections, a strong TH2 type response is induced. This is evidenced by the presence of high Immunoglobulin E (IgE) and oesinophil levels. At the time of egg production, production of TH2 cytokines [Interleukin-4 (IL-4), IL5, and IL-10] is seen, with a down regulation in the secretion of TH1 cytokines (IL-2 and IFN-γ); (Grzych *et al.*, 1991).

This study was performed to examine the parasitological, pathological, and immune responses that occur in mice, during a schistosomiasis-malaria co-infection.

1.2 Literature review

1.2.1 Schistosomiasis

Schistosomiasis is responsible for extensive morbidity and mortality. It is estimated that worldwide more than 200 million persons are infected with schistosomes, with 85% of the cases occurring in Africa (Thomas *et al.*, 2003). This is being increasingly recognized as a significant public health problem, particularly in developing countries (WHO, 2004).

Schistosomiasis is caused by parasitic blood flukes *Schistosoma haematobium, S. mansoni, S. intercalåtum, S. mekongi* or S. *japonicum* (Charles *et al.*, 2005). This study focused on *S. mansoni.*

1.2.2 *Schistosoma mansoni*

S. mansoni is found in Africa, Middle East, South and Central America, distributed among 50 countries (WHO, 1994). In Kenya, it is found in particular foci along the shores of Lake Victoria, Lake Jipe, Machakos, Kitui, Kirinyaga, Kiambu and Murang'a.

1.2.3. Life cycle and Hosts

The life cycle of *S. mansoni* involves two hosts: an intermediate host which is a fresh water snail of the genus B*iomphalaria*, and definitive hosts which include man, rodents and primates (Macsween, 1980).

Once laid, eggs of *S. mansoni* commonly break out of the venules in the submucosal and mucosal layers of the intestines and escape into the lumen, from where they are passed out in faeces. The egg has a yellowish-brown transparent shell and a characteristic lateral spine. It is usually fully embryonated by the time it is discharged in the faeces, and hatches soon after contact with water to release a miracidium.

The released miracidium swims freely in water for about 5-6 hours, within which it infects a *Biomphalaria* snail by use of anterior penetration glands, secreting proteolytic enzymes. The attraction of the miracidium to the snail host is due to chemicals like macromolecular glycoproteins. These chemical attractants enable the miracidium to distinguish between snails of different strains during their chemotaxis towards the host (Haas *et al.*, 1995).

During penetration, the ciliated outer covering is shed and the miracidium elongate and become a tubular sporocyst. This makes its way through the viscera to the digestive gland at the innermost extremity of the snail and transforms into a

mother sporocyst (Chandler, 1961). After about two weeks, daughter sporocysts are produced by the mother sporocyst, which in turn produce fork-tailed cercariae.

The cercaria is the stage infective to the definitive host. One miracidium is capable of producing 200,000 cercariae. The cercariae leave the snail host and become temporarily free living (Wakelin, 1996), showing characteristic behavior patterns like hanging from the surface film of the water body, detaching, sinking, then moving upwards again. This increases their chances of encountering a suitable host.

Infection of the final host by the cercariae is by direct penetration of the skin, using the histolytic and hyaluronidase-bearing secretions of their penetration glands (Macsween, 1980). Skin penetration requires only a few minutes and may be accompanied by a prickling sensation and subsequent dermatitis. The cercarial head penetrates the skin while the tail is left behind. The head undergoes physiological changes to become schistosomula, finds its way into the blood stream, and is carried by the blood to the lungs via the heart. The schistosomula of *S. mansoni* accumulate in the lungs on the 2^{nd} and 3^{rd} days, and by the 6^{th} day, they appear in the liver where they establish by the 15^{th} day. The schistosomules feed on the portal blood and once in the liver they grow rapidly, mature and the adult worms pair up (male and female) before migrating into the veins of the mesenteries (Fausts *et al*., 1970).

The adult worms can survive for as long as five to six years (Wakelin, 1996), during which time they release a very large number of eggs, about 300 per day. The eggs then break out of the venules and into the lumen of the intestines from where they are passed out in faeces.

1.2.4 Pathogenesis, Pathology, and Symptomatology

Infection by *S. mansoni* causes the production of lesions on the intestinal walls and delayed production of granulomas. The initial egg laying is accompanied by Schistosomiasis dysentery, with blood and mucus in the faeces. Frequent stools accompanied by abdominal pain are common. After tissue reaction around eggs infiltrated into the intestinal wall, dysentery becomes less conspicuous. The intestinal wall becomes inflamed, thickened and fibrosed, with abscesses opening through the mucosa (Fausts *et al.*, 1970).

As the condition progresses, there is development of papillomata and prolapse of the rectum (Fausts *et al.*, 1970). The mesenteric lymph nodes become enlarged due to cellular reaction against infiltrated eggs. The liver and spleen become enlarged and tender. Eggs at times escape via the blood system into the tissues of the lungs, pancreas, spleen, kidneys, adrenals, myocardium or even the spinal cord, and initiate pathological processes in these organs with corresponding symptoms.

During the period of tissue proliferation and repair, fibrosis and thickening of the intestinal wall continues, with the development of papillomata along the entire bowel. This is characteristic in the hyper endemic areas in Africa. The liver gradually becomes cirrhosed and the spleen tremendously enlarged. Due to periportal fibrosis, ascites occurs. The ultimate severity of the infection is dependent on the worm burden since the number of worms dictates egg production.

Pathology in schistosome infections is primarily due to the eggs, both in the intestines and those escaping back to other organs such as lungs, and especially the liver. More than 50% of the eggs laid remain trapped in the body. Infection by only a single sex of schistosome causes little pathology since no eggs are produced (Sadun *et al.*, 1970).

1.2.5 Immunopathology

The most intensively studied immunopathological reactions are those associated with chronic *S. mansoni* infections, which are responsible for the gross changes seen in the liver (Wakelin, 1996). The pathology associated with this infection is caused by the immunological and inflammatory responses of the host, rather than by the direct activities of the parasite.

The worms evoke both an antibody response and cell-mediated immunity in the host. In experimental animal infections there is evidence that the adult worms may be coated by a layer of mucopolysaccharide which may be of host origin, and which acts as an immunological barrier and prevents hypersensitivity reactions. However, when ova are laid in the tissues, they lack this coating and stimulate a delayed hypersensitivity reaction characterized by accumulation of epithelioid macrophages, lymphocytes, eosinophils, and eventually dense fibrosis (Macsween, 1980).

Some of the immunopathological symptoms associated with infection include dermatitis following cercarial penetration, acute allergic phase caused by parasite migration through the lungs, and immune complex disease. Potent immunogens are released by miracidia through the pores in the eggshell and these elicit a state of delayed hypersensitivity. Each egg then becomes the focus for the production of a granuloma.

Certain components of the soluble egg antigens (SEA) become recognized by T-helper cells and also by the cytokines released by these T cells. This recognition results in the focal accumulation of lymphocytes, macrophages, eosinophils and other inflammatory cells around each egg. This accumulation results in production of inflammatory focus known as granuloma that may reach about 400μm in diameter. The release of tumor necrosis factor (TNF) from the macrophages is what initiates

granuloma formation. SEA promotes the selective development of TH-2 cells which release IL-5. This cytokine is responsible for the accumulation of eosinophils that take part in granuloma formation. The cells around the granuloma also release factors that stimulate production of fibroblasts and collagen, causing the development of fibrosis in the liver (Macsween, 1980).

1.3 Malaria

Malaria is one of the commonest and most important of the parasitic diseases. It is estimated that about 1 billion people are at risk of infection and about 200 million are actually infected. Africa experiences 1 million deaths due to malaria annually, majority being children (Wakelin, 1996).

The major foci of distribution occur in Africa, India, Asia, Central and South America. This follows the distribution of anopheline mosquitoes, which are the vectors for malaria. A parasitic protozoan of the genus *Plasmodium* causes malaria. There are four species of *Plasmodium* that are known to cause malaria in man: *P. ovale,* which is rare though present in West Africa, South America, Russia, Palestine and New Guinea (Chandler, 1961). The other three: *P. malariae, P. vivax and P. falciparum,* are common, and widely distributed.

Although intensive vector control programmes have been undertaken in some countries with great success, no significant global reduction in malaria infection has been achieved. In fact, in some countries, serious resurgences have occurred due to breakdown of control measures and the emergence of insecticide and drug resistance (Wakelin, 1996).

Apart from the human host, malaria also affects other mammals, birds, reptiles, monkeys, and rodents. *P. rhodaini, P. cynomolgi* and *P. knowlesi* are species that

affect non-human primates. *P. berghei* and *P. vinckei* infect various rodents like rats and mice. This study however focuses on *P. berghei* only.

1.3.1 *Plasmodium berghei*

P. berghei is a species of malaria parasite, which is geographically localized in a small region of Central Africa, where its vector, *Anopheles dureni* is found (Garnham, 1966). The natural hosts for *P. berghei* are various wild rodents like rats and mice.

The parasites are always in low density in natural infections, but are quite lethal especially to white mice, with death occurring between the seventh and twenty-fourth days depending on the strain and number of parasites present.

P. berghei is easily maintained and transmitted by *Anopheles stephensi* in the laboratory. It serves as a good model for the human parasites with which it shares high homology in most essential aspects of structure, biochemistry and lifecycle (Carter and Diggs, 1977).

1.3.2 Life cycle

1.3.2.1 Sporozoites and pre-erythrocytic development

An infection starts with the bite of an infected mosquito, which inoculates the haploid sporozoites in the blood stream of the vertebrate host. The sporozoites move to the liver and invade hepatocytes, and can be observed inside these cells a few minutes to several hours after inoculation. Hepatocyte invasion is mediated by invagination of the host cell plasma membrane to form a parasitophorus vacuole, which surrounds the invading sporozoites (Mota *et al*., 2001).

Within the hepatocyte, the sporozoite develops within 47-52 hours through the trophozoite stage into the mature schizont containing merozoites. A mature schizont may contain 1500-8000 merozoites but the number varies in different hosts.

The liver cells then rupture and the merozoites of the pre-erythrocytic schizont are released into the blood stream where they invade erythrocytes. In *P. berghei,* there is no evidence of hypnozoite stage in the liver (Cogswell, 1992).

1.3.2.2 Erythrocytic development

The haploid merozoites released from the liver schizont invade red blood cells, especially the reticulocytes. Within the erythrocytes, the merozoites develop into a trophozoite characterized by an increase in cell size and cytoplasm. This takes about 16 hours (Mota *et al*., 2001).

The trophozoite then enters into a state of schizogony during which the parasite replicates its DNA and divides its nuclei a number of times. The total duration of the asexual blood stage development is 22-24 hours. Immature and mature schizonts disappear from the peripheral circulation and sequester in the capillaries of inner organs such as the spleen and lungs. After rupture of the schizonts, the free merozoites invade new red blood cells, resulting in an increase in the parasitaemia.

The blood stage development of *P. berghei* in laboratory rodents is usually asynchronous. The different developmental stages such as rings, trophozoites, and schizonts are simultaneously present in the blood during the course of infection (Cogswell, 1992).

1.3.2.3 Sexual Development

In each asexual cycle, a small proportion of parasites stops asexual multiplication and differentiate into sexual cells called gametocytes. These haploid macrogametocytes and microgametocytes are the precursors of the female and male gametes.

In *P. berghei,* the merozoites of the liver schizonts are able to differentiate directly into gametocytes after invasion of the erythrocyte (Suhrbier *et al.*, 1987). The period of development of a merozoite into a mature gametocyte takes only 26-30 hours (Mons, 1986). There is no evidence that developing gametocytes of *P. berghei* sequester in capillaries of inner organs during development, as is the case with gametocytes of *P. falciparum.* It has been proposed that certain stages of gametocytes sequester preferentially in blood capillaries from which mosquitoes take their blood meal (Landau and Chabaud, 1994).

1.3.2.4 Fertilization and Zygote Development in the Mosquito

When a mosquito feeds on an infected host, only the mature gametocytes can undergo further development in the mosquito midgut. Gametocytes actively escape from erythrocytes and undergo gametogenesis. The female gametocyte differentiates into a single, spherical gamete (macrogamete), whereas the male gametocyte produces eight 'sperm-like' gametes.

Three environmental triggers have been described that induce the differentiation of the gametocytes into gametes:

i. A drop in temperature of the infected blood to at least 5°C below that of vertebrates.

ii. A rise in pH from 7.3 to 7.8-8.0.

iii. The presence of Gametocyte Activating Factors (GAF); (Bilker *et al.*, 1997).

In *P. berghei,* the mosquito-derived GAF is Xanthurenic acid.

Fertilization takes place by penetration of the haploid male gamete into the haploid female gamete, resulting in a diploid zygote. The spherical zygote develops into a banana-shaped, motile ookinete within a period of 18-24 hours.

1.3.2.5 Oocyst and Sporozoite Development

Mature, motile ookinetes traverse the midgut epithelium by invasion of the cells of the epithelium and settles between the basement cell membrane and the basal lamina of the midgut wall. Most probably the *P.berghei* ookinete does not invade a specific midgut cell type and ookinetes traverse the cytoplasm of several midgut cells before entering and migrating through the basal lateral intercellular space to access the basal lamina. The invaded cells undergo apoptosis (self suicide) (Vlachou *et al.*, 2004).

After emerging from the epithelial cell, the ookinete attaches to the basal lamina, from where the parasite rapidly rounds up and develops into an oocyst. After asexual mitotic replication, a mature oocyst containing thousands of sporozoites is formed. The oocyst ruptures and the haploid sporozoites are released into the haemocoel. The sporozoites invade the salivary glands. The number of oocysts produced and the number of sporozoites found in the salivary glands is dependent on the *Anopheles* species that is used to transmit *P. berghei.* In *A. stephensi* infected with ANKA strain of *P. berghei,* an average 8000 sporozoites per oocyst have been recorded (Sinden, 1997).

The first sporozoites reach the salivary gland at around 13 to 14 days after the infectious blood meal. They migrate through cells of the gland and get into the

extracellular secretory space where the sporozoites can persist for many weeks (female mosquitoes can live up to 14 weeks), before being injected into a new host.

1.3.3 *P. berghei* in rodents

P. berghei ANKA infection in mice leads to the development of cerebral malaria that kills 80-90% of the animals in 6-9 days (Carvalho *et a*l., 2000). In some mouse strains, the parasite causes a neurovascular syndrome, experimental cerebral malaria, involving immunopathological reactions (Veronique *et al.*, 1998).

1.3.4 Immunopathology

The immune response is vital for pathogenesis of *P. berghei* malaria. Elevated levels of inflammatory cytokines are detected in sera of *P. berghei*-infected mice, and endothelial cell adhesion molecules are also upregulated (de Kossodo and Grau, 1993). Petechial hemorrhaging is observed in brains of *P. berghei*-infected mice, and there is a breakdown of the blood-brain barrier (Neill and Hunt, 1992). Type 1 cytokines (IL-2, IFN-γ, and TNF-α) are required for pathogenesis of experimental cerebral malaria (Yanez *et al.*, 1996).

1.4 Justification and significance of the study

Areas endemic for schistosomiasis have malarial infections and those endemic for malaria also have schistosome infections. For example in Kenya, areas like Mwea, Lake Victoria region, along the Coast and Ukambani, are known for this. Not much information is available on the consequences of having the two infections together. Such knowledge is of importance for the rational design and optimization of vaccination protocols and treatment programmes (Helena *et al.*, 1998). At the same

time, study of co-infection in humans may not allow for much manipulation. For example, one may not know when the infections started.

1.5 Objectives of the study

1.5.1 General objective

To generate information that may be useful in the control and/or treatment of malaria and schistosomiasis, through vaccination and/or chemotherapy.

1.5.2 Specific objectives

1. To determine the degree of *P. berghei* parasitemia in co-infected and singly infected mice at different stages of infection.
2. To determine the worm burden of *S. mansoni* in both co-infected and singly infected mice.
3. To evaluate the immune responses in *P. berghei and S. mansoni* co-infected mice in terms of immunoglobulin G responses.
4. To analyze the gross pathology and histopathology of the co-infected and singly infected mice.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Design

The study was carried out using BALB/c mice as models for human malaria and schistosomiasis. The study entailed an infection and analysis experiments on the immune, parasitological, and pathological responses.

At the beginning of the study, eighty mice were infected with *S. mansoni* cercariae on day 0 (zero) of the experiment. Each mouse received an aliquot containing 150 cercariae

Experiment 1 (Table 1): Thirty-five randomly selected mice out of the eighty infected with *S. mansoni*, served as *S. mansoni* single-infection control (SmC), ten for each sampling week. Another fifteen mice with *S. mansoni* infection were picked at random and super-infected with *P. berghei* at week 4 post- *S. mansoni* infection (SmPb4). Each mouse was injected with 5.5µl of blood containing 2.5 x 10^6 *P. berghei* parasitized red blood cells. At the same time, fifteen naive mice were infected with a similar number of *P. berghei* parasitized red blood cells, from the same pool as for the co-infection. These served as the *P. berghei* single-infection control (PbC4).

Experiment 2 (Table 2) : The set up was similar to Experiment 1 except that the co-infected group (SmPb5), and the malaria single-infection control group (PbC5) were infected at week 5 post- *S. mansoni* infection. The number of *P. berghei* parasitized red blood cells was the same as for Experiment 1.

Experiment 3 (Table 3): This was set up as in Experiments 1 and 2 except that the co-infected group (SmPb6), and the malaria single-infection control group (PbC6) were infected at week 6 post- *S. mansoni* infection. The number of *P. berghei* parasitized red blood cells was the same as for Experiments 1 and 2.

For each of the three experiments, sampling was done at Day 4 and Day 7 post-*P. berghei* infection. At Day 4, sampling entailed preparation of blood smears, heart puncture, gross pathology, and histopathology. At Day 7, perfusion was carried out in addition to all the other procedures as for Day 4. Five mice from each experimental group were sacrificed at each sampling point and the relevant analysis done (perfusion, blood smears, gross pathology, harvesting livers for histopathology, and heart puncture).

The experimental design is shown in Tables 1, 2, and 3.

EXPERIMENTAL DESIGN TABLES

Table 1: EXPERIMENT 1

EXPERIMENTAL GROUP	DAY 0	DAY 0	DAY 4	DAY 7
Sm C	I Sm		s	S+p
SmPb4	I Sm	I Pb	s	S+p
PbC4		I Pb	s	S

Table 2: EXPERIMENT 2

EXPERIMENTAL GROUP	DAY 0	DAY 0	DAY 4	DAY 7
Sm C	I Sm		s	S+p
SmPb5	I Sm	I Pb	s	S+p
PbC5		I Pb	s	S

Table 3: EXPERIMENT 3

EXPERIMENTAL GROUP	DAY 0	DAY 0	DAY 4	DAY 7
Sm C	I Sm		s	S+p
SmPb6	I Sm	I Pb	s	S+p
PbC6		I Pb	s	S

KEY

Sm- *Schistosoma mansoni* **p-** Perfusion

Pb- *Plasmodium berghei* **C-** Control

I- Infection **s-** Sampling

4, 5, 6- Week 4, Week 5, Week 6 post-*S. mansoni* infection.

SmPb- Co-infection

Day 0, 4, 7 I Pb- Days post-*P. berghei* infection.

2.2 Laboratory Maintenance of Snails

Biomphalaria pfeifferi snails were collected from Kangundo, Machakos District and screened for cercaria shedding. The screening was done by exposing them to strong light (100 watts) for two hours to induce the release of cercaria. This process was repeated every week for five weeks until the pre-patent period (4-5 wks) of *S. mansoni* in snails was over. Snails found schistosome-free were selected and maintained for subsequent exposure to infection with *S. mansoni* miracidia.

The snails were maintained in a temperature-controlled room at the Institute of Primate Research (IPR), Nairobi. The temperature was maintained at 25-28°C, and the room subjected to 12 hours of light / 12 hours of darkness.

Rectangular plastic trays measuring 40cm x 25cm x 12cm were used for housing the snails. The trays were first washed in 3% hydrochloric acid to sterilize them and subsequently rinsed thoroughly with chlorine-free water.

Soil from the riverbed where the snails were collected was obtained and sterilized by heating at 150°C for 11 hours in an oven. The soil was allowed to cool and layered at the bottom of the snail trays. Chlorine-free water was then added to the trays, up to three quarters full and the snails distributed in them. Snails were fed on blanched lettuce throughout the experimental period. This maintenance is according to already established protocols at the Institute of Primate Research.

2.3 Hatching of Miracidia

Eggs were obtained from faeces of *S. mansoni* chronically infected baboons maintained at IPR Animal Resource Department. The baboon faecal sample was placed in a beaker and topped with 1 litre of saline. This was mixed thoroughly using a wooden spatula to obtain a thin suspension of saline and faecal material. The

suspension was then poured through a sieve (mesh size 250μm) into urine jars. Using a wash bottle, the sieve was sprayed with saline to ensure that no eggs remained on the mesh, before filling up the urine jars with saline.

The urine jars were left in the dark for 30-45 minutes and the supernatant poured off without disturbing the deposit. The deposit was topped with fresh water, re-suspended, and again left in the dark for another 30-45 minutes. This was repeated 3 times until the supernatant became clear. Once clear, the supernatant was discarded without disturbing the deposit. The deposit was re-suspended in a small volume of water and transferred onto petridishes. The petridishes were then left under artificial light at a temperature of 20-25°C for 30 minutes. After this time, the emergence of miracidia was observed using a dissecting microscope (X 10 - X 40 magnifications).

2.4 Infecting Snails with Miracidia

The schistosome-free snails were infected individually with 3-6 miracidia, artificially hatched from harvested eggs of *S. mansoni* from infected baboon faeces as described in 2.3 above. The miracidia were picked from the petridish by use of a pipette mounted with a rubber bulb, and transferred into each well of a 24-well culture plate. The snails were then transferred individually into separate wells, plates covered with plate lids and left for 30 minutes to allow miracidia penetration.

The infected snails were then maintained under the conditions described in section 2.2, for four weeks. After the fourth week, the snails were placed in the dark to ensure that the snails did not shed cercaria before they were required.

2.5 Shedding of cercaria for infection

To obtain cercaria for infection of mice, the infected snails from the dark were transferred into 100 ml beakers containing 20 ml chlorine-free water and exposed to

artificial light (100 watts) for one to three hours (Smithers and Terry., 1965). This induced them to shed cercariae, which were then used to infect the mice. Three aliquots of 20 µl of cercarial suspension were placed on a Petri dish separately in droplets and the number of cercariae in each aliquot counted. The average number of cercariae was worked out and the total number of cercariae in 1 ml of suspension determined. The volume containing 150 cercariae was calculated and used for infection.

2.6 Laboratory maintenance and Infection of mice

2.6.1 Maintenance of mice

One hundred and ten, six-week-old BALB/c mice bred at the Institute of Primate Research (Nairobi, Kenya), were caged in groups of 10 and fed on commercial pellets and water provided *ad libitum*. They were kept under a natural light-dark cycle of 12/12 hours, at an ambient temperature of 25°C and relative humidity 50-60%.

2.6.2 Infection of mice with *S. mansoni*

Eighty mice were anaesthetized with a 1:2 mixture of Xylaxine (Rompun® 2%) and Ketamine (100 mg/ml; Rotex Medica GMBH Tritau-Germany). Each mouse was injected with 0.02 ml of anaesthesia per 30 g body weight intra-peritoneally, using a 1 ml syringe.

Each mouse was shaved on the abdomen, the abdominal area wiped with wet cotton wool, and a metal ring, 1 cm in diameter, secured in place on the shaved wet region using masking tape. A specified volume of aliquot (80µl) containing 150 cercariae was dispensed into the metal ring. The parasites were allowed 30 minutes to penetrate the abdominal skin and thus infect the mice (Smithers and Terry, 1965).

2.6.3 Infection of mice with *P. berghei*

2.6.3.1 Mouse infection with cryopreserved parasites

Contents of a cryotube containing *P. berghei*-parasitized blood suspension (from IPR parasite bank) were thawed at room temperature. A mouse was swabbed with 70% alcohol and injected with 0.03 ml of the suspension intra-peritoneally using 1ml syringe fitted with 27G needle.

2.6.3.2 Mouse infection with parasites from an infected mouse (Mechanical passage)

After 3 days blood was drawn from the mouse infected with cryopreserved parasites. A volume of blood containing 2.5 x 10^6 parasitized red blood cells was calculated and three separate volumes used to infect three naive mice. These were used as a source of parasites for the experiments. Each mouse was placed in a Bowman's restrainer. The tail vein was located (runs down right under the tail from the anus), and 0.28µl of the infected blood containing 2.5 x 10^6 parasitized red blood cells injected slowly until the whole amount flowed into the vein.

2.7 Parasitaemia analysis

2.7.1 Preparation of Giemsa stained blood films from tail blood

Two separate thin blood films from tail blood were made on standard microscope slides, and air-dried before fixing the films in methanol for 5 seconds. Fresh Giemsa stain solution was prepared in distilled water (10% v/v) and poured over the films (approximately 2 ml per slide), and the slides left to stain for 15 minutes before rinsing the excess stain in tap water. The slides were then left in an upright position to air dry. The stained blood films were observed under a standard light microscope using the x100 objective lens with immersion oil. Infected and uninfected red blood cells (RBCs) in different fields of view were identified and

counted. A total of at least 2000 RBCs were counted per slide. The percentage parasitemia was then determined using the following formula:

$$\text{Percentage parasitaemia} = \frac{\text{Total No. of infected RBCs}}{\text{Total No. of RBCs counted}} \times 100$$

2.8 Collection of blood for serum preparation

2.8.1 The heart puncture

Mice were anaesthetized by intra-peritoneal injection of 0.02 ml of Ketamine/xylaxine (2:1) mixture (as described in 2.6.2). 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 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, and extended up to the sternum and through the cartilage of the sternum to the left side. The ribs on either side of the sternum were trimmed carefully to prevent puncture of the lungs or the heart. Care was taken to avoid puncturing the two vessels on the sternum. The left ventricle was located and a 28G needle connected to a 1 ml syringe inserted into it. Blood was sucked in small jerks in order to create a vacuum and to 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.

2.8.2 Serum Preparation

Blood collected from each group of mice using the heart puncture technique was pooled in a centrifuge tube, allowed to clot by standing at room temperature for at

least 2 hrs. The clotted blood was left at 4°C overnight. The following day, it was spun in a centrifuge at 2 500 revolutions per minute for 10 minutes. The clear supernatant (serum) was then collected in clean Eppendorf tubes and stored at –20°C for use in ELISA.

2.9 Preparation of Antigens

2.9.1 Schistosome Worm Antigen Preparation (SWAP)

Adult *S. mansoni* worms were obtained from infected baboons perfused at week five post-infection (Yole *et al.,* 1996). The worms were washed thoroughly in PBS, placed in a tube containing PBS, and sonicated 923 KHZ, 16 uM amplitude) for 10 minutes and the homogenate centrifuged for 1hr at 100 000g, at 4°C to obtain the soluble protein. The protein estimation was based on the Bio-Rad method of Bradford 1976. This method utilizes bovine serum albumin (BSA) as a standard protein (see Appendix1). The optical densities at 595 nm, of serial dilution of BSA were read using a Cecil spectrophotometer (Cecil Instruments CE 6600R, England). Optical densities of different dilutions of SWAP were then calculated using the equation of the curve given by the spectrophotometer. The protein was aliquoted and sterilized by exposure to UV light (10 minutes, 5 cm from a 30 watt ultra violet OSRAM bulb) before use in *in vitro* assays. The aliquots were stored at -70°C. Protein concentration was adjusted to 100 µg/ml in PBS, before use in ELISA.

2.9.2 0-3 hr Antigen

S. mansoni cercaria were obtained from infected snails with a patent infection of five weeks by shedding (as described in section 2.5). Heads and tails of the cercariae were separated and the heads isolated on a discontinuous Percoll gradient and washed three times in complete media - RPMI 10 (RPMI 1640 in 10% foetal serum, 0.1%

gentamycin and 5x 10^{-5} β-mecarptoethanol). Gradient for separating heads and tails was prepared of two concentrations of Percoll: 70% and 45%. A 50 ml tube was filled up with 21 ml of Percoll mixed with 11 ml of RPMI 1640. Three drops of Hepes were added to each tube to keep the pH constant. The 45% Percoll was layered over the 70% Percoll to create a discontinuous gradient. Cercarial suspension was chilled for one hour in the cold room to make them settle at the bottom of the beaker. A 5% glucose solution was made in double distilled water. Excess water from the chilled cercarial suspension was discarded and the cercariae re-suspended by gently sucking in and out using a Pasteur pipette. The cercariae were placed in chilled glass tubes and centrifuged for 10 seconds at 100 g. The supernatant was sucked out and 0.5 ml of glucose added. The chilled cercariae were vortexed for 90 seconds to separate the heads from the tails. The separated heads and tails of cercariae were dispensed gently on the Percoll gradient using a Pasteur pipette and centrifuged at 450 g for 10 minutes. The heads formed a band at the top of the interface of the gradient. These were aspirated and washed three times in RPMI/10. The heads were re-suspended in RPMI/10 and transferred to Bijou tubes and incubated at 37°C, 5% CO_2 for 3 hrs. After 3 hours of incubation, the suspension was centrifuged for 10 minutes at 450 g, at 37°C. The supernatant was discarded. The pellet containing the proteins released by penetrating schistosomula between 0-3 hrs of penetration was re-suspended and aliquoted in cryovials. The protein was sterilized by exposure to ultraviolet light by placing the vials 5 cm from a 30 watt ultraviolet OSRAM bulb for 10 minutes. Aliquots were stored at -20°C until required for ELISA.

2.9.3 Malaria Antigen Preparation (Soluble *P. berghei* Antigen)

At a parasitemia of 4%, mice infected with *P. berghei* were bled by cardiac puncture using a 23-G needle attached to 1ml syringe containing 0.1 ml heparin.

Blood from five mice was pooled into a 50 ml tube containing 10 ml complete culture medium (RPMI 1640, pH 7.3, containing 25% FCS v/v) and spun at 1500 rpm for 10 minutes. The supernatant was discarded and the cells re-suspended in 40 ml complete culture medium. The mixture in the flasks was gassed for 2 minutes using 5% CO_2, 5% O_2, and 90% N_2 and incubated for 18 hrs at 37°C. The procedure up to just before incubation was carried out in a sterile hood.

After 18 hrs, slides were prepared to confirm the presence of parasites in the culture as well as to rule out any contamination. The confirmation was done by putting 200 µl of the culture into a vial. The vial was spun at x1000 g for 1 min and the supernatant discarded. The pellet was used to make slide smears as described in section 2.7.1. After confirmation, the culture was then spun at 3000 rpm for 10 minutes and the supernatant containing soluble *P. berghei* antigen harvested. The supernatant was aliquoted into 2 ml cryovials and stored at -20°C until use in ELISA.

2.9.4 Crude P. berghei Antigen

Infected blood at 50% parasitemia was spun at 3000 revolutions per minute (rpm) for 10 minutes and the supernatant discarded. The pellet was mixed with 0.15 % saponin in RPMI (w/v) in the ratio 1:4 and mixed thoroughly. This was incubated in ice (4°C) for 10 minutes shaking every two minutes to mix the suspension thoroughly and enhance lysis. The mixture was spun again at 3000 rpm for ten minutes and the supernatant discarded. The same procedure as above was repeated three times. The pellet was finally re-suspended in 4.5 ml of RPMI and aliquoted in three-1.5 ml cryovials. These were stored at -20°C awaiting use.

2.10 Gross Pathology and Perfusion

On day seven post malaria super-infections, 5 mice from each of the co-infected and *S. mansoni* single-infection groups were perfused to recover the adult worms. The perfusion was done using a modified method of Yole *et al.*, 1996.

Each mouse was anaesthetized with 0.02 ml per 30 g body weight Ketamine/Rompun® (2:1) mixture. A transverse mid-ventral nick was made on the skin of the abdomen using a pair of scissors and the skin peeled off upwards and downwards. The abdominal wall was opened up without cutting the viscera. Gross pathology of the liver and spleen was observed. Observations included: liver enlargement, adhesions and presence of granulomas. Inflammation of the spleen was also considered. The gross pathology of the liver was then subjectively categorized as none (no granulomas), few (one or two) moderate (three to ten granulomas per section), and severe (more than ten granulomas per section).

After the observation of the gross pathology, the thoracic cavity was opened and ribs on either side of the sternum snipped off leaving the two veins on either side of the sternum intact. The hepatic portal vein was then located and cut. A perfusion needle containing perfusion fluid (containing 0.85% Sodium chloride and 1.5% Sodium Citrate) was inserted in the left ventricle of the heart and perfusion carried out until the liver and the mesenteries were clear. Worms were recovered from the perfusate using the method of Yole *et al* 1996.

Briefly, the perfusate was collected in a glass Petri dish (20 cm in diameter) and then transferred to urine jars to settle. After settling, the supernatant was carefully sucked out. Phosphate Buffered Saline (PBS) was dispensed into the urine jars and the worms allowed to sediment before sucking out the supernatant. This process was repeated until the perfusate became clear. Once clear, the supernatant was discarded

and worms transferred to a 10 cm plastic Petri dish, ruled at the bottom, on the outside to ease counting.

The worms were counted and the mean and S.E.M for each group calculated. The livers were collected and fixed in 10% buffered formalin awaiting histological examination.

2.11 Histopathological examination of tissues

The fixed (in 10% buffered formalin) livers were dehydrated using ethyl alcohol, and embedded in paraffin wax. Sections, 7μm thick, were cut using a rotary microtome. Tissue sections were placed on slides and stained with haematoxylin and counter-stained with eosin. Slides were observed under X25 and X40 objective lenses. The number of granulomas was noted and the sizes established by measuring (using a stage micrometer) the length and width of each granuloma with a centrally placed schistosome egg, and then getting the average (Farah *et al, 2000).* Ten granulomas were measured for each of the two schistosome-infected groups: the co-infection and *S. mansoni* control.

2.12 IgG ELISA

Specified wells of Nunc-Immuno™ plates (MaxiSorp ™ Surface) ELISA plates were coated overnight with 50 μl of soluble *P. berghei* antigen (4 % parasitemia), SWAP 10 μg/ml, 0-3 hr antigen, or crude P. berghei antigen (50% parasitemia), diluted in bicarbonate buffer pH 9.6. The plates were incubated overnight at 4°C. The antigen was 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 off the non-specific binding sites with 100 μl 3% BSA in PBS for 1hr at 37°C and washing off unbound BSA six times with washing buffer. Diluted (1:200) serum

samples (50 µl) were dispensed into different wells in duplicates and incubated for 1hr at 37°C, and then washed as above. After washing the unbound serum, 50 µl of 1:2000 peroxidase conjugated rabbit anti-mouse IgG was dispensed in all the wells and incubated for 1hr at 37°C. The unbound conjugate was washed off.

After washing off the unbound conjugate, 50 µl orthophenyldiamine substrate (0.4 µg/ml) in citrate buffer was added to each of the wells, and the plates incubated at 37°C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader.

2.13 Statistical analysis

Experimental and control values were analyzed for significant differences by Student's t-test using Microsoft Excel. A probability of less than 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 Parasitological findings

3.1.1 Parasitemia analysis

Groups of mice with a 4-week, 5-week, and a 6-week *S. mansoni* infection, and their controls, were infected with 2.5 x 10^6 blood-stage *P. berghei parasites*, and the malaria parasitemia monitored using Giemsa-stained thin blood smears. Parasitemia was recorded as mean ± standard error of the mean [SEM].

Experiment 1: At week 4 post-*S .mansoni* infection, a group of mice was super-infected with malaria. A naïve group was also infected with malaria to serve as a control. The malaria-only group recorded significantly higher parasitemia at day 7 (4.82 ± 0.846%) than at day 4 (0.728 ± 0.184%, $P<0.05$).

The co-infected mice also developed a significantly higher malaria parasitemia (Fig. 1), at day 7 (17.12 ± 0.871%) than at day 4 (5.38 ± 0.626%, $P<0.01$).

Parasitemia developed significantly faster in the co-infected mice, being 5.38 ± 0.626% on day 4, compared to 0.728 ± 0.184% in *P. berghei*-only-infected group on the same day [$P<0.01$].The co-infected mice developed a significantly higher malaria parasitemia than mice infected with *P. berghei* alone at both sampling points [$P<0.01$].

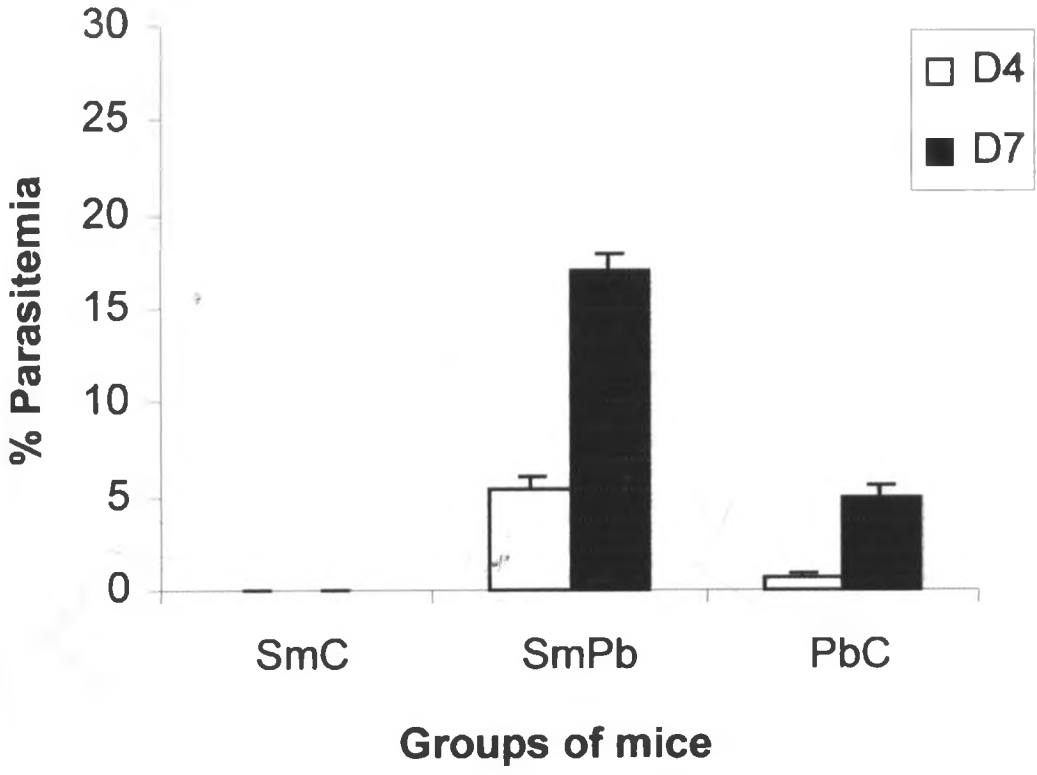

Fig 1: Parasitemia in single and co-infected mice (Exp. 1)

Experiment 2: At week 5 post-*S .mansoni* infection, a group of mice was super-infected with malaria. A naïve group was also infected with malaria to serve as a control. The results are shown in Fig.2.

In the single infected group, parasitemia at day 7 (19.66 $\pm$ 1.465%) was significantly higher than at day 4 (0.535 $\pm$ 0.056%, $P<0.01$). The parasitemia in the co-infected group was also higher at day 7 (6.03 $\pm$ 3.086%) as compared to day 4

(1.395 ± 0.395%). However, the difference was not significant [$P>0.05$]. A comparison between the two groups showed that the *P. berghei*-only-infected mice developed significantly higher parasitemia (19.66 ± 1.465%) than the co-infected ones (6.03 ± 3.086%, $P<0.05$) at day 7. At day 4, though the co-infected group showed higher parasitemia than the malaria-only group, the difference was not significant [$P>0.05$].

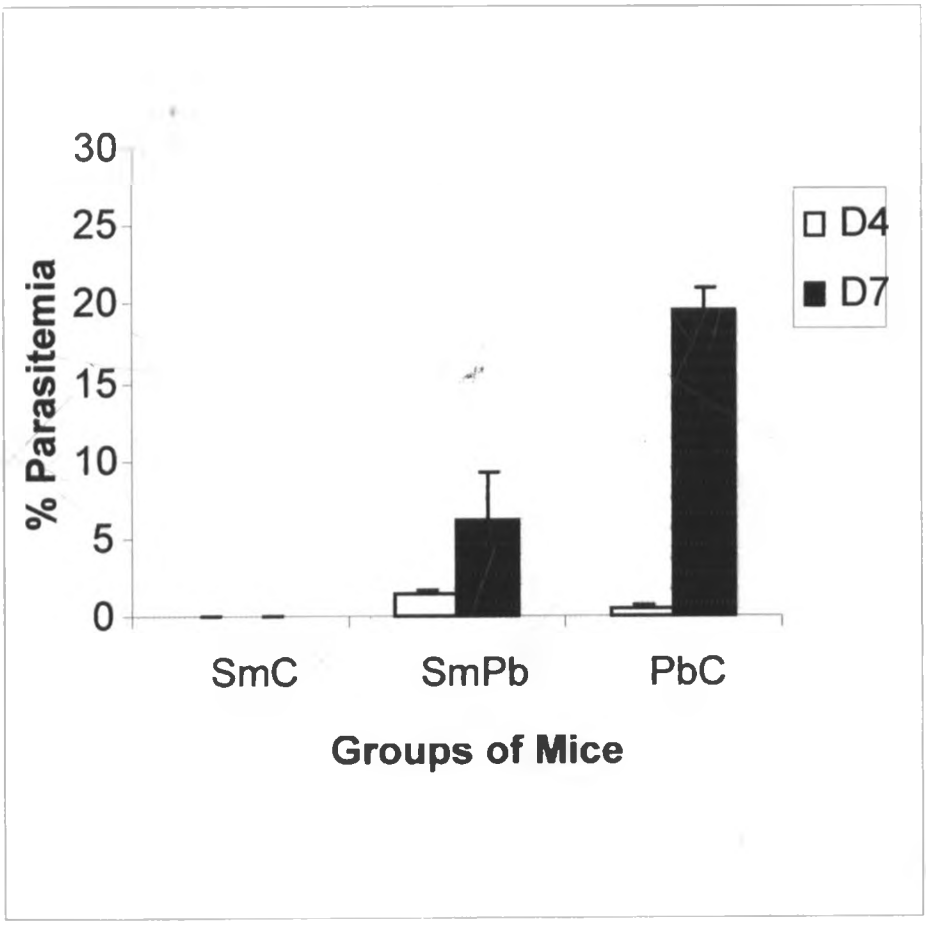

Fig 2: Parasitemia in single and co-infected mice (Exp. 2)

Experiment 3: At week 6 post-*S .mansoni* infection, a group of mice was super-infected with malaria. A naïve group was also infected with malaria to serve as a control. The parasitemia in the co-infected group (Fig 3), was significantly higher at

day 7 (23.94 ± 3.575%), as compared to day 4 (1.308 ± 0.193%, $P<0.05$). In the single infected group, parasitemia at day 7 (19.018 ± 2.887%) was significantly higher than at day 4 (4.68 ± 0.741%, $P<0.05$).

The parasitemia in the co-infected group was higher at day 7 than in the malaria-only group although the difference was not significant [$P>0.05$]. At day 4, the malaria-only group exhibited a significantly higher parasitemia than the co-infected group [$P<0.05$].

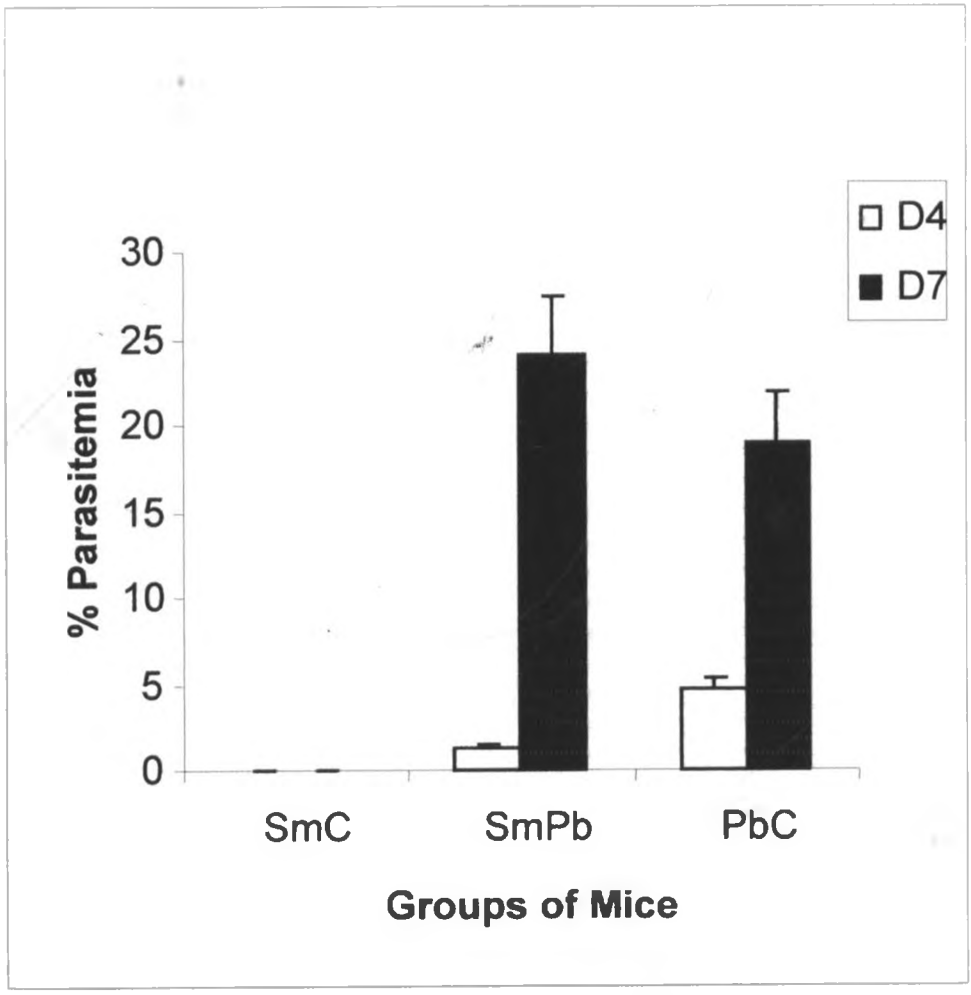

Fig 3: Parasitemia in single and co-infected mice (Exp. 3)

3.1.2 Analysis of Worm load

The number of worms present in the single and co-infected groups was monitored through perfusion and counting (Fig.4). Perfusion and counting was carried out once every week- 7 days post-*P. berghei* infection, beginning at the fourth week of *S. mansoni* infection.

Experiment 1: For the group of mice super-infected with malaria at week 4 of *S. mansoni* infection, and their controls, the *S. mansoni*-only group showed a higher mean worm load of 13.6± 2.223, as compared to 13 ± 2.041 in the co-infected group. The difference was however not significant [$P>0.05$].

Experiment 2: For the group of mice super-infected with malaria at week 5 of *S. mansoni* infection, the mean worm load was significantly higher in the *S. mansoni*-only group (25.2 ± 3.815), than in the co-infected group (14 ± 3.206, $P<0.05$).

Experiment 3: For the group of mice super-infected with malaria at week 6 of *S. mansoni* infection, the trend in experiments 1 and 2 was replicated-with the single-infected group having a significantly higher worm load (40.4 ± 2.948) than the co-infected ones (24.2 ± 3.234, $P<0.05$).

A comparison between the weeks showed a significant difference for the *S. mansoni*-only group between week 4 and 5 [$P<0.05$], and between week 4 and 6 [$P<0.01$], However, the difference was not significant between week 5 and 6 [$P>0.05$]. For the co-infected groups, the difference between the weeks was not significant.

Generally, the number of worms present in both the co-infected and the single-infected groups increased over time. The increase was however higher in the *S. mansoni*-only groups than in the co-infected groups, with significant differences at week 4 and 5 (as shown under each experiment above).

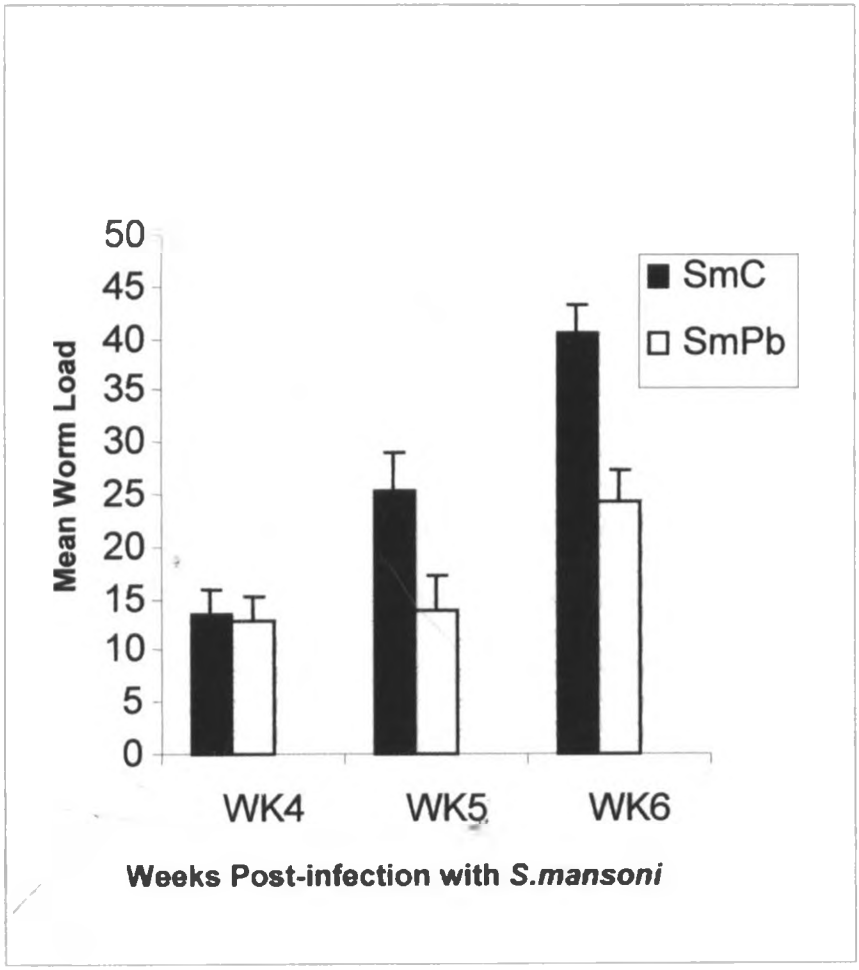

Fig 4: Worm burden in single and co-infected mice

3.2 Immunological findings

3.2.1 Antigen-specific IgG responses

Comparative specific IgG responses for groups SmPb (co-infection), SmC (Schistosome only infection), and PbC (malaria only infection) are shown in Figures 1- 12 for the antigens 0-3 Hr, SWAP, Soluble *P. berghei*, and Crude *P. berghei*, for the different infections at each of the two sampling points: day 4 and 7. The antibodies were measured by ELISA. The IgG levels were determined and presented as absorbance (OD) at 630 nm. Results were obtained from serum dilution of 1: 200.

Mice infected with *S. mansoni* were super-infected with *P. berghei* at different times. In Experiment 1, they were super-infected at week 4 of *S. mansoni* infection. In Experiment 2, they were super-infected at week 5, and in Experiment 3, they were super-infected at week 6.

3.2.2 0-3 hr antigen- specific IgG responses

Experiment 1: Mice in this experiment were super-infected with *P. berghei* at 4 weeks post-infection with *S. mansoni*. The results (Fig.5) indicate that IgG antibody response to 0-3 Hr antigen was low in the malaria-only-infected group at both sampling points. It was however higher at day 4 (O.D 0.024) than at day 7 (O.D 0.003). IgG level in the co-infected group was higher at day 4 (O.D 0.191) than at day 7 (O.D 0.064). In the *S. mansoni*-only group, the IgG level was higher at day 4 (O.D 0.193) than at day 7 (O.D 0.113). All the treatments showed a higher response than the negative group (O.D 0.019) except for malaria-only group at day 7. In comparison, the malaria-only group showed the lowest IgG response, whereas the groups with schistosomiasis-both single-infected and co-infected, showed a higher response. The response was however higher in the *S. mansoni*-only group than in the co-infected group.

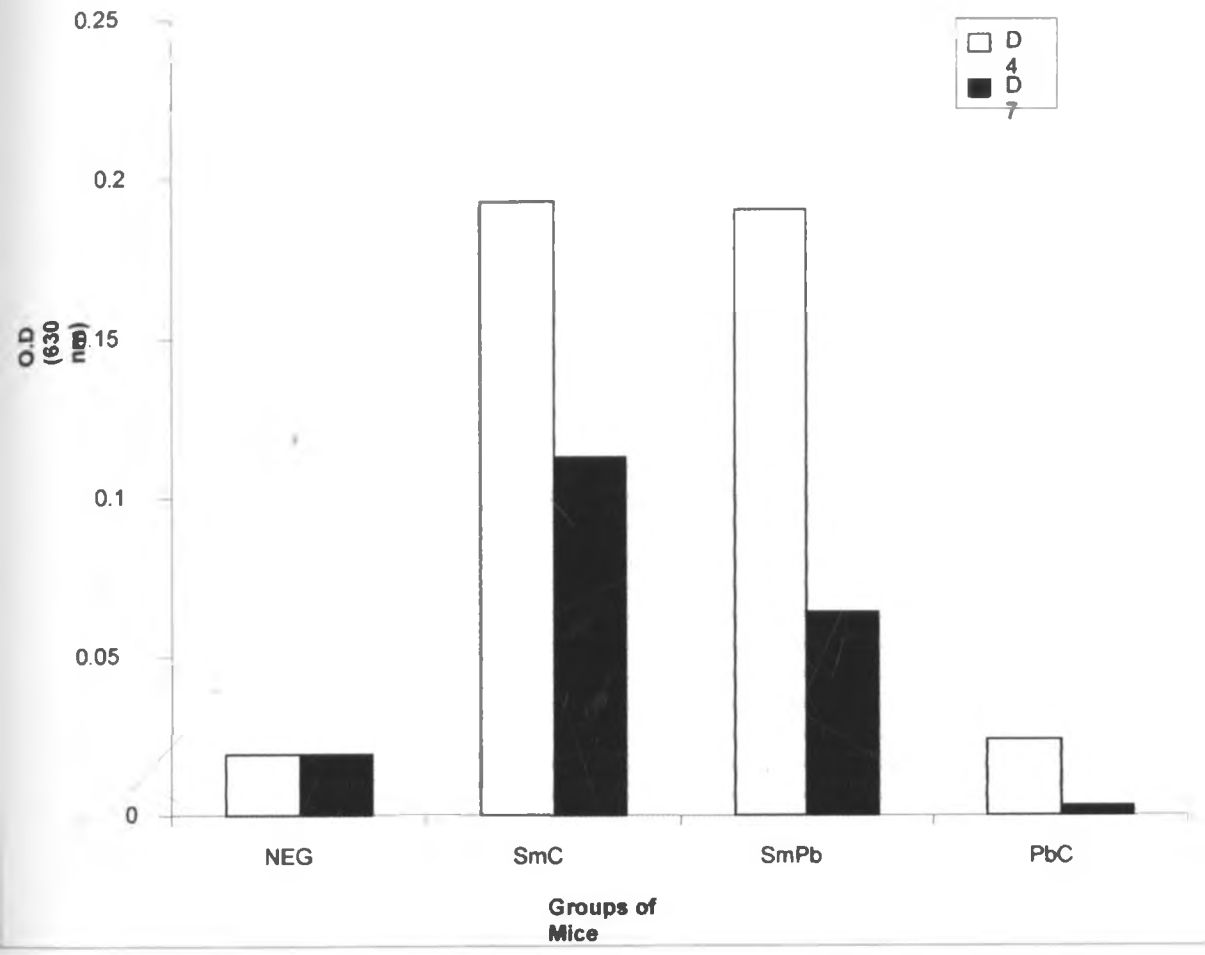

Fig 5: 0-3hr antigen specific IgG responses in single and co- infected mice (Exp. 1)

KEY

NEG - Negative control

O.D – Optical density

PbC - *Plasmodium berghei* control

SmC - *Schistosoma mansoni* control

SmPb - *Schistosoma mansoni* / *Plasmodium berghei* co-infection

D4 - Day 4

D7 - Day 7

Experiment 2: Mice in this experiment were super-infected with *P. berghei* at week 5 post-*S. mansoni* infection. The malaria-only-infected group showed a lower IgG response (Fig.6) with slightly higher levels at day 7 (O.D 0.034) than at day 4 (O.D 0.032). In the co-infected group, the IgG response at day 7 was higher (O.D 0.141) than at day 4 (O.D 0.084). The *S. mansoni*-only group showed a lower IgG response at day 4 (O.D 0.089) than at day 7(O.D0.098).

All the treatments showed a higher IgG response than the negative control (O.D 0.019), and day 7 responses were higher than day 4 in all the groups.

0-3 hr-specific IgG levels were generally lower than in experiment 1 and the malaria-only-infected group still showed a lower IgG level than both groups having *S. mansoni*.

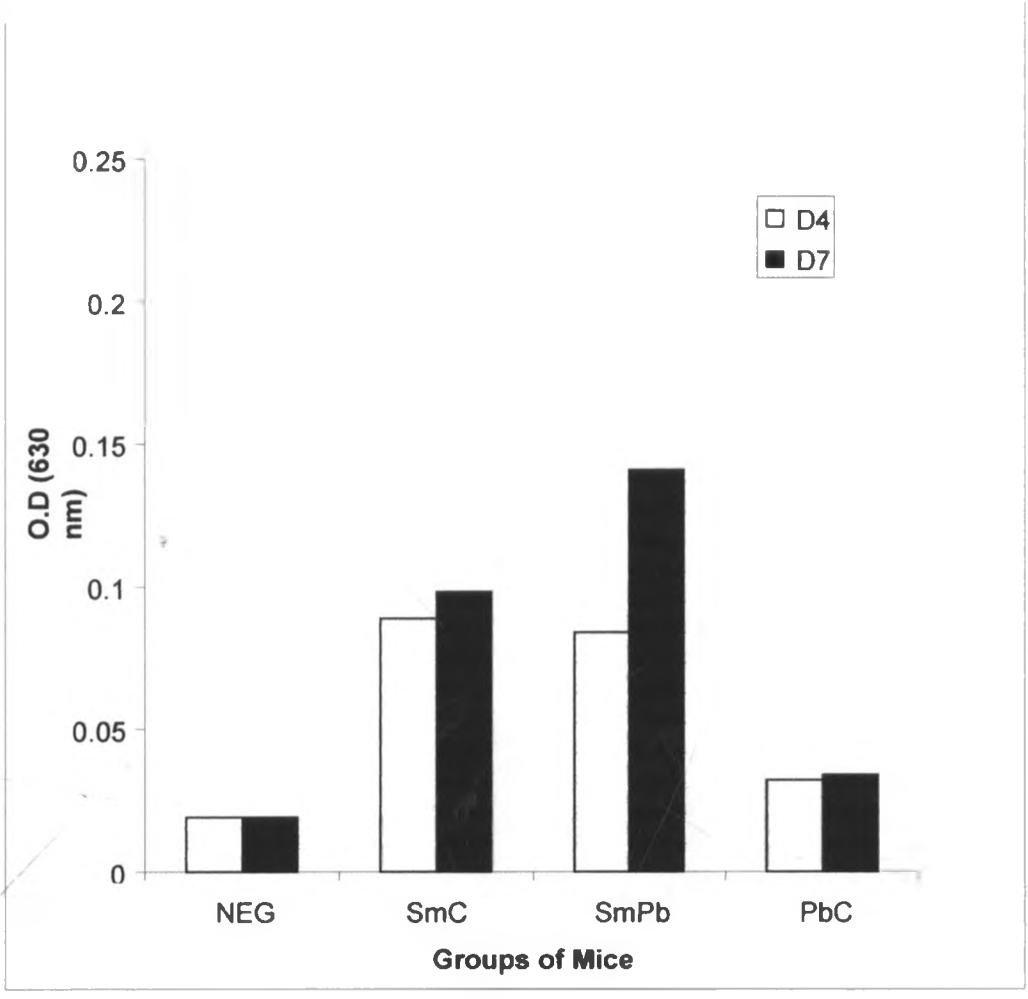

Fig 6: 0-3hr antigen specific IgG responses in single and co- infected mice (Exp. 2)

Experiment 3: Mice in this Experiment were super-infected with *P. berghei* at week 6 of *S. mansoni* infection (Fig.7). For the malaria-only group, the IgG levels were low with recordings of O.D 0.064 at day 4 and O.D 0.063 at day 7. The co-infected group showed the highest IgG level (O.D 0.136) at day 4, and a lower level at day 7 (O.D 0.089). For the *S. mansoni*-only group, the IgG level was lower at day 4 (O.D 0.106) than at day 7 (O.D 0.114). The co-infected group showed higher IgG

level at day 4 than the *S. mansoni*-only group on the same day. However, on day 7, the *S. mansoni*-only group showed a higher IgG level than the co-infected group.

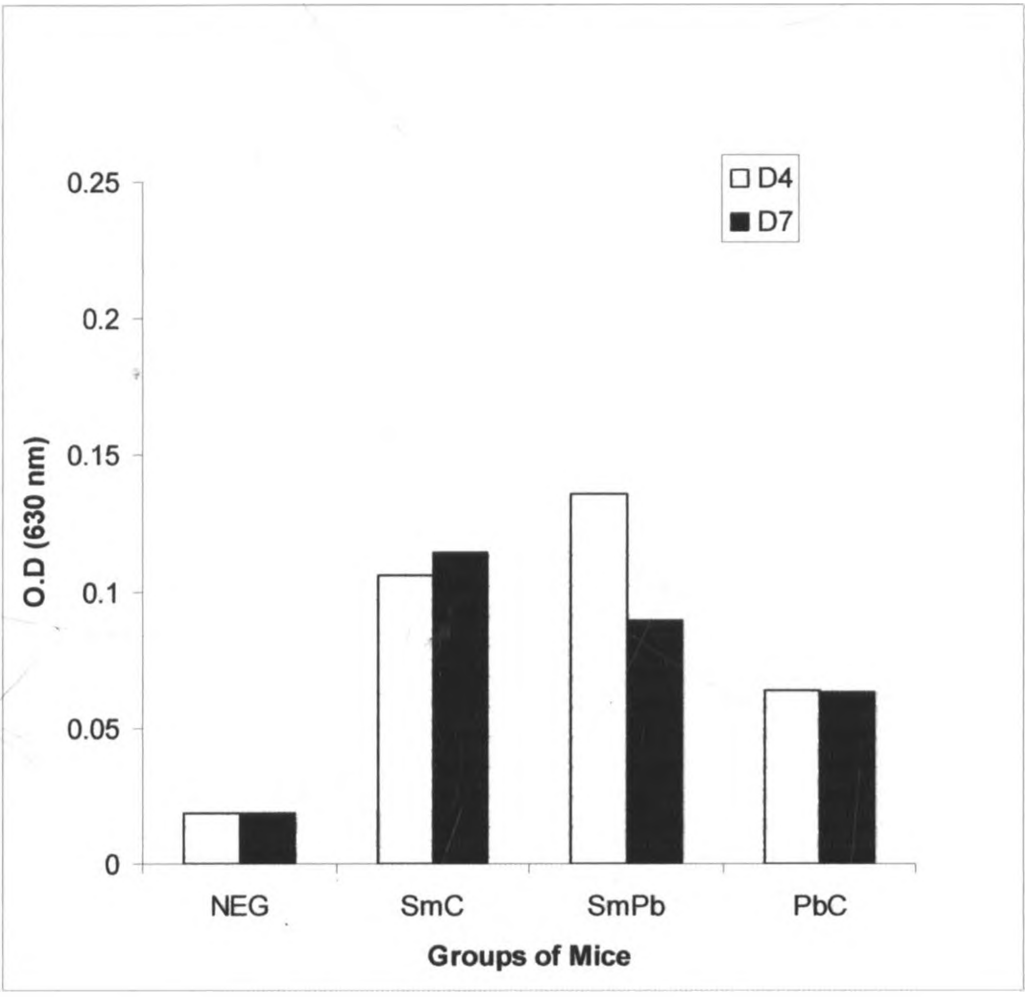

Fig 7: 0-3hr antigen specific IgG responses in single and co- infected mice (Exp. 3)

Generally, the IgG levels in Experiment 3 were similar to those in Experiment 2, except for the co-infected group where in Experiment 3; day 4 recordings were higher than in Experiment 2. However, the malaria-only group showed higher IgG levels in experiment 3 than in Experiments 1 and 2 but still showed the lowest recording just

like in the other experiments. The *S. mansoni*- infected groups showed lower recordings than in Experiments 1 and 2.

3.2.3 SWAP-Specific IgG responses

Experiment 1: The group in this experiment was super-infected with malaria at 4 weeks post-infection with *S. mansoni.* IgG antibody response to SWAP is shown in Figure 8 for the co-infected and the control animals. In the malaria-only-infected group, an optical density of 0.083 was recorded on day 4 while the value rose to 0.104 on day 7. The co-infected group had higher levels at day 4 (O.D 0.437), than at day 7 (O.D 0.33). For the *S. mansoni*-only group, the O.D at day 4 was 0.385, which was higher than at day 7 (O.D 0.308).

The IgG antibody response to SWAP was lower in the malaria-only-infected group than it was in the co-infected or *S. mansoni*-only-infected groups. In the co-infected group, the response was higher than in the *S. mansoni*-only group at both sampling points though the trend was similar. All the treatments also showed a higher response than the negative control (O.D 0.095) except for the malaria-only group at day 4.

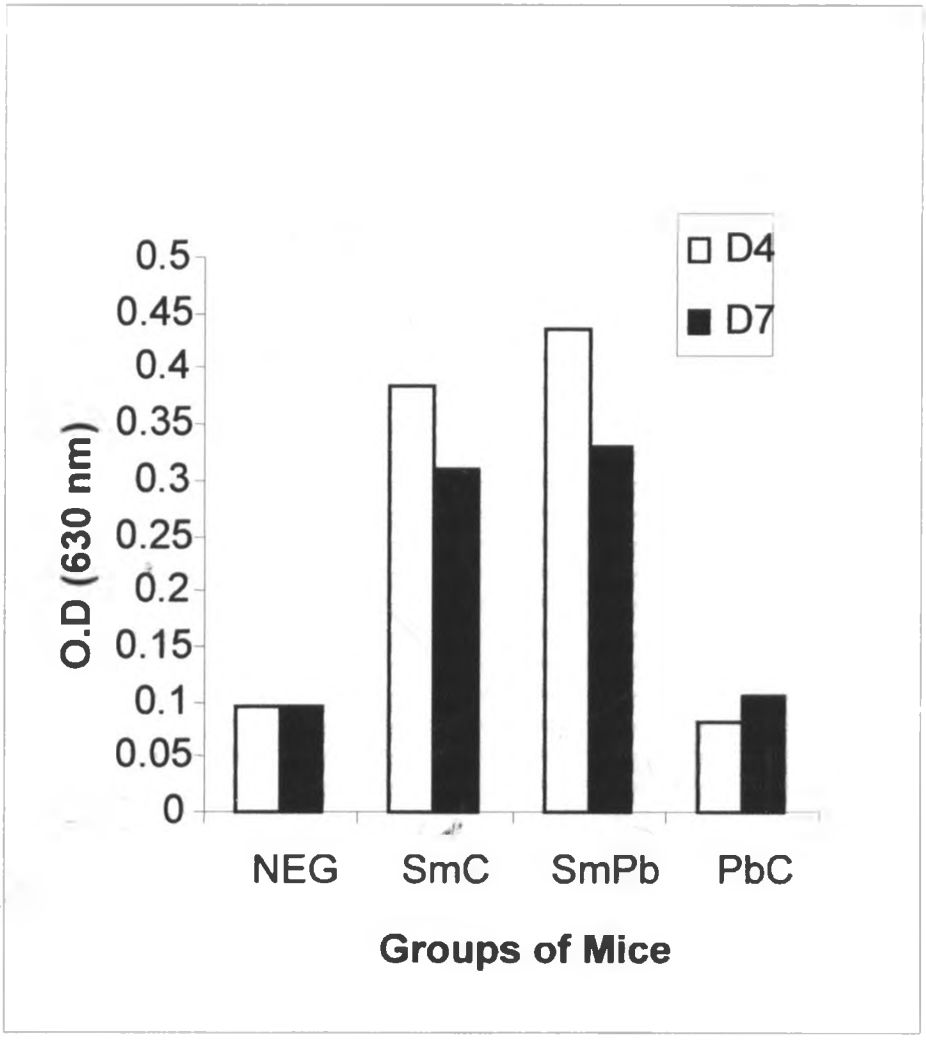

Fig 8: SWAP specific IgG responses in single and co- infected mice (Exp. 1)

Experiment 2: For the group that was super-infected with malaria at week 5 of *S. mansoni* infection, the SWAP-specific IgG levels are recorded in Figure 9. The malaria-only-infected group showed a slightly higher level at day 4 (O.D 0.124) than at day 7 (O.D 0.116). In the co-infected group, the IgG response was higher on day 7 (O.D 0.287) than on day 4 (O.D 0.221). In the *S. mansoni*-only group, IgG response was higher at day 4 (O.D 0.239), than at day 7 (O.D 0.218).

The SWAP-specific IgG levels were generally lower than in experiment 1 (super-infected at week 4). The malaria-only-infected group still showed a lower IgG

level than the groups having *S. mansoni* just like in Experiment 1, and in the 0-3hr-specific IgG responses. The response in all the treatments, just like in the 0-3hr-specific IgG responses, remained higher than in the negative control group (O.D 0.095).

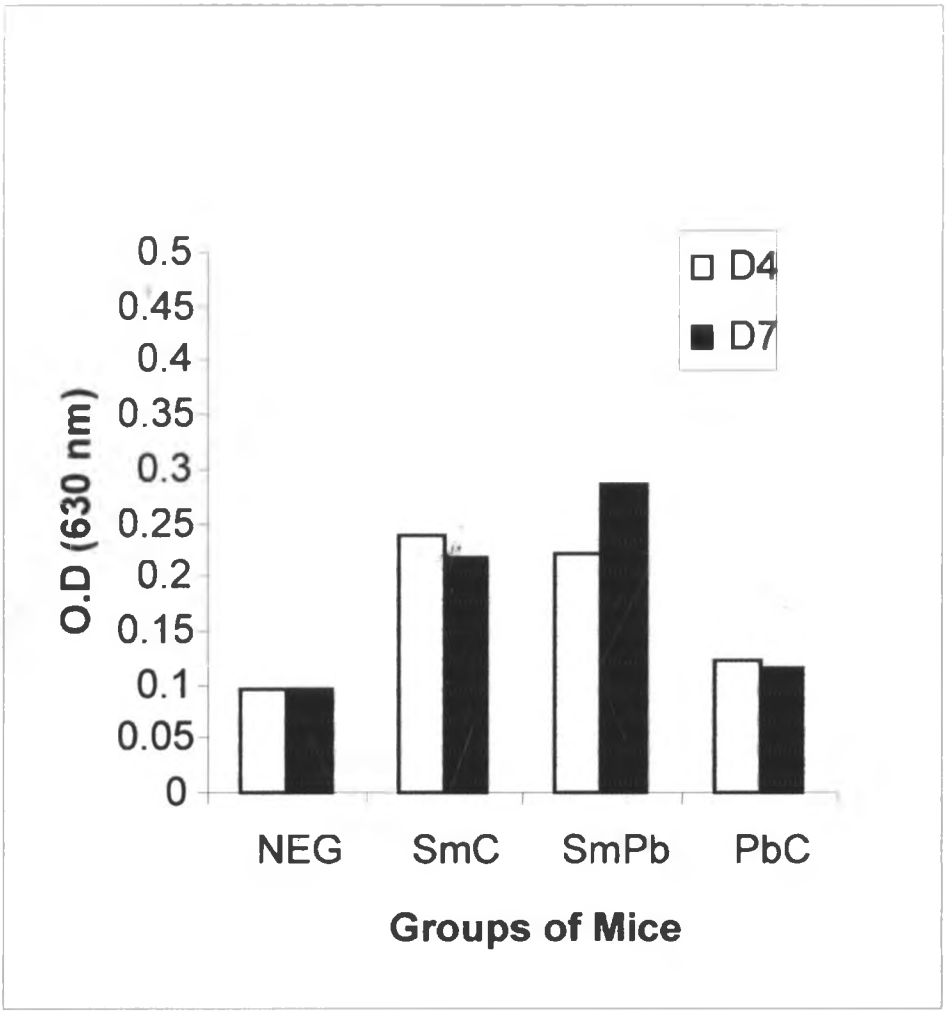

Fig 9: SWAP specific IgG responses in single and co- infected mice (Exp. 2)

Experiment 3: For the group that was super-infected with malaria at week 6 of *S. mansoni* infection, the SWAP-specific IgG levels are recorded in Figure 10. The malaria-only group showed a lower response at day 7 (O.D 0.159) than at day 4 (O.D 0.169). The co-infected group showed a higher response at day 7 (O.D 0.336), than at

day 4 (O.D 0.323). The *S. mansoni*-only group showed a higher response at day 7 (O.D0.273) than at day 4(O.D0.26).

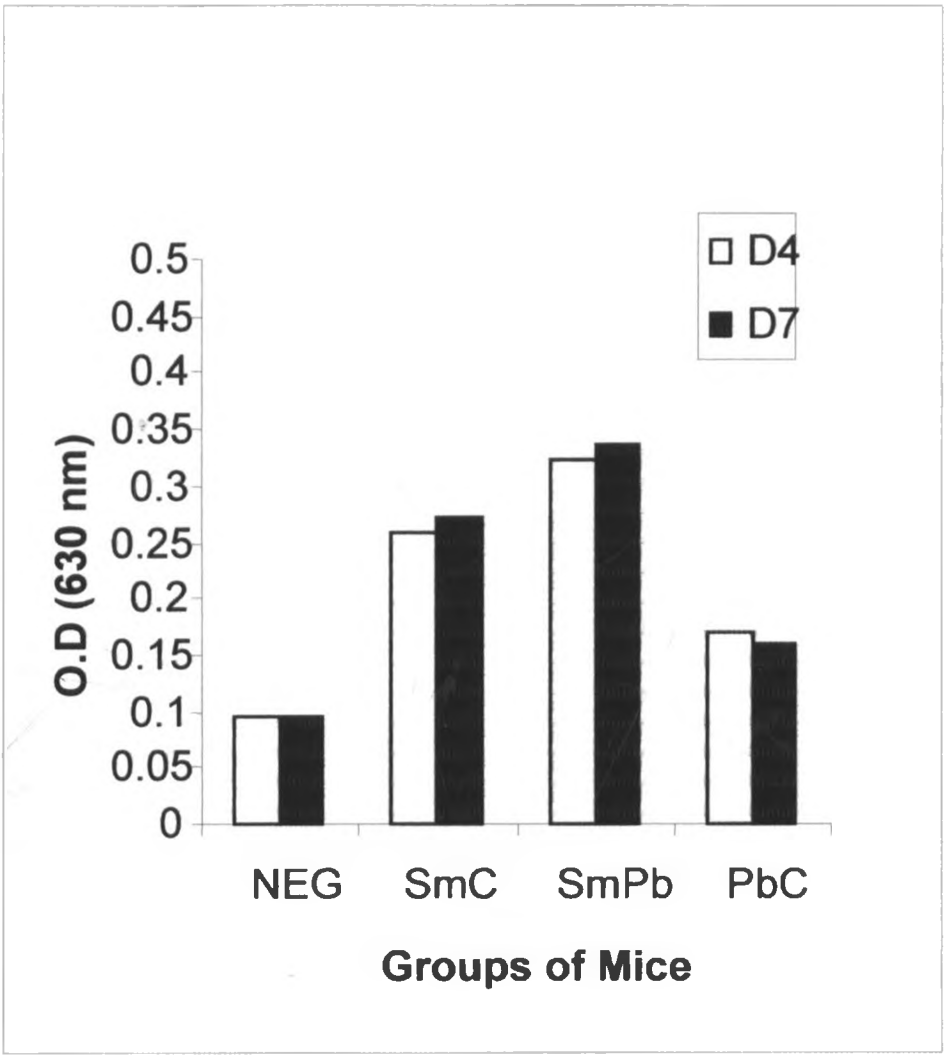

Fig 10: SWAP specific IgG responses in single and co- infected mice (Exp. 3)

The malaria-only group still showed a lower response than the groups bearing *S. mansoni* at both day 4 and day 7. The co-infected group still showed a higher response at both sampling points, than the *S. mansoni*-only group. All treatments still remained higher than the negative group (O.D 0.095). There was a general increase in the IgG levels in the co-infected as well as in the *S. mansoni*-only groups as compared

to Experiment 2. The two groups however showed a lower response as compared to those in Experiment 1.

3.2.4 Soluble *P. berghei*-specific IgG responses.

Experiment 1: This experiment had groups of mice that were super-infected with *P. berghei* at week 4 of *S. mansoni* infection. IgG antibody response to soluble *P. berghei* antigen are shown in Figure 11. In the malaria-only group the response was higher at day 4 (O.D 0.077) than at day 7 (O.D 0.054). In the co-infected group, the response was higher at day 4 (O.D 0.25), than at day 7 (O.D 0.054), while in the *S. mansoni*-only group, the response was higher at day 7 (O.D 0.195), than at day 4 (O.D 0.148).

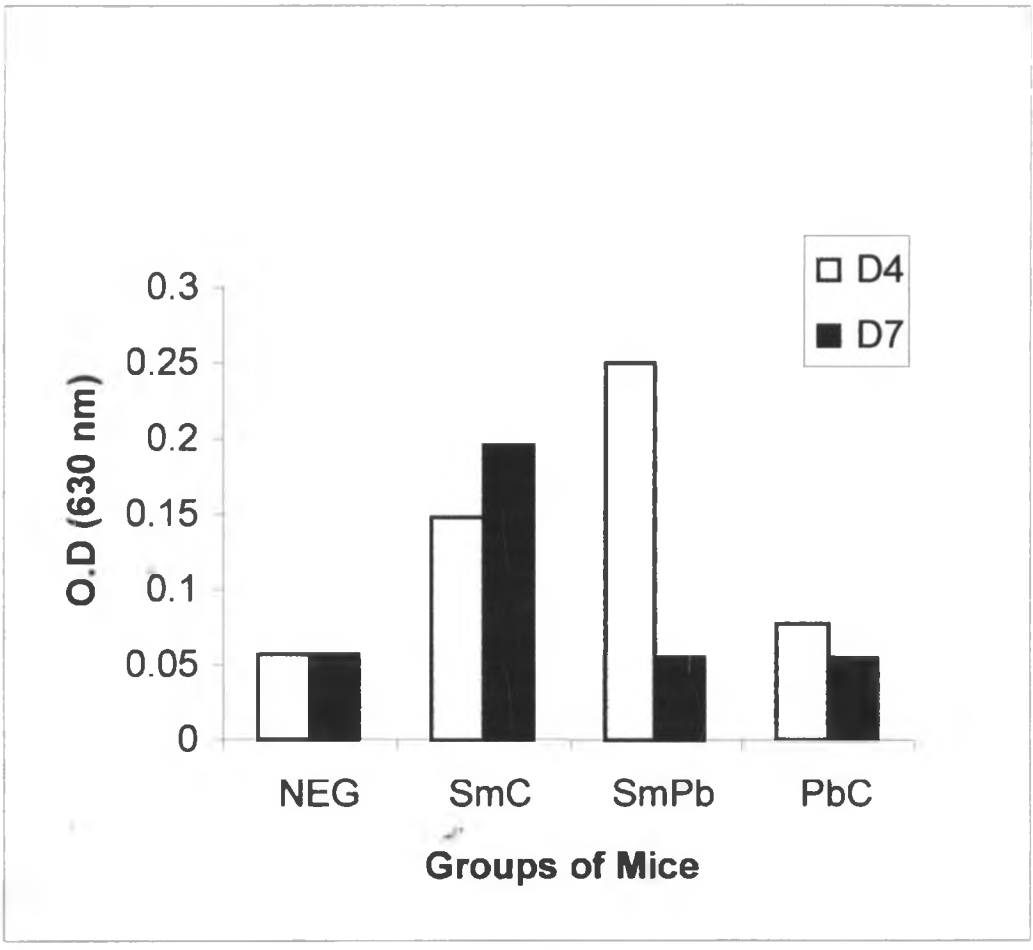

Fig 11: Soluble P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 1)

IgG antibody response to soluble *P. berghei* antigen was lower in the malaria-only group than in the groups bearing *S. mansoni* infection (similar to preceding experiments). The co-infected group had the highest response at day 4, compared to the *S. mansoni*-only group, but day 7 of the *S. mansoni*-only group recorded a higher response than for the co-infected group. All the treatments recorded higher optical densities than the negative control (O.D 0.056), except for day 7 of the malaria-only group and the co-infection which showed a slightly lower recording (O.D 0.054).

Experiment 2: In the group that was super-infected with malaria at week 5 post-*S. mansoni* infection (Fig.12), the malaria-only-infected group showed a lower IgG

level at day 4 (O.D 0.087) than at day 7 (O.D 0.103). In the co-infected group, the IgG response was higher on day 7 (O.D 0.159) than on day 4 (O.D 0.131). In the *S. mansoni*-only group, IgG response was higher at day 4 (O.D 0.136), than at day 7 (O.D 0.094).

The soluble *P. berghei* antigen-specific IgG levels were again generally lower in the malaria-only-infected group than in the groups having *S. mansoni* though day 7 response was higher than that of the *S. mansoni*-only group for the same day. The co-infected group showed a generally higher response than in the *S. mansoni*-only group. The response in all the treatments remained higher than in the negative control group (O.D 0.056).

The general trend in this experiment was similar to that of Experiment 1 where by the malaria-only group showed the least response, the co-infected group the highest and the *S. mansoni*-only group was in between.

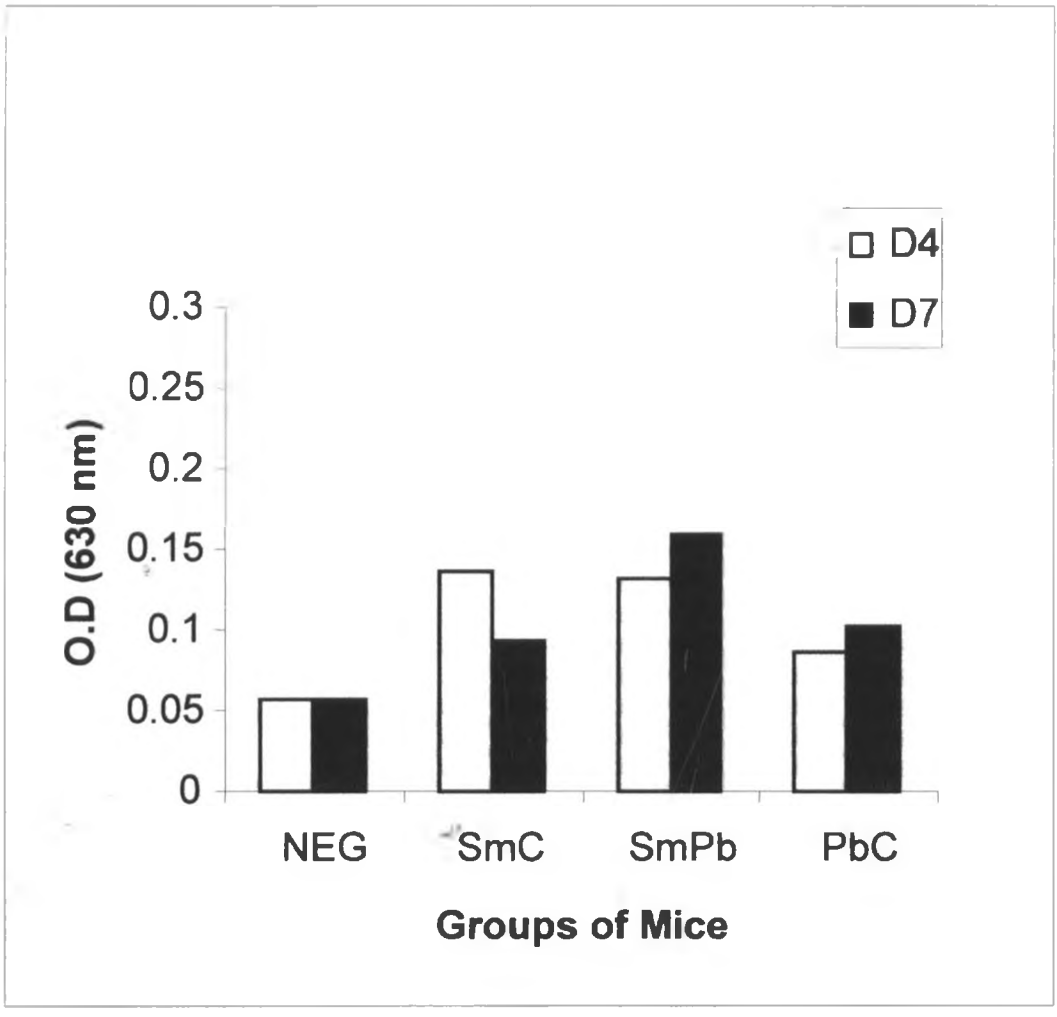

Fig 12: Soluble P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 2)

Experiment 3: For the group that was super-infected with *P. berghei* at week 6 of infection with *S. mansoni* (Fig.13), the malaria-only group showed a lower response at day 4 (O.D 0.083) than at day 7 (O.D 0.127). The co-infected group showed a higher response at day 4 (O.D 0.167), than at day 7 (O.D 0.101). The *S. mansoni*-only group showed a lower response at day 4 (O.D 0.099) than at day 7 (O.D 0.128).

The malaria-only group still showed a lower response than the groups bearing *S. mansoni* at day 4 while day 7 showed a higher response (O.D 0.127). The co-infected

group showed a higher response at day 4 than the *S. mansoni*-only group. However, day 7 recorded a higher response for *S. mansoni*-only group than for the co-infected group. All treatments still remained higher than the negative group (O.D 0.056).

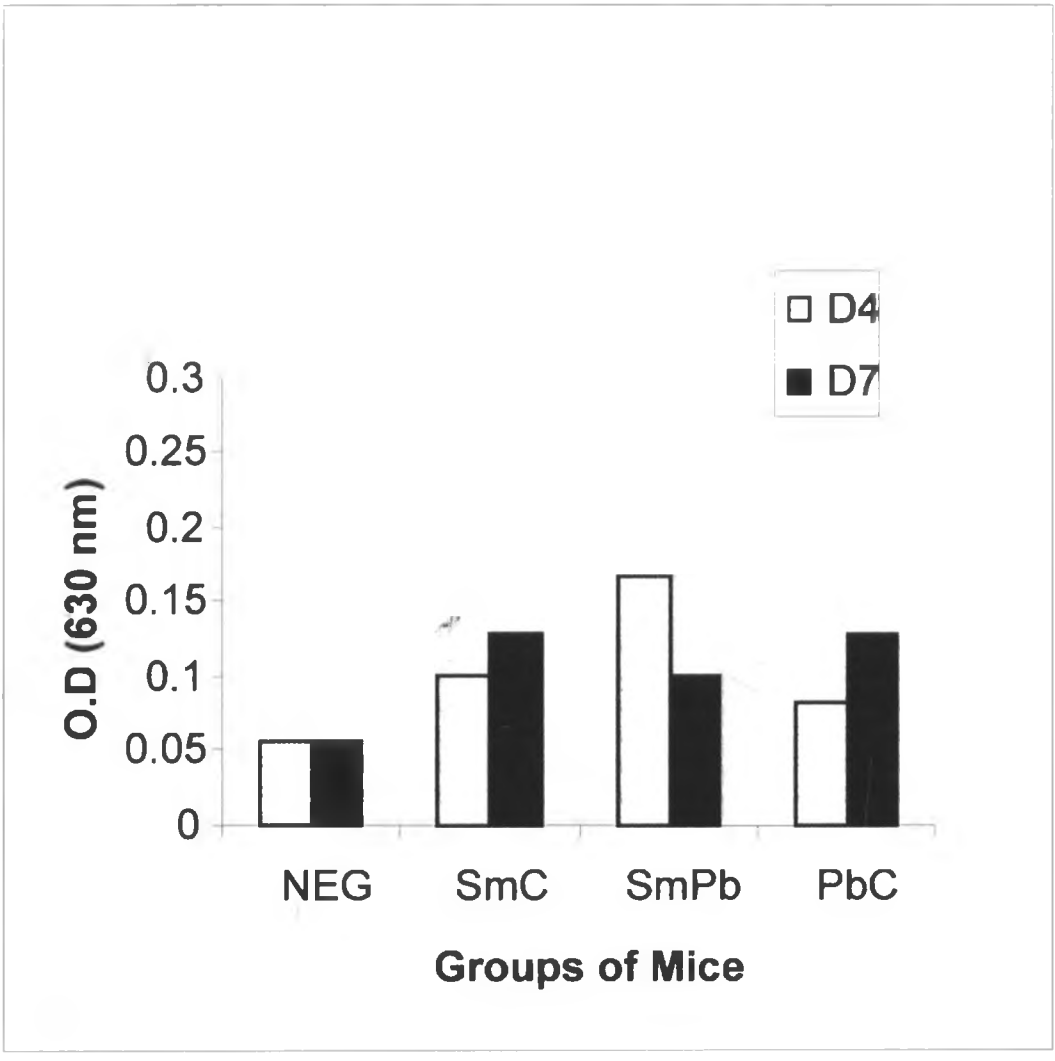

Fig 13: Soluble P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 3)

3.2.5 Crude *P. berghei*–specific IgG responses

Experiment 1: For the group of mice that was super-infected with *P. berghei* at 4 weeks post-infection with *S. mansoni* (Fig.14*)*, The IgG response in the malaria only group was higher at day 4 (O.D 0.122) than at day 7 (O.D 0.104). In the co-infected

group, the response was much higher at day 4 (O.D 0.451) than at day 7(O.D 0.13). In the *S. mansoni*-only group, the response was lower at day 4 (O.D 0.329) than at day 7(O.D 0.409).

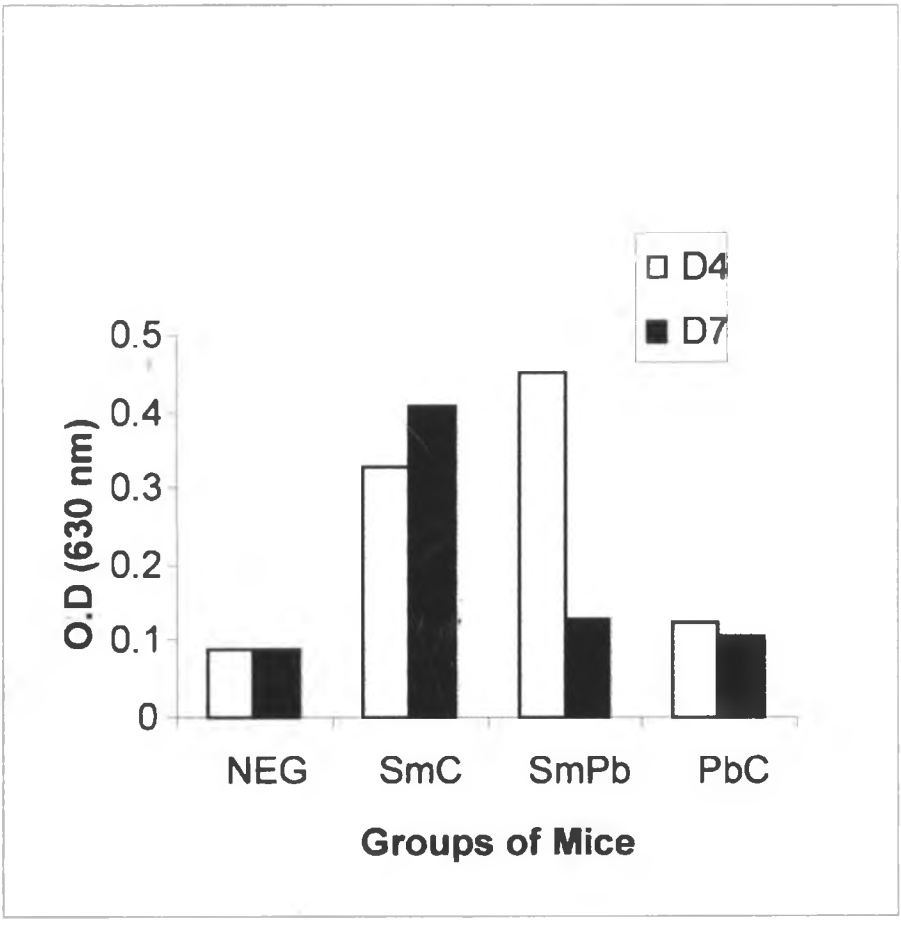

Fig 14: Crude P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 1)

IgG antibody response to Crude *P. berghei* antigen was generally lower in the malaria-only-infected group than it was in the groups having *S. mansoni*, both single and co-infected. This was the same trend recorded in response to the other antigens in the previous experiments. In the co-infected group, the response was higher at day 4 than in the *S. mansoni*-only group. However at day 7, the *S. mansoni*-only group had a higher IgG level than the co-infected group. All the treatments showed a higher response than the negative control (O.D 0.087), just like in the other groups.

Experiment 2: In the group that was super-infected with malaria at 5 weeks after *S. mansoni* infection (Fig.15), the malaria-only infected group showed a lower IgG level at day 4 (O.D 0.143) than at day 7 (O.D 0.158). In the co-infected group, the IgG response on day 4 (O.D 0.227), was higher than at day 7 (O.D 0.163). In the *S. mansoni*-only group, the response was similar on the two days.

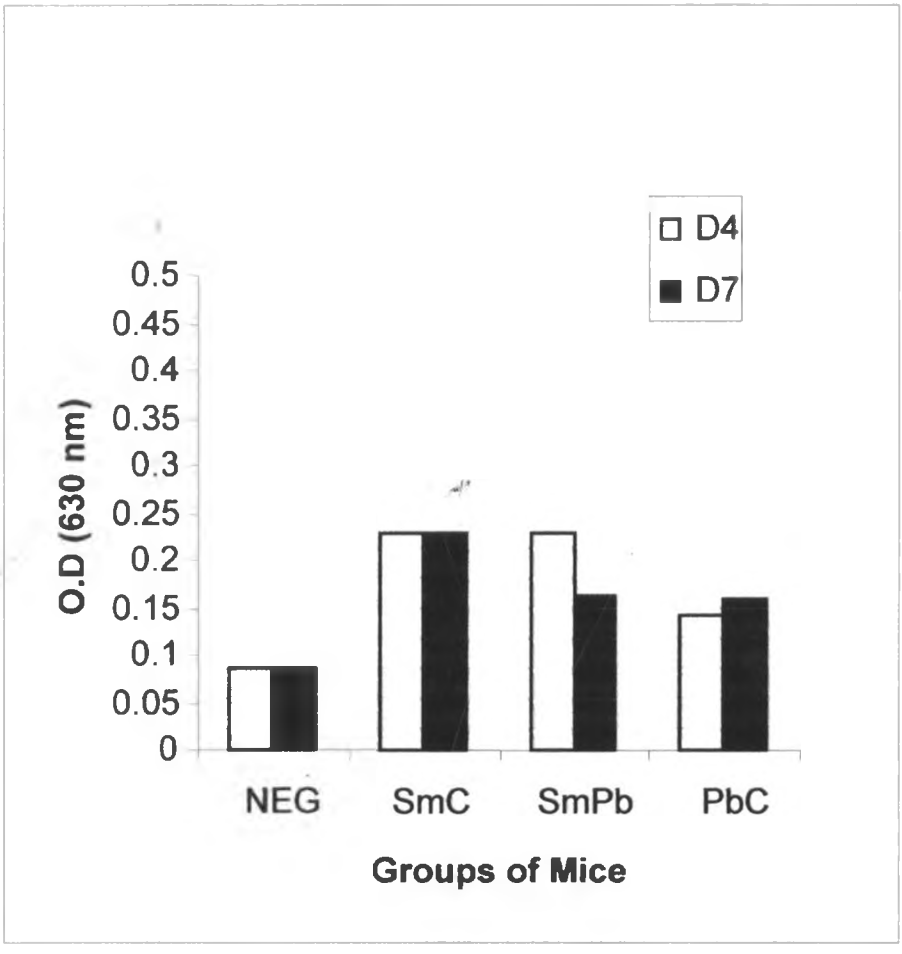

Fig 15: Crude P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 2)

The Crude *P. berghei* -specific IgG levels were generally lower in the malaria-only-infected group than in both groups having *S. mansoni.* In the co-infected group, the IgG response on day 4 was the same as in the *S. mansoni*-only group (O.D 0.227). However, it was lower at day 7 in the co-infected group than in the *S. mansoni*-only

group. The responses for the groups bearing *S. mansoni* were high in Experiment 1 as compared to Experiment 2 except for day 7 of the co-infected group. The malaria-only group recorded a higher response in Experiment 2 than in Experiment 1. The negative control still recorded a lower O.D (0.087), than the rest of the treatments.

Experiment 3: In the group that was super-infected with malaria at 6 weeks post-infection with *S. mansoni* (Fig.16), the malaria control group had a higher response at day 4 (O.D 0.224) than at day 7(O.D 0.173). The co-infected group also showed a higher response at day 4 (O.D 0.294), than at day7 (O.D 0.2). The *S. mansoni*-only group showed a higher response at day 7 (O.D 0.276), than at day 4 (O.D 0.23).

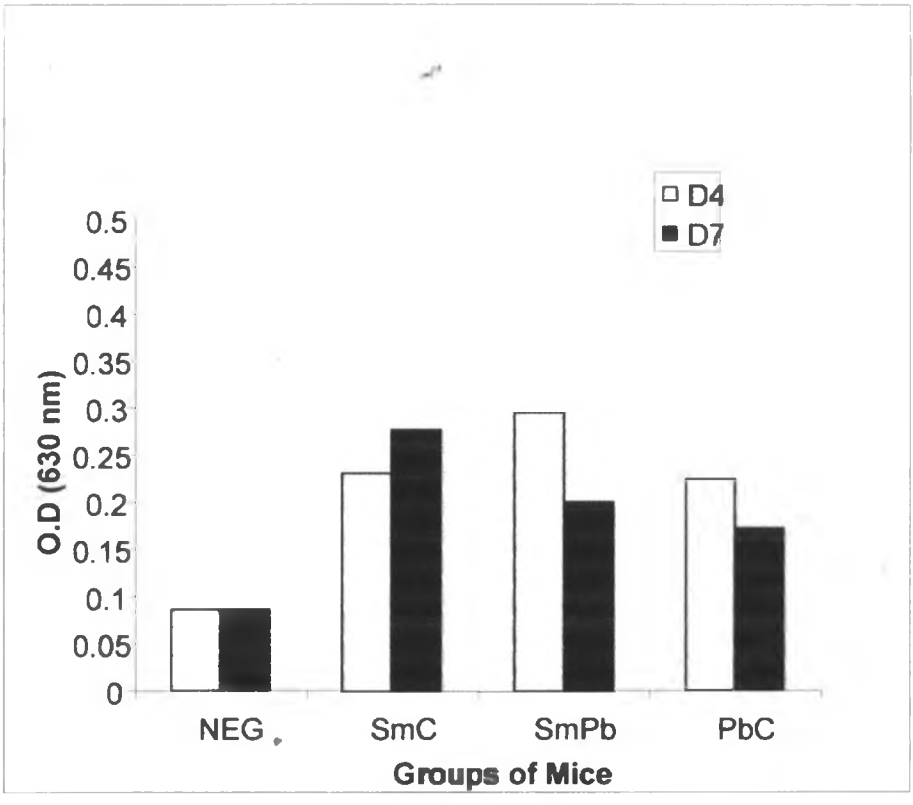

Fig 16: Crude P. berghei antigen specific IgG responses in single and co-infected mice (Exp. 3)

In comparison to Experiments 1 and 2, the responses were more or less similar. The malaria control group still had a generally lower response than the groups with *S. mansoni*. The co-infected group showed a higher response at day 4 than the *S. mansoni* control group and a lower response at day 7. All treatments had a higher response than the negative control group (O.D 0.087).

3.3 Pathological findings

3.3.1 Gross pathology

Gross pathology examination was focused on the general and overt appearance of the liver and spleen. The observations considered in the liver were presence or absence of granulomas, presence or absence of adhesions, and whether the liver was inflamed or not. The spleens were checked for inflammation or lack of it. Granulomas appeared as whitish spots on the surface of the liver lobes. The presence of granulomas affected the color and texture of the liver. The usually smooth surface of the liver was replaced with a rugged surface and the red-pink hue of normal livers turned pale.

Experiment 1: Four weeks after infection with *S. mansoni*, gross pathology of the livers from both the single infected and co-infected groups showed no presence of granulomas though they all showed slight inflammation. The spleens from all the groups also showed some enlargement.

Experiment 2: Five weeks after infection with *S. mansoni,* the spleens from the co-infected group were grossly enlarged, while those with single infections of *S. mansoni* and *P. berghei* showed slight inflammation. The livers of the co-infected mice showed presence of few granulomas while the single infected ones had no visible granulomas. Granulomas appeared as whitish spots on the surface of the liver lobes.

Experiment 3: Six weeks after infection with *S. mansoni*, spleens from the co-infected group were severely inflamed while the single-infected ones showed only slight inflammation. The livers from the co-infected group had moderate (3-10 granulomas) to severe (>10 granulomas) lesions covering almost the entire surface. The livers were enlarged and edematous with adhesions connecting the different lobes. The mice in the *S. mansoni*-only group had few (1-2), to moderate (3-10) granulomas per lobe, and the livers were inflamed and had adhesions. The malaria-only group had slightly inflamed livers.

3.3.2 Histopathological findings

Granulomas appeared as centrally placed schistosome eggs surrounded by immunological cells and fibrotic changes (Fig 18). Apart from the granulomas, the histopathological sections also showed the presence of portal triads (Fig 19) with the accumulation of immune cells around them. Portal triads are collagenous zones in the liver lobules, surrounded by a circumferential layer of hepatocytes. They contain intra-hepatic branches of the bile ducts, hepatic artery, and portal vein.

Granuloma sizes were determined by measuring the length and width of each granuloma with a centrally placed egg, and then getting the average of ten granulomas (Fig 17).

Experiment 1: Four weeks after *S. mansoni* infection, only two out of five co-infected mice, and one out of five single-infected mice showed small granulomas (125 μm). The rest did not show any granulomas. Therefore no graphical representation was given since the granulomas were not enough.

Experiment 2: Five weeks after *S. mansoni* infection, none of the single-infected mice showed the presence of granulomas. One out of five of the co-infected

mice showed a small granuloma (160 μm). Again no graphical representation was given since the granulomas were less than the required ten.

Experiment 3: Six weeks after *S. mansoni* infection, the granulomas were large and florid with a conspicuous egg at the centre (Fig 18). The single- infected group had significantly larger granulomas (323 ± 10.21 μm), than the co-infected group (245 ± 9.081 μm, $P<0.01$) [Fig.17].

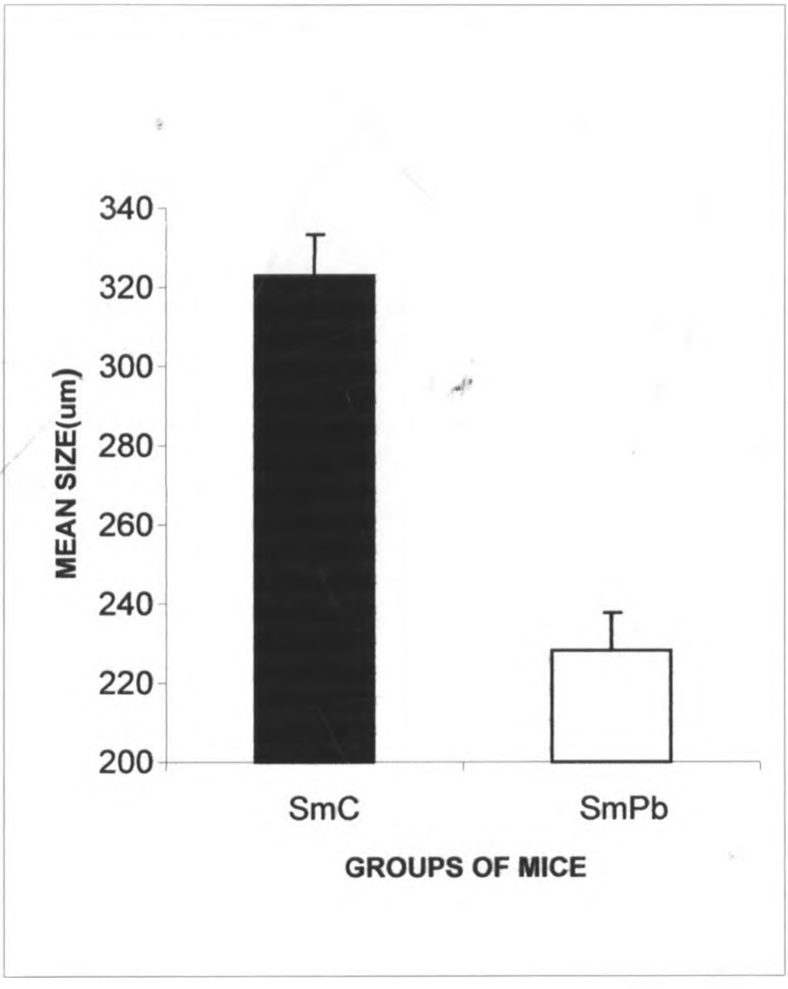

Fig 17: Mean granuloma sizes in single and co-infected mice

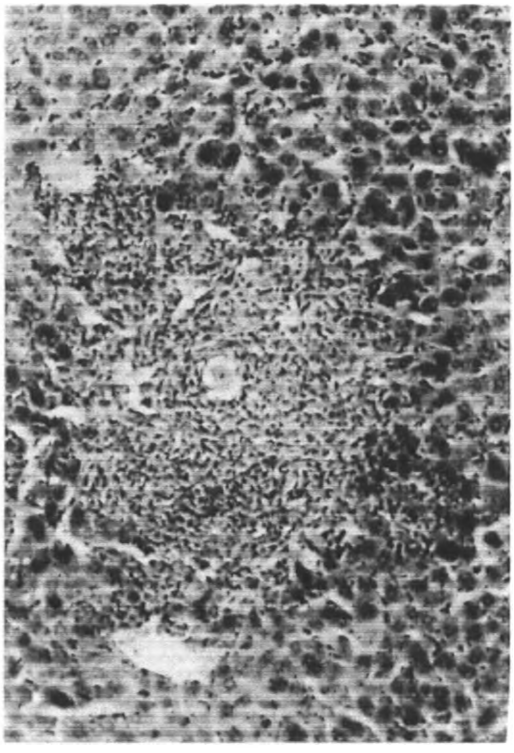

Fig.18 Histological section of the liver showing granuloma from infected BALB/c mice in the single-infected group. The micrograph was taken at x25 magnification.

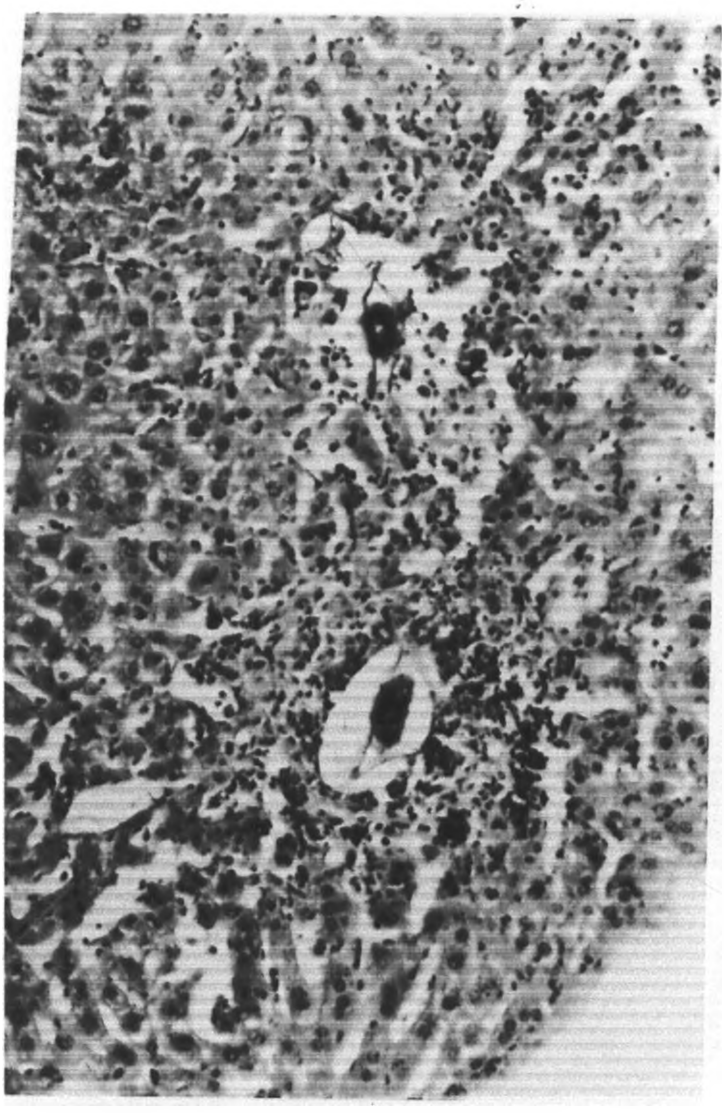

Figure 19: Histological section of the liver showing granuloma from infected BALB/c mice in the co-infected group. The micrograph was taken at x40 magnification.

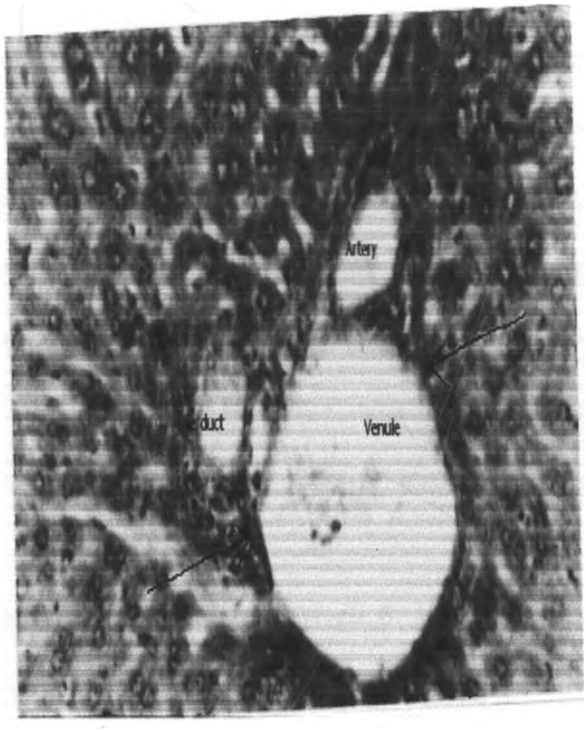

Fig. 20: Histological section of the liver (from co-infected mice) showing a portal triad with cellular infiltration around the vessels. The arrows point at some of the cellular infiltration. The micrograph was taken at x40 magnification.

CHAPTER 4. DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Parasitemia

In mice super-infected with malaria at week 4 post *S. mansoni* infection, the co-infected mice had significantly higher parasitemia than the single infected ones ($p<0.01$). In mice super-infected with malaria at week 5 post *S. mansoni* infection, the co-infected mice showed lower percentage parasitemia than the single infected ones.

This might however not be the true indication of the results since some of the mice in the co-infected group died before sampling. The results given are therefore based on only three mice that remained in the co-infected group, versus five in the single infected group. The deaths could have been caused by severe anemia associated with increased malaria parasitemia. More studies may be required in order to elucidate the mortality factor.

In mice super-infected with malaria at week 6 post *S. mansoni* infection, the parasitemia in the co-infected group was higher than in the single infected group though the difference was not significant ($p>0.05$). In all the experiments, the parasitemia at day 4 was lower than at day 7 in both the single and co-infected mice. This shows that parasitemia increases with time.

Mice carrying a patent *S. mansoni* infection and infected with blood-stage *P. berghei* parasites developed a more rapid and severe course of malaria, indicative of a defect in the initial control mechanism. A low but consistent number of co-infected mice died during each experiment. The increased malaria parasitemia in the co-infected mice could be due to a defect in the capacity of the macrophages to produce TNF-α, known to reduce malaria parasitemia in both mice and humans and to enhance

survival in mice (Jacobs *et al.*, 1996). Another likely explanation for the increased parasitemia seen in the co-infected mice might be an *S. mansoni*-induced suppression of macrophage activation, probably through IL-10 and possibly also IL-4 and/or transforming growth factor β (Oswald *et al.*, 1992). This would lead to an inability of the macrophages to respond to IFN-γ and thus a defect in their capacity to kill parasitized red blood cells at an early stage.

A number of previous studies have also demonstrated higher parasitemia and severe conditions in mice co-infected with various strains of rodent malaria and other parasites than mice infected with plasmodium alone. A study by Yoshida *et al.*, (2000) demonstrated higher susceptibility to *P. chabaudi*, increased mortality and elevated *P. chabaudi* parasitemia in *S. mansoni-P. chabaudi* co-infected resistant strain mice than mice infected with *P. chabaudi* alone. Helena *et al.*, (1998) also observed remarkably higher parasitemia in *S. mansoni-P. chabaudi* co-infected mice, than in mice infected with *P. chabaudi* alone. Strickland *et al.*, (1972) observed higher mortality and persistent parasitemia in mice co-infected with *Toxoplasma gondii* and *P. berghei yoelii* than in mice infected with *P. berghei yoelii* alone.

It is therefore of paramount importance to consider schistosome infections in clinical and therapeutic management of malaria patients in areas where the two diseases are co-endemic.

4.1.2 Worm Count

Worm loads after perfusions were subjected to Student's t-test. At week 4, the *S. mansoni*-only group had a higher worm load than the co-infected group. The difference in worm maturation between the co-infected and single-infected mice was however not significant ($P>0.05$).

The worm count in the groups co-infected at week 5 and 6 also revealed that the single-infected mice had significantly higher worm loads ($P<0.05$) than the co-infected ones. There was also a general increase in the number of worms present in both groups over time. The one week delay between super- infections seemed to contribute to this increase in the number of worms.

These results clearly show that malaria infection reduces the number of worms in the host during a co-infection with *S. mansoni.* The presence of malarial parasites in the co-infected group could have had an effect on the immune response of the host against *S. mansoni.* The high antigen-specific IgG levels seen in co-infected mice might have led to inhibited maturation, establishment, and/or death of the worms.

The wide array of antigens presented by the malaria and helminth parasites may also have triggered non-specific antibody responses which contributed to the death of worms. This shows that a malaria infection would confer an advantage to the co-infected host by reducing the number of worms that would establish in the host although there are known complications like anemia due to malaria..

To the knowledge of the author, no data has been published on the effect of a co-infection on worm loads. However, Ashford *et al.*,(1992) showed a positive correlation between egg load of *Ascaris lumbricoides* and *Plasmodium* density, while Briand *et al.*, (2005) observed no association between intestinal helminth infection (*A. lumbricoides, Hymenolepis nana, Strongyloides stercoralis*, and *Trichuris trichiura)* and *P. falciparum* density.

4.1.3 IgG Responses

The effects of concomitant infections on the development as well as the maintenance of an immune response remain largely unknown (Helena *et al.*,1998). The murine models of *P. berghei* and *S. mansoni* have been well studied and

characterized in terms of both cell-mediated immunity and humoral immunity. Thus, BALB/c mice should be ideal for studying the interaction between two different parasitic infections with regard to parasite-specific immune responses. Little information regarding concurrent *S. mansoni* and malaria infection has been published (Helena *et al.*, 1998).

The immune response to patent *S. mansoni* infection is generally directed into a systemic Th2 type of response at the onset of egg production, with elevated production of IL-4, IL-5, and IL-10 in response to SEA as well as non-parasite antigens (Grzych *et al.*, 1991).

The present study was undertaken to investigate the effect of an *S. mansoni*-directed IgG response on the development of a protective IgG response to *P. berghei* in mice, and vice versa. In this study, 4-week, 5-week, and 6-week *S. mansoni*-infected mice were challenged with *P. berghei* and the IgG response monitored during the development of the malaria infection. Serum antibody analysis was done using the antigens 0-3 hr, SWAP, soluble *P. berghei*, and crude *P .berghei*.

The results revealed that co-infected mice exhibited a high IgG response as compared to the malaria-only and *S. mansoni*-only-infected groups. The malaria-only-infected groups showed the lowest IgG response in all the weeks. In the responses to the different antigens, there is no clear difference between day 4 and day 7. These findings indicate that there is no general decrease in B-cell activity in the co-infected mice. This might suggest that malaria infection generally boosts the IgG response against *S. mansoni.* The low levels of soluble *P. berghei* and crude *P .berghei* antigen-specific IgG levels in the malaria-only-infected mice could be suggestive of a strong immune suppression seen during malaria infection. The decrease in circulating soluble *P. berghei*, and crude *P .berghei*, antigens-specific IgG, may be due to

reduced IFN-γ production, known to influence B-cell differentiation and immunoglobulin production (Snapper and Paul, 1987), or an increased turnover of plasma cells or antibodies.

This high immune response in co-infected mice is in line with an observation by Diallo *et al.*, (2004). They observed that the level of IFN-γ, a pro-inflammatory cytokine, was significantly higher in children and adults, co-infected with *S. haematobium* and *P. falciparum.* Helena *et al.*, (1998) in a co-infection with *S. mansoni* and *P. chabaudi* also observed that malaria-specific IgE response was not induced following a single infection with malaria, but that the response was induced either by repeated exposure to the parasite or during a co-infection with schistosomiasis. Mutapi *et al.*, (2000) reported that children with schistosomiasis who were also infected with malaria parasites had higher levels of schistosome-specific IgG3 than did children who were free of malaria. They suggested that a plausible explanation for this would be that malaria infection influences the cytokine environment and generally favors the production of IgG3.

The observation that mice infected with *S. mansoni* had responses towards malaria parasites indicates cross-shared antigens between schistosome and malaria (Yole *et al.*, 1996). This was also evident in *P. berghei* single infection though at a lower magnitude as compared to *S. mansoni.* The occurrence of cross-reactive antibodies to *P. berghei* and *S. mansoni* has not been reported elsewhere, although Naus *et al.*, (2003) reported cross-reactivity between *P. falciparum* and *S. mansoni.*

Data from the present study demonstrate that a murine *P. berghei* malaria infection clearly affects the *in vivo* IgG response to *S. mansoni* antigen in mice with a patent *S. mansoni* infection. Following the results on immunological findings, it is evident that malaria has got a protective effect against schistosomiasis. This implies

that if a patient with a concomitant malaria- *S. mansoni* infection is treated for malaria only, there is the risk of aggravating the helminth infection. Hence, concurrent schistosome infection should be considered before treatment is started and if present, both diseases should be treated simultaneously.

4.1.4 Gross Pathology

In this study, the general and overt appearance of the liver and spleen was observed in all groups of mice (single-infected and co-infected), at 4, 5 and 6 weeks post-infection with *S. mansoni.*

At week 4, the liver and spleen were slightly inflamed but no granulomas were seen. This was just the beginning of egg-laying and therefore only a low grade immune response had been mounted. This explains the absence of granulomas and the presence of slight inflammation in the liver and spleen.

At week 5, the livers and spleens of the co-infected mice were grossly enlarged and a few granulomas were present, while those of the single infected ones showed only slight inflammation and no visible granulomas. This indicates that the combined effect of malaria and schistosomiasis elicited a greater immune response than the separate single infections, thus the gross inflammation and presence of granulomas in the co-infected group.

At week 6, the inflammation of the livers and spleens was severe in the co-infected group, with an equally severe case of granulomas. The single infected ones were less inflamed and had few to moderate granulomas. The results showed that the severity of inflammation and granulomas increased over time. This could again be explained in terms of increased immune responses due to the wide array of antigens exposed to the host by the parasites.

Evidently, malaria is aggravating the gross pathology, but reduces the granuloma sizes. The increase in the humoral immune responses as evidenced by the increased IgG levels and increased inflammation seems to counter cellular responses against schistosome eggs, thus reduced granuloma sizes.

Booth *et al.*, (2004) observed in children that the degree of splenomegaly was greatest among children living in an area where both *S. mansoni* egg counts and IgG3 responses to malaria schizont antigen were significantly elevated. The children also had hardened spleens which were associated with relatively high egg count and a greater degree of splenomegaly. The study suggested that children with relatively high levels of exposure to *S. mansoni* and malaria were worse affected in terms of spleen enlargement. This observation lends support to the present study.

4.1.5 Histopathology

In this study, hepatic granulomas were measured from two groups of mice (single-infected and co-infected), at 4, 5 and 6 weeks post-infection with *S. mansoni*

At week 4, only one single-infected mouse and two co-infected ones were found to have granulomas. This could have been because the egg laying had just commenced, therefore granuloma formation had also just been initiated on the few eggs. Egg deposition begins about 4 to 5 weeks after infection, with detectable granulomas present by about 6 weeks.

At week 5, none of the single-infected mice showed presence of granulomas, but one of the co-infected mice did. This suggests that the presence of malaria during an *S. mansoni* infection leads to an early initiation of granuloma formation. It also suggests that malaria boosts the cellular response against *S. mansoni*.

At week 6, there was an increase in number of granulomas as well as a significant increase ($P<0.01$) in size of granulomas in the single-infected group as

compared to the co-infected ones. Concurrent *S. mansoni- P. berghei* infection led to reduced granuloma sizes. *P. berghei* seems to modulate granuloma sizes in co-infection which suggests that malaria boosts the cellular response against *S. mansoni in vivo*. This agrees with a report that *P. yoelii* infections reduced granuloma formation in the lungs of mice injected with *S. mansoni* eggs (Abdel-Wahab *et al.*, 1974). Furze *et al.*, (2006) also noted that co-infection of mice with influenza virus during the early phase of trichinosis resulted in a reduced inflammatory infiltrate in the lungs (pulmonary pathology). They explained that the pulmonary pathology and cellular infiltration observed in their study could be due to a variety of factors, such as immune exhaustion as a result of ongoing intestinal inflammation.

4.2 Summary

The present study shows that co-infection with *P. berghei* and *S. mansoni* in mice favored rapid *P. berghei* development and high parasitemia. It also indicates that a malarial infection may influence granuloma formation in co-infected patients. *P. berghei* in the co-infected mice reduced the worm counts as well as the severity of histopathology. It therefore shows that *P. berghei* reduces the severity of *S. mansoni* infection. Fewer worms in the host means that fewer eggs will be laid and conceivably less pathology. Reduced pathology means reduced severity of schistosomiasis.

On the other hand, *S. mansoni* exacerbates malaria parasitemia. A patent *S. mansoni* infection does not seem to inhibit the *P. berghei* Th1 response but may render macrophages unresponsive. This observation has important public health implications since people in endemic areas in many parts of Africa, are at risk of mixed parasitic infections. It is therefore important to consider intestinal helminthic

infections (and as per this study *S. mansoni*) in clinical and therapeutic management of malaria patients in areas where the two diseases are co-endemic.

Animal studies will help define further the components of the immune response that are crucial for helminth-plasmodium interaction and help identify single molecules from helminths that dampen immune responses to plasmodium and vice versa. For instance, some molecules with immunoregulatory properties have been identified in filariae (Marshall *et al.*, 2005) and schistosomes (Chatterjee and Van marck, 2001). If helminth co-infection allows higher plasmodium loads at lower levels of pathology, what are the practical consequences for control of helminth and malaria infection for human beings? As the world is engaged in integrated efforts to control several helminth infections simultaneously (WHO, 2005), one would not consider stopping helminth control on the basis of the available co-infection data.

However, the data lend support to the integration of more field studies into helminth and malaria control programmes. These studies should assess malaria pathology by use of relevant endpoints such as anemia and cerebral malaria, and should also assess helminth control. If the field studies show that concomitant helminth infections have a benefit against malaria pathology, and that this benefit would become unavailable to human beings infected with plasmodium after schistosome elimination, the call is even stronger to integrate malaria with helminth control. Only a concerted approach should be pursued 'if we do not want to rob Peter to pay Paul'.

4.3 Conclusion

It was found out from this study that *P. berghei* infection reduces the severity of *S. mansoni* infection, by reducing the worm load as well as the granuloma sizes. Even

though inflammation of the liver and spleen seems to be exacerbated by the malaria infection; the end result is reduced severity of *S. mansoni* infection.

S. mansoni worsens malaria infection as shown by the increased parasitemia in the co-infected mice as well as severe gross pathology in the co-infected mice. It was also demonstrated that concurrent infection with the two parasites, a helminth and a protozoan, alters the host's IgG response.

4.4 Recommendations

1. More field studies should be integrated into helminth and malaria control programmes.

2. Intestinal helminthic infections (and as per this study *S. mansoni*) should always be considered in clinical and therapeutic management of malaria patients in areas where the two diseases are co-endemic and vice versa. If both diseases are present concurrently, they should be treated simultaneously.

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APPENDIX 1

Determination of protein concentration (Dye-binding method).

Principle.

The Bio-Rad protein assay, based on the method of Bradford (1976) is a simple and accurate procedure for determining concentration of solubilized protein. Acidic and basic groups of proteins interact with the dissociated groups of organic dyes to form colored precipitates. Bradford developed the assay by using Coomasie brilliant blue G-250, which is an acidic dye that binds to proteins. The dye binds primarily to basic and aromatic amino acid residues, especially arginine. The maximum absorbance for the dye shifts from 465 nm (red form) to 595 nm (blue form) when binding to protein occurs. Comparison to standard curve provides a relative measurement for protein concentration.

Procedure.

The dye reagent (Bio-Rad protein assay, Germany) was prepared by mixing the dye and distilled water in the ratio 1:4. Standard protein (Neat Bovine Serum Albumin), SWAP (soluble antigen), and a blank were prepared for the assay. The tube for the blank contained PBS and dye reagent only. Five tubes were used for the standard group. Tube 1 contained 100 µl of neat BSA and 100 µl of PBS. Serial dilution of the mixture was done by transferring 100 µl into test tubes 2,3 4 and 5, which already contained 100 µl of water. The test sample SWAP was also mixed with PBS and the resulting mixture serially diluted in to five other tubes. In to each tube was added 5 ml of the dye reagent and mixed thoroughly. The tubes were incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm against a blank prepared from a mixture of 0.1M of sample buffer and 5 ml of dye reagent. The standard readings were printed out and the curves used for comparing concentration.

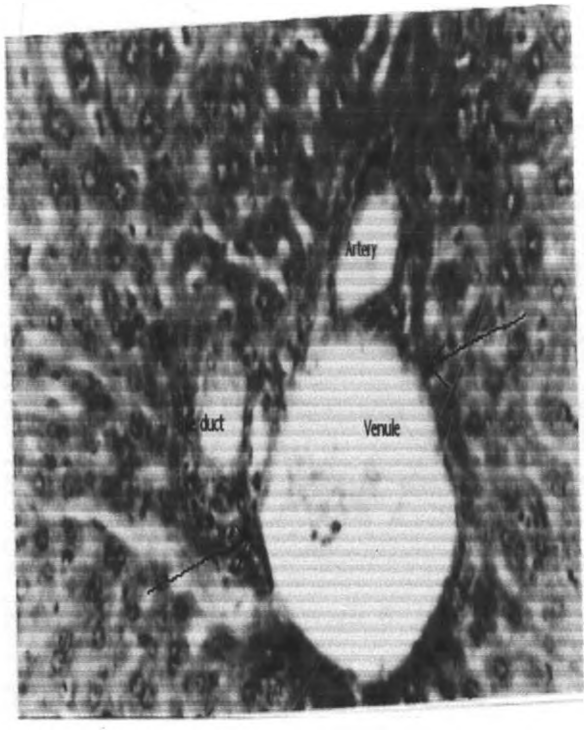

Fig. 20: Histological section of the liver (from co-infected mice) showing a portal triad with cellular infiltration around the vessels. The arrows point at some of the cellular infiltration. The micrograph was taken at x40 magnification.