

PRELIMINARY INVESTIGATION ON IMMUNE
RESPONSE OF *SCHISTOSOMA MANSONI* IN GRIVET
MONKEYS (*CERCOPITHECUS AETHIOPS AETHIOPS*)
INFECTED WITH DIFFERENT CERCARIAL DOSES

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TARIKU DEMELASH

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9. Declaration

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“This thesis is my own work, it has not been presented as a thesis work for a degree in this or any other University and that all sources of material used in this thesis have been duly acknowledged.”

This thesis has been submitted for examination with my approval as University advisor

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LIST OF ABBREVIATIONS

B7-CD28	B cell cluster of differentiation 28
B7RP-1-ICOS	Inducible co-stimulator-B7RP-1
BSA	Bovin serum albumin
CD 154	Cluster of differentiation 154
CD1	Cluster of differentiation 1
CD4	Cluster of differentiation 4
CIC	Circulating immune complex
CPA	Cercariae per monkey
DC	Dendritic cells
ECMP	Extracellular matrix proteins
ELISA	Enzyme linked immunosorbent assay
EPG	Egg per gram stool
ES	Excretory secretory
IC	Immune complex
ICAM-1	Intra cellular adhesion molecule-1
IF	Immuno-fluorescence
IFN-g	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IL-10	IL-10 Interleukin-10
IL-12	Interleukin-12
IL-12 Rb2	Inerlukin-12 receptor b2
IL-13	Interleukin 13
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-23	Interleukin-23
IL-27	Interleukin-27
IL-4	Interleukin-4

IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
NKT cells	Natural killer T cells
NO	Nitric oxide
PAMP	Pathogen associated microbial patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
Pi	post infection
pNPP	Para-nitro phenyl phosphate
PPF	Periportal fibrosis
SEA	Soluble egg antigen
SG	Schistosomal glomerulopathy
SPSS-11.5	Statistical package for social scientists version 11.5
SWAP	Soluble worm antigen preparation
TGF- β	Transforming growth factor-beta
Th1	T helper 1
Th2	T helper 2
TIMP	Tissue inhibitors of metalloproteases
TMP	Tissue metalloproteases
TNF-a	Tumour necrosis factor alpha

ABSTRACT

In order to evaluate grivet monkey (*Cercopithecus aethiops aethiops*) as an animal model for the immune response of mansonian schistosomiasis, groups of three or four grivet monkeys were exposed individually to 100, 200, and 300 cercariae per monkey. Infection and clinical manifestations were observed; EPG of stool was determined and different cytokines (IL-12, IFN- γ , TNF- α , IL-4 and IL-10) were measured. Infected monkeys began to release eggs 7 weeks post infection. Most of them became ill 7 to 9 weeks after initial exposure and exhibit most of the clinical manifestations typical of human acute schistosomiasis. The fecal egg count confirmed there is significant ($P<0.05$) variation between groups in terms of numbers of eggs released in stools, where the group that receives the lowest cercariae dose exhibiting the highest egg output. There was a significant difference in the level of cytokine production between those infected and the controls. Th1 cytokine (IL-12, INF-gamma, and TNF-alpha) levels reached their highest before their Th2 counterpart (IL-4). Cytokine levels, however, were not found to be dose-dependent as anticipated. Comparison of this study with studies on related primates suggests that grivet monkey closely resemble the baboons in their immune response to *Schistosoma mansoni* infections. Thus, this work may underline the potential of the grivet monkey to provide a cheaper, more readily available primate model for the study of the immune response and the acute stage of infection by *S. mansoni*.

1. INTRODUCTION

1.1. Background

Schistosomiasis, also called bilharziasis (after the German physician Theodor Bilharz), is one of the world's oldest helminthic diseases. It ranks high among parasitic diseases in terms of socioeconomic and public health importance in tropical and subtropical areas. Schistosomiasis is a major health risk in the rural areas of central China and Egypt and ranks high in other developing countries

Schistosomiasis is mainly a rural occupational disease that affects people engaged in agriculture or fishing. In many areas, a large proportion of children are infected by the age of 14 years; in other areas, women face the highest risk because of domestic and occupational contact with fresh water. Increased population movements help to propagate schistosomiasis, as evidenced by its introduction into an increasing number of periurban areas of northeastern Brazil and Africa and among refugees in Somalia, Zimbabwe and Cambodia.

The disease is known to be caused by five species of the parasitic trematodes belonging to the sub class Digenea: the intestinal species *Schistosoma mansoni*, *S. japonicum*, *S. mekongi*, and *S. intercalatum* and the urinary species *S. haematobium*.

1.2. Epidemiology

Schistosomiasis is the second most important parasitic disease in the tropics next to Malaria. At least one form of schistosomiasis is now endemic in 74 tropical developing countries (Chitsulo *et al.* 2000). It is estimated that over 200 million people residing in rural agricultural and periurban areas are infected, of whom 120 million are symptomatic and 20 million have severe disease (Chitsulo *et al.* 2000). An estimated 500–600 million people in the world run the risk of becoming infected as a result of poverty, substandard hygiene in poor housing, and inadequate public infrastructure (Chitsulo *et al.* 2000). The disease is more prevalent in Africa than any other part of the world; Africa accounting for more than 85% of the total number of patients.

1.2.1 Epidemiology in Ethiopia

Schistosomiasis is also one of the most important parasitic diseases in Ethiopia. Temperature appears to be the major factor that affects the distribution of both *Schistosoma species* (*S. mansoni* and *S. haematobium*) in Ethiopia. *S. mansoni* is found mainly at altitudes between 1200-1900m. There is a sharp increase at about 1300m and sudden fall around 2000m indicating the lower and upper altitudinal limits of its transmission. The parasite, however, has also been recovered from both human and intermediate hosts at Abobo and Pigindo, Gambella (500m) and Desse (1248m) while *S. haematobium* is found at lower altitudes (300-850m) (Eshete and Tsehai, 1989).

Most of the major valleys in Ethiopia are believed to be endemic to schistosomiasis. For example, schistosomiasis is locally prevalent in the Omo Valley (Fuller *et al.*, 1979). It has been repeatedly reported from the Awash Valley (Birre, 1986). Other foci were discovered gradually in areas of great agricultural potential: in the Baro Basin (Mekasha, 1982), in the Beles Valley (Tedla and Jemaneh, 1988) and in the Wabi-Shebelle Valley (DeSole *et al.*, 1978). Prevalence studies done in as many parts of Ethiopia as north western Ethiopia (Teklehaimanot and Fletcher, 1990), north east Ethiopia (Birre *et al.*, 1998), north Ethiopia (Jemaneh, 2000), and South Ethiopia (Erko *et al.*, 2002) indicate an over all prevalence that range from as low as 16.4% in north Ethiopia (Jemaneh, 2000) to as high as 86% in north eastern Ethiopia, namely Kemise (Birre *et al.*, 1998); the prevalence being higher among children of age between 10-14 years of age. Over all an estimated 29.89 million people in Ethiopia are at risk of acquiring infection and of these 4 million are infected (Chitsulo *et al.* 2000). Now, with the rise in population and the expansion of dams and irrigation schemes in schistosomiasis endemic areas, the figure is expected to be higher.

1.3. Life Cycle of *Schistosoma mansoni*

Human infection by *S. mansoni* is initiated by penetration of intact skin with infective cercariae. These organisms are released from infected snails in fresh water bodies: once in the subcutaneous

tissue, the cercariae transform into the next stage: the schistosomula. Schistosomula begin their migration within 2 to 4 days via venous or lymphatic vessels, reaching the lungs and finally the liver parenchyma. Sexually mature worms descend in pairs into the venous system at intestinal veins. Adult gravid females then travel against venous blood flow to small tributaries where they deposit their ova intravascularly.

Aided by enzymatic secretions through mini pores in eggshells, ova move through the venous wall, traversing host tissues to reach the lumen of the intestinal tract, and are voided with stool or urine. Many eggs, however, fail in their attempt to be transported to the outside environment and are either retained in host tissues locally (intestines or urinary tract) or carried by venous blood flow to the liver and organs. Schistosoma ova that reach fresh water bodies hatch, releasing free-living miracidia.

The miracidium searches for a suitable fresh water snail (*Biomphalaria Sp.*) to act as an intermediate host and penetrates it. Following this, the parasite develops via a so-called mother-sporocyst and daughter-sporocyst generation to the cercaria. From a single miracidium results a few thousand cercaria. The cercariae merge from the snail during day light and they propel themselves in water with the aid of their bifurcated tail, actively seeking out their final host, human.

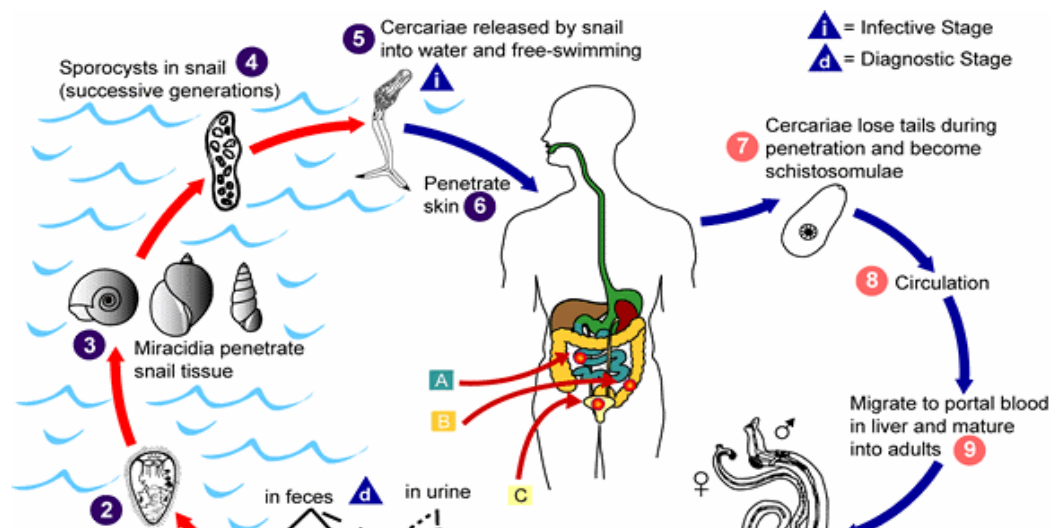


Fig.1 Life Cycle of *S. mansoni*

Source: <http://www.dpd.cdc.gov/dpdx/html/schistosomiasis.htm> accessed June, 2 005.

1.4. Clinical Presentation of Schistosomiasis by S .mansoni

In the definitive mammalian host, eggs that fail to be transported to the outside environment are either retained in host tissues locally (intestine or urinary tract) or are carried by venous blood flow to the liver and the lungs and other organs and are

surrounded by the granulomatous inflammatory responses of the host (Cheever *et al*, 1994b). In the same way, the variety of antigens secreted by the worms or shed during the different developmental stages of the worm life cycle (cercariae, schistosomula, adult male and female and eggs) provide strong, sustained stimuli to the host humoral and T- cell mediated immune systems. This resulted in a vigorous host response mounted on parasite eggs and antigens, which may lead to the development of pathologic events that characterize the different stages of the disease.

1.4.1 Dermatitis

This is a type of skin reaction that develops within a few hours after infection. Schistosome infection is initiated after cercariae penetrate through the skin and transform into schistosomula, the larval form suited for existence in the host milieu. Infected individuals exposed to *S. mansoni* cercariae experience itching within 1 hr of water contact (Amer, 1982). In mild cases, a skin rash appears that have rounded, discrete, and 1 to 2mm erythematous papules. The itchy sensation and papular eruption may take days to disappear, and the healed papules may leave behind a pigmented spot. Severe dermatitis is manifested by an evenly distributed confluent papular reaction. If papules are infected with bacteria, pustules will appear (Amer, 1982).

Dermatitis is common in migrants or tourists that come from a place where the disease is not endemic, or who have got the infection for the first time. The dermatitis is similar to, but less severe than, swimmers' itch, which develops in sensitized persons when they are re-infected by species of schistosomes that do not colonize human -usually the types that colonize birds (Baird and Wear, 1987).

1.4.2. Acute schistosomiasis

This includes clinical manifestations that often occur from 4 to 8 weeks after infection. It is a serum sickness, katayama fever like syndrome with symptoms as varied as fever, malaise, diarrhea, intense eosinophilia and occasionally allergic manifestations such as asthma or angio edema hives (Lambertucci; 1993, Lambertucci *et al.*, 1997; Rabells *et al.*, 1997). Symptoms may appear before the onset of oviposition and are accentuated after oviposition.

Acute schistosomiasis is a severe disease, and the mechanisms involved in its clinical manifestations are not completely understood. Both immune and non-immune mechanisms may participate in the pathogenic process. Experiments in vitro demonstrated that schistosomula directly activate the alternate pathway of complement (Santoro *et al.*, 1979). Thus, conceivably, the antigens released in large amounts during the migration, maturation, or death of schistosomula may trigger serum complement that results in one's anaphylactic reactions. Because IgE antibody levels are elevated at the acute phase of the infection and may correlate positively with the magnitude of the infection (Haitt *et al.*, 1979), newly infected individuals may develop anaphylactic reactions following parasite antigen - induced degranulation of IgE coated mast cells (Otteson *et al.*, 1981). Clinical manifestation seen in patients i.e. skin rash, eosinophilia, pulmonary edema or diarrhea, are consistent with this conception (Fallon, 2000).

Acute disease is not common in residents of the endemic areas, which is presumably because of their pre -and postnatal exposures to schistosome antigens via transplacental, lactation, and repeated infections at an early age (Montesano *et al.*, 1999a; Montesano *et al.*, 1999b; Abdelfattah *et al.*, 2003).

1.4.3. Chronic Schistosomiasis

This is the stage of the infection which is believed to occur 10 weeks and above after infection and may last for years. The stage is characterized by intestinal and hepatosplenic diseases, as well as several manifestations associated with portal hypertension. During the intestinal phase of the disease, which may begin a few months after the infection and may last for years, symptomatic patients characteristically display colicky abdominal pains and bloody diarrhea (Mahmoud, 2001).

Patients may report fatigue and an inability to perform daily routine functions. The severity of intestinal manifestations is often related to the intensity of worm burden.

The hepatosplenic phase may manifest early, during the first year of infection particularly in children. It is usually, but not invariably, associated with enlargement of the liver and spleen, and reversible hepatosplenomegaly may occur in early infestations not complicated by the development of portal hypertension (De Cock, 1986). In subsequent phases of infection, presinusoidal blockage of blood flow leads to portal hypertension and splenomegaly (Mahmoud, 2001). Portal hypertension might lead to varices at the lower end of the esophagus and at other sites. Bleeding from esophageal varices may, however, be the first clinical manifestations of this phase, and patients may experience repeated bleeding.

In the last stage of the disease, typical fibrotic changes occur along with liver function deterioration and the onset of ascites, hypoalbuminemia and defects in coagulation (Mahmoud, 2001). The liver may gradually decrease in size, but increases in hardness as fibrosis is gradually extended into the parenchyma, resulting eventually in liver cirrhosis in severe cases. The enlarged spleen may reach the level of the umbilicus or even at times expand to fill most of the abdomen (DeCock, 1986). Features of hepatocellular failure, ascites often being the most obvious clinical sign, may complicate the end stage hepatosplenic schistosomiasis. Nutritional deficiencies and concurrent viral infections of the liver may as well accelerate or exacerbate the deterioration of hepatic function.

1.5. Diagnosis

Identifying human schistosome infections is crucial at all stages of schistosome control programmes, and for comparing control programmes. Diagnostic methods are the key to such comparisons and, not surprisingly, a great many tests have been developed over the years.

1.5.1. Parasitological diagnosis

Adult worms lie hidden in the blood system and parasitological diagnosis therefore serves as a good evidence of their presence within a patient, relying on the detection of eggs in faecal samples. It is, though, absolutely specific because an egg is a direct proof of infection. Today, the most widely used methods are the Kato thick smear (Katz *et al.* 1972) and the Nuclepore filtration techniques for faecal and urine examination, respectively.

A standard, 50 mg Kato thick smear has a detection threshold of 20 eggs per gram (epg) of faeces (1 egg per slide): smaller Kato smears have higher thresholds e.g., 50 epg for a 20 mg smear. Nuclepore filtration is conventionally used to examine 10 ml of urine collected between 11.00 and 14.00 h at the peak of the diurnal egg count cycle. There is considerable day-to-day variation in egg counts, both in urine and faecal samples and there is little point trying to estimate absolute worm burden from egg counts for an individual because there are so many possible sources of variation. However, classification of egg counts from a *community* on a relatively coarse scale will detect overall trends after a control intervention: in this context, egg counts are a quantitative, or at least a semi-quantitative, measure of infection – a few eggs indicating a few worms and a lot of eggs, a lot of worms. The main deficiency of egg counts is missing very light infections at or below the threshold of detection. For quantitative purposes, examining duplicate samples on three or more days increases the chance of finding eggs, but at increased costs in time and labour.

1.5.2. Immunodiagnostic methods

Immunodiagnosis of schistosomiasis relies mainly on antibodies to detect antigens and *vice versa*. In some, though not all, instances, their levels in man are more stable than those of egg counts. Antibody detection tests provide only indirect proof of exposure because they are molecules produced by the host's immune response to the parasite. Prepatent and early infections may not have stimulated a detectable antibody response, resulting in false negative tests if the wrong antigen is used. Positive tests do not necessarily denote an active (living) infection. Antibody tests are also prone to cross reactions with other infections (especially tissue dwelling parasites), which diminish their sensitivity (for detecting infected cases) and specificity (for detecting normal, uninfected people). However, improved reagents provided by molecular biological techniques have revived interest in antibody detection, particularly for specific isotypes and sub-classes associated with different stages of infection. Two of these immunological tests are: falcon assay screening

test-ELISA (FAST-ELISA) and confirmatory enzyme –linked immuno electro transfer blot (EITB) (Mahmoud, 2001). Both are highly sensitive and 96% specific (Mahmoud, 2001).

Antigen tests, by definition, detect molecules produced by the parasite and therefore are better proof of infection than antibody detection tests. Antigen levels are often significantly lower than those of antibodies, but, theoretically, are directly related to the number of worms present. Continuously improving techniques now allow detection of very low antigen levels. Levels tend to be more stable than egg counts but the relationship between egg counts and antigen levels is not always perfect. Although antigen tests often confirm suspected light infections with sparse eggs, negative antigen tests still occur in subjects passing eggs. Despite claims to the contrary, egg counts remain the gold standard for diagnosis (Hagan *et al.* 1998).

1.6. Immunopathology of *S. mansoni*

1.6.1. Egg-Induced Granuloma.

Granulomatous inflammation around parasite eggs clearly constitutes the most important disease manifestation in schistosomiasis. The magnitude of the initial inflammatory event and its fibrous repair, offset by the relative efficacy and strength of the subsequent immunomodulation process, are the key elements in dictating the overall course and severity of the disease. Granulomas are aggregates of mixed inflammatory leukocytes embedded in an increasingly fibrous extracellular matrix. Most granulomas are composed of cells such as eosinophils, macrophages, lymphocytes, neutrophils, mast cells, and fibroblasts (Metwali *et al.*, 1996). Mast cells are infrequent in 8- wk granulomas in most mouse strains and become more frequent in chronic infections (Weinstock *et al.*, 1983) and these may be important because they secrete fibrogenic mediators and interact with hepatic stellate (Ito) cells (Brito and Borojevic, 1997). Circulating “fibrocytes” are thought to be infiltrated into granulomas, and it is speculated that these cells may be important for attracting CD4+ lymphocytes as well as for collagen formation (Brito and Borojevic, 1997).

The granulomatous response is maximal at the 8th week, during the acute stage of the disease. Soon after, the immune reaction starts to down modulate, and granulomas around recently deposited

eggs become progressively smaller (Fallon, 2000). Although the response to new eggs is down regulated, cumulative damage occurs as older lesions involute to leave fibrosis scars. Thus, the rate of damage decreases but accumulated damage may increase, the balance being determined by the variable ability of the host to kill worms, to inhibit worm fecundity, and to destroy eggs, and repair tissue damage (Cheever *et al.*, 2002).

Most granulomas develop at the sites of maximal accumulation of eggs — the intestine and the liver. However, periovular granulomas have been found in many types of tissue, including the skin, lung, brain, adrenal glands, and skeletal muscle (King, 2001).

Evidences indicate that the vigorous host granulomatous responses, rather than the direct action of the parasite, are responsible for the pathologic tissue manifestations that characterize this stage. Eggs of *S. mansoni* embolize to the liver, where the granulomatous inflammatory response induces presinusoidal inflammation and periportal fibrosis. Periportal collagen deposits lead to the progressive obstruction of blood flow, portal hypertension, and ultimately varices, variceal bleeding and, hepatosplenomegaly (Allen *et al.*, 2002; Mahmoud, 2001).

Besides causing vascular obstruction and subsequent fibrosis, shistosome granulomas may be potentially harmful to adjacent parenchymal cells. The cell populations that comprise the granulomas are found at metabolically active state and are capable of secreting a number of tissue destructive substances. During phagocytosis, cell activation, or cell death, such substances may diffuse from the granulomas and damage tissue parenchyma cells. Granulomas isolated from murine livers and cultured under *in vitro* conditions have been shown to secrete lysozyme and B-glucuronidase, as well as collagenase and catalase (Perotto *et al.*, 1976; Truden and Boros, 1985; Fallon, 2000). Macrophages isolated from dispersed hepatic granulomas were found to secrete neutral proteases and super oxide anions (Chensue *et al.*, 1983). Neutral proteases and super oxide anion can degrade or oxidize cell membrane constituents with potential tissue damage.

Considerable number of evidences, however, seems to indicate the tissue protective capacity of the inflammatory response by sequestering the toxic and antigenic substances secreted continuously

from the *Schistosoma* eggs, thereby, preventing further diffusion of these harmful materials. T-cell depleted animals, thus, failed to form granuloma, and showed liquifactive necrosis in the liver and gut with severe destruction of tissue parenchyma, releases of hepatic enzymes and death (Buchanan *et al.*, 1973). Hepatic necrosis adjacent to the smaller granulomas was also observed in infected mice (Bryam and Lichtenberg, 1977) and in animals treated with anti-L3T4 anti serum, which suppressed T-helper activity and granuloma (Mathew and Boros, 1986).

1.6.2. Hepatic Fibrosis

Egg-derived products and mediators released at the site of inflammation stimulate the differentiation of stellate cells (Ito) into myofibroblasts. Fibroblasts secrete extracellular matrix proteins (ECMP) that deposit in the periportal space following immune modulated granulomas (Mahmoud, 2001). In most infected subjects living in endemic areas, ECMP accumulation is well controlled, and these individuals develop minor pathological manifestations. However, in 5–10% of *S. mansoni*-infected subjects, ECMP accumulate in the portal space, due to imbalance between fibrogenesis and fibrolysis, leading to extended periportal fibrosis (PPF), also called Symmers' pipestem fibrosis. PPF is a major cause of portal hypertension and its attendant sequel, which include varices and abdominal ascites. Subjects with advanced PPF may die of hematemesis, coinfection, or heart failure (Mahmoud, 2001).

The production of collagen and ECMP is stimulated by a group of cytokines and growth factors: IL-1, IL-4, IL-13, IL-7, TNF- α , monocyte chemo tactic protein1 (MCP-1), platelets derived growth factors (PDGF), and transforming growth factor-beta (TGF- β). All these have been investigated by measuring cytokine expression in lymphoid and peripheral tissues, by blocking or eliminating cytokines or their receptors by using antibodies or knock out mice respectively, and by administering cytokine along with sensitizing antigen as part of strategies for immunization (Cheever *et al.*, 1994a; Chou *et al.*, 1996; Gressner, 1995; Khalil *et al.*, 1995). General conclusions from these studies have been that these cytokines and growth factors stimulate fibroblast differentiation and the production of ECMP by fibroblasts, Kupffer cells, and endothelial cells. Other cytokines, such as IFN- γ , seems to have the opposite effect. Analysis of the immune response of the subjects with severe PPF has demonstrated an association of advanced PPF with a reduction in IFN- γ production (Henri *et al.*, 2002). These observations are supported by various

studies that demonstrate that IFN- γ is a key antifibrogenic and profibrotic cytokine. Indeed, IFN- γ inhibits stellate cell differentiation and collagen production by myofibroblasts (Mallat *et al.*, 1995). Moreover, IFN- γ stimulates ECM degradation by increasing tissue metalloproteases (TMP) and by inhibiting tissue inhibitors of metalloproteases (TIMP) (Tamai *et al.*, 1995).

The regulation of fibrosis is often independent of the regulation of granuloma size, i.e. larger granulomas are not always associated with greater hepatic fibrosis (Cheever *et al.*, 1994 a) and compared with hepatic granulomas, and much less collagen was accumulated in the ileal and the colon (Cheever *et al.*, 2002).

1.6.3. Glomerulopathy

Patients with chronic *S. mansoni* infection (involving portal hypertension and hepatosplenic schistosomiasis) have an increased frequency (15%) of renal disease (Andrade and Van Marck, 1984). This syndrome may sometimes be attributed solely to concomitant bacterial infections such as salmonellosis (Barsoum *et al.*, 1977), which may increase the incidence of circulating immune complex (CIC) formation and directly or indirectly cause renal disease. However, the helminthic infection alone is sufficient to initiate the nephritic syndrome.

To establish the immune etiology of schistosomal glomerulopathy (SG), the sera and renal tissues of both infected humans and experimental animals have been examined for CIC and the schistosome antigen components that participate in IC formation (Andrade and Van Marck, 1984; De Brito *et al.*, 1999; Van Velthuysen and Florquin, 2000). The results showed the presence of specific antibodies of all major immunoglobulin classes-IgM, IgA, IgG, IgE- circulating immune complexes containing schistosomal antigens in the sera of both humans and experimental animals, and the presence of schistosomal worm antigens in the glomerular deposits. Therefore, a glomerular inflammatory injury, which occurs as a consequence of glomerular immune-complex deposition, was suggested as the more probable mechanism of the glomerulopathies detected in mansonian schistosomiasis (Andrade and Van Marck, 1984; Couser, 1985).

Cure of schistosomiasis does not stop the progression of glomerular disease in humans or experimental animals (Barsoum, 1993). Besides, there is lack of correlation between the severity of

the nephritis and the intensity of the parasite infection (Van Velthuisen and Florquin, 2000). These data may suggest that, despite the importance of the schistosomal antigen/s in the initiation of glomerular injury, other factors such as autoimmunity may play a role in the further evolution of the lesions (Barsoum, 1993). The severity of the glomerular lesions and proteinuria is correlated with the impairment of hepatic macrophage function (Barsoum *et al.*, 1988). This macrophage function may involve the clearance of circulating immune complexes and eventually the clearance of other nephritogenic factors.

Differential diagnosis of SG was found to be difficult owing to false positive reactions for schistosomal antigens by both immuno-fluorescence (IF) and IM-EM in patients with a positive serology for hepatitis B and C viruses (Johnson *et al.*, 1994; Nussenzveig *et al.*, 2002; De Brito *et al.*, 1999).

1.7. The Immunology of *S. mansoni*

Infective schistosome cercariae gain entry to the mammalian host via a percutaneous route and use a number of proteolytic enzymes to digest a route through the skin prior to their exit via blood capillaries or lymphatic vessels (Mountford and Trottein, 2004; McKerrow and Salter, 2002). Ordinarily, schistosomes traverse the skin of their primary host within days, and the vast majority enters the circulation to reach their final site of parasitization. The precise amount of time the larva spend in the skin has recently been debated (McKerrow and Salter, 2002; Curwen and Wilson, 2003; Whitfield *et al.*, 2003), although evidence from tracking studies of *S. mansoni*, using both rodent (Mountford *et al.*, 1988) and primate (Wilson *et al.*, 1990) models, show that migration out of the skin can occur within 48–72 h, with the epidermal basement membrane providing the major obstacle for the larva to negotiate.

Following penetration, the cercaria undergoes a number of changes such as the loss of the tail and shedding off the glycocalyx as it transforms into schistosomula. This is to be followed by the release of the excretory secretory (ES) products from post and pre acetabular glands (the head glands of the skin stage schistosomula), and binding of schistosomal PAMPs to PPRs (e.g. TLRs/ and C-type lectins) on innate immune cells.

Although much is not known about this stage of the immune reaction, various evidences indicate that the schistosomula in the natural host starts to produce antigens, which initiate a series of sensitization process. In most cases in general, where proved by animal experiments, an extensive infiltration of neutrophils and lymphocytes occurs during the first 30 hours of exposure, which is augmented by the influx of eosinophils by day 2 (Colley *et al.*, 1972). The early wave of cell infiltration is invoked by antibodies, which trigger the complement pathway, which in turn generates polymorphonuclear cells infiltration. The subsequent activity is attributed to T-lymphocytes, which, especially on secondary or repeated experimental exposures, trigger memory T-lymphocytes that elicit both humoral and T-cell mediated, accelerated reaction against the cercariae (Colley *et al.*, 1972; Machado *et al.*, 1975).

From studies in a classical experimental murine model, it is recognized that the adaptive immune response leading to immunopathology in schistosomiasis is principally mediated and orchestrated by major histocompatibility complex (MHC) class II-restricted CD4⁺T lymphocytes specific for schistosome egg antigens (SEA) (Mathew and Boros, 1986; Hernandez *et al.*, 1997; Pearce *et al.*, 2004) (See Fig 2 and 3). After infection, an initial pro-inflammatory CD4⁺T-helper-1 (Th1)-type polarized response, with elevated interferon- γ (IFN- γ) and interleukin- 2 (IL-2) levels, continues into the period of oviposition at around 5 weeks post-infection, when perioval granulomatous inflammation begins (Stadecker and Hernandez, 1998). This Th1 response follows an early

production of IL-12 by accessory cells (Stadecker and Hernandez, 1998; Miguel *et al.*, 2004), which varies considerably among mouse strains in intensity and duration.

Within the next 1–2 weeks, granuloma formation increases amid a change in the cytokine environment, which under normal circumstances becomes dominated by anti-inflammatory Th2-type cytokines, including IL-4, IL-5, IL-10, and IL-13 (Pearce and Mac Donald, 2002; Stadecker and Hernandez, 1998; Pearce *et al.*, 1991; Wynn *et al.*, 1993) (Fig. 2). Coincident with the development of Th2 response, there are notable increases in plasma IgE levels and circulating eosinophil numbers, which reflect the production of interleukin (IL)-4 and IL-5, signature cytokines of Th2 cells, that, respectively, help B cells to class switch Ig isotype production to IgE and act as a growth and survival factor for eosinophils (Mosmann, 1992). After about 3 months of mixed-sex infection, a significant diminution of the Th2 response is apparent, and this of comparative hyporesponsiveness persists for the remainder of the infection (Grzych *et al.*, 1991). This owes much to the regulatory role of IL-10. IL-10 inhibits lymphocyte activation, proliferation and inflammatory cytokine production, including IL-12 (Matthias *et al.*, 2004). In contrast, in animals infected with single sex cercariae, where egg production does not occur, the antigen-specific Th1 response persists (Grzych *et al.*, 1991).

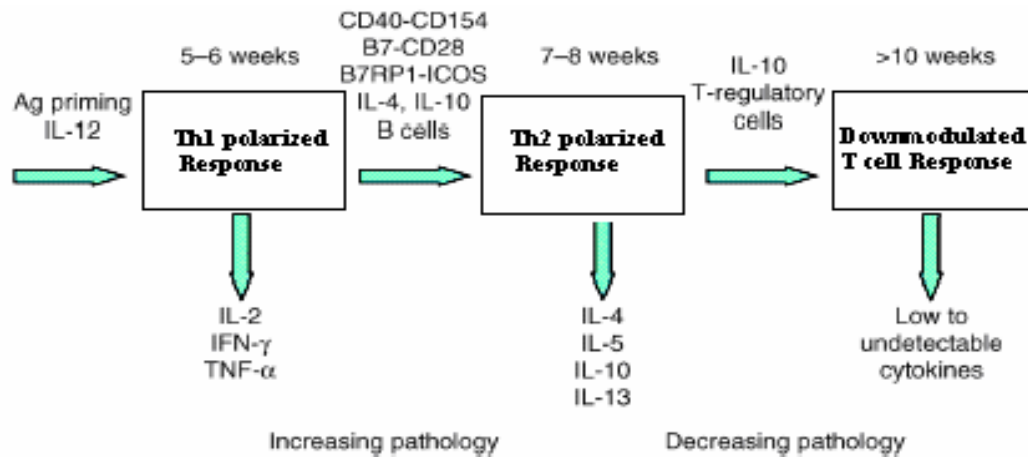


Fig 2. Evolution of the immune response in murine schistosomiasis. Depicted is the normal evolution of the pathogenic CD4⁺ T-cell response during infection with schistosomes. After contact with the parasite, the host develops an early Th1-polarized response, determined by the presence of interleukin-12 (IL-12) and characterized by the cytokines IL-2, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α). Following oviposition, which precipitates an escalating hepatic immunopathology, there is a prompt transition to a Th2-type response; this switch is variously dependent on co-stimulatory systems, as well as IL-4, IL-10, and B cells. A firmly established Th2 milieu, marked by the cytokines IL-4, IL-5, IL-10, and IL-13, is critical for the subsequent gradual overall downmodulation of T-cell response and immunopathology. IL-10, produced by regulatory T cells but also by other lymphocytes and nonlymphocytes, plays a major role in the immunomodulation process. In certain cases, an excess of IL-12 or a deficiency in any of the critical switching or regulatory mechanisms will upset the normal course of events, resulting in persistently elevated Th1 responses and the failure to adequately control the immunopathology, which can lead to death. The approximate time frames of these events are indicated in weeks after infection (adapted from Stadecker *et al.*, 2004).

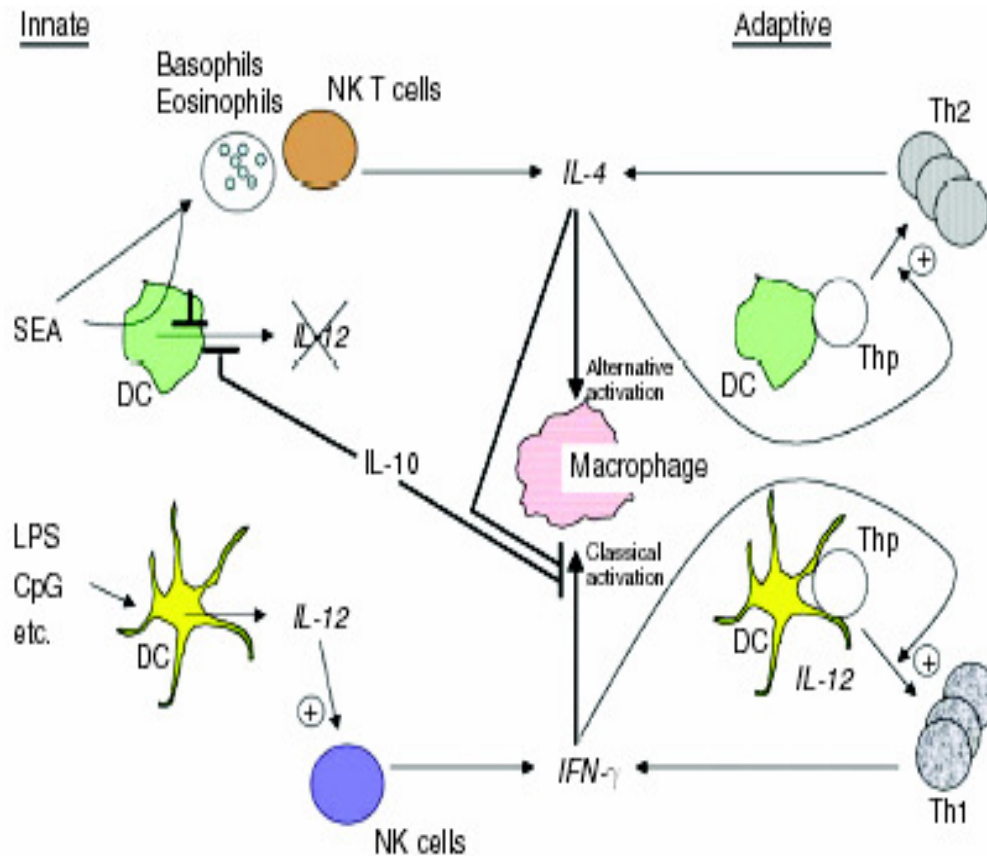


Fig. 3. Innate and adaptive responses initiated by soluble egg antigen (SEA) are characterized by the production of interleukin (IL)-4. Schistosome eggs and SEA activate innate cells to produce IL-4, either directly or via dendritic cells (DCs), and drive polarization of T-helper (Th) responses in a Th2 direction. IL-4 plays an important role in suppressing pathways that can lead to the production of inflammatory mediators, including nitric oxide (NO) and tumor necrosis factor- α (TNF- α), by cells such as macrophages, and promotes the development of alternatively activated macrophages in infected animals. IL-10, from a variety of sources including Treg cells, myeloid cells, and Th2 cells, plays an important additional role in the regulation of inflammation. These responses contrast with those induced by pathogen associated microbial patterns (PAMPs), such as lipopolysaccharide (LPS) and CpG, which strongly induce DC activation via Toll-like receptor ligation, leading to IL-12 production, the induction of interferon (IFN)- γ production by innate and adaptive cells, and the promotion of proinflammatory mediator production by macrophages and other cells, NK (natural killer) cells (Adapted from Pearce *et al.*, 2004).

Oviposition by itself is not sufficient to induce the Th1 to Th2 switch, as it fails to fully materialize in the absence of a number of well-defined molecular mechanisms. Significantly, among the T-cell co-stimulatory molecules, the B7-CD28 (Wilson and Coulson, 1999) and CD40-CD154 (Mosmann, 1992) pathways are required for Th2 development. Their absence, however, has a variable effect on the remaining Th1 response, which is either augmented in the former or unaffected in the latter. By comparison, blockage of the inducible co-stimulator (ICOS)-B7RP-1 system results in failure to downmodulate the Th1 response, as measured by IFN- γ levels, but does not significantly affect Th2 development (Dunne *et al.*, 1991). The lack of IL-4 (Doenhoff *et al.*, 1981) or of B cells (Dunne and Doenhoff, 1983) also precludes the induction of Th2 cells, perhaps in part because of their dependence on intact B7-CD28 co-stimulatory signaling (Brunet *et al.*, 1997).

Overall, the Th1 to Th2 switch is critical to prevent excessive host morbidity and promote enhanced survival; however, the absence of the various conversion mechanisms has dissimilar effects on the resulting immunopathology. Of greatest consequence is the lack of intact CD40-CD154 and B7-CD28 signaling as well as of IL-4, as their absences can result in lethal disease characterized by severe hepatic inflammation with hepatocellular injury and necrosis (Hernandez *et al.*, 1999; MacDonald *et al.*, 2002; Brunet *et al.*, 1997).

Under normal conditions, CD4 T-cell responses are essential to prevent hepatocyte damage during *S. mansoni* infection. Schistosome eggs secrete molecules that are hepatotoxic (Dunne *et al.*, 1991), and Th-dependent antibodies along with Th cell orchestrated granulomatous lesions that develop around tissue-trapped eggs cooperate to prevent these toxins from reaching surrounding hepatocytes (Doenhoff *et al.*, 1981; Dunne and Doenhoff, 1983). During natural infection in the mouse, the dominant Th response is Th2 like, and thus it is assumed that these cells control granuloma formation. Animals that are genetically incapable of making Th2 responses, for example IL-4^{-/-} mice, whose IL-4 gene have been knocked out, mount egg antigen specific Th1 responses and continue to develop granulomas around eggs trapped in their livers, but they start to lose weight shortly after the onset of egg production and fail to survive long past week 9 of infection (Brunet *et al.*, 1997; Fallon *et al.*, 2000).

Th1 responses and disease severity are increased if IL-10, in addition to IL-4, is absent (Hoffmann *et al.*, 2000). Morbidity and mortality in these animals is linked to increased serum transaminase

levels (Fallon *et al.*, 2000; Hoffmann *et al.*, 2000), which are considered to be indicative of hepatocyte damage caused by excessive pro-inflammatory mediator production (La Flamme *et al.*, 2001). These data highlight the crucial roles of IL-4 and IL-10 in the prevention of severe inflammatory disease during schistosome infection and suggest that Th2 cells normally mediate the spectrum of protective mechanisms that allow hosts to survive infection with *S. mansoni*, including granuloma formation, the development of an antibody response against egg antigens, and the regulation of inflammatory processes that have the potential to cause lethal disease (Pearce and Mac Donald, 2002; Hoffmann *et al.*, 2002). It is also increasingly apparent that in the longer term there are deleterious consequences associated with chronically polarized Th2 responses. One of the Th2 product, IL-13, has potent profibrotic effects and is largely responsible for the hepatic fibrosis that develops within the liver as a consequence of the repeated insults associated with granuloma development and resolution (Fallon *et al.*, 2000; Hoffmann *et al.*, 2002).

A complex balance of factors controls Th response polarization. Upon initial activation, naive Th cells enter the cell cycle and begin to produce the autocrine growth factor IL-2 plus one or more effector cytokines, such as IFN- γ and IL-4 (Murphy and Reiner, 2002). Thereafter, under the influence of a variety of factors, including co-stimulatory molecule engagement, strength of signal, and most importantly the cytokines in the environment responses polarize to become dominated by IFN- γ -producing Th1 cells or IL-4/IL-5/IL-13-producing Th2 cells (Murphy and Reiner, 2002).

Th1 polarization owes much to the continuous production of IL-12. IL-12 is made by DCs and macrophages, cell types that can process and present antigens to Th cells, and additionally by neutrophils (Trinchieri, 2003). The involvement of these cells in the innate response to Th1 response-inducing pathogens during the period preceding the initiation of adaptive immunity can create an environment rich in IL-12, which promotes the production IFN- γ and the outgrowth of Th1 cells. IFN- γ itself stabilizes the expression of the IL-12R β 2 on Th cells and in this way helps consolidate the Th1 response (Murphy and Reiner, 2002). Other cytokines, such as IL-18, IL-23, and IL-27, can support this pathway (Robinson and O'Garra, 2002; Trinchieri *et al.*, 2003). Thus, the prevailing view of Th1 response development is one in which innate cells produce factors that promote Th1 polarization of the adaptive response. Why the egg antigen induces Th2 response simply reflects a failure of SEA to induce the production of these factors (Fig. 3).

For Th2 response development, IL-4 rather than IL-12 is a crucial polarizing factor. Schistosome infection in mice that lack IL-4 fails to induce egg antigen-specific Th2 responses, whereas the absence of IL-12 has no effect on egg-induced Th2 response development (Patton *et al.*, 2001). Independently of its growth factor effects, IL-2 also plays an important role for Th2 development (Cheever *et al.*, 1992; Cote-Sierra *et al.*, 2004). Neutralizing IL-2 inhibits *in vitro* and *in vivo* priming for IL-4 production and *in vitro* priming for production of essentially all Th2 cytokines, even when no reduction in cell proliferation or total cell expansion occur (Cote-Sierra *et al.*, 2004).

The cellular source of IL-4 important for Th2 response development is generally unclear. Eggs and antigens released from them innately activate mast cells (Sabin *et al.*, 1996) and basophils (Haisch *et al.*, 2001; Schramm *et al.*, 2003), cell types that are able to make IL-4. Moreover, as part of an early innate response in mice that have been injected with eggs, mast cells elicit the recruitment of IL-4-producing eosinophils (Sabin *et al.*, 1996). Interestingly then, eggs induce a Th2-like pattern of responsiveness in the innate as well as the adaptive arm of the immune response. However, the innate Th2-like components of this response appear to be dispensable for the adaptive response, because eggs will induce Th2 responses of normal intensity in the absence of either mast cells or eosinophils (Sabin *et al.*, 1996; Brunet *et al.*, 1999).

Moreover, the production of IL-4 by cells, DCs (d'Ostiani *et al.*, 2000) and NKT cells, which have been implicated for Th2 response development, proved to be not essential for Th2 responses to SEA (Brown *et al.*, 1996; MacDonald and Pearce, 2002). Thus, it seems likely that, as was originally suggested a decade ago (Schmitz *et al.*, 1994), the IL-4 necessary for Th2 response polarization is being provided by Th cells themselves. A caveat to this conclusion is studies with CD1-/- DCs have shown them to be incapable of inducing SEA-specific Th2 responses, suggesting a scenario in which SEA-pulsed DCs activate both Th cells and NKT cells, and the IL-4 produced by the latter promotes the outgrowth of a Th2 response (Faveeuw *et al.*, 2002).

1.8 Animal Models Used for the Study of Infection by *S. mansoni*

Most of our knowledge about schistosomiasis infection and immunology came as a result of studies done on different experimental models (mice, rats, rodents, primates, etc). Of particular importance for the laboratory studies are the different strains of mice. Schistosome infected mice develop a Th1 polarized response before egg production; form granuloma in a Th2 polarized environment following an egg release by the female worms 5 weeks *pi*; down modulate granuloma afterwards, and showed a remarkable production of ECMP thus contributing to hepatic fibrosis. Besides, because of their availability, fecundity, low cost and susceptibility to experimental infections, mice proved to be the most often used animal models for both *in vitro* and *in vivo* study of schistosome infection.

Mice, however, might not serve as good models to study human schistosomiasis for a number of reasons: First, most experimental infections in murine models are given as single heavy infections while in humans infections are acquired by repeated, slight exposure to cercariae gradually over years. Second, the intensity of experimental murine infection is generally extremely high. A single *S. mansoni* worm pair in a mouse may be equivalent to more than 1,000 pairs in an infected person (Cheever 1969, Gryseels and de Vlas 1996). Third, most murine models have been performed in highly inbred strains of mice and may not reflect the heterogeneity of response expected for human populations. And finally, great anatomic, genetic, and immunologic differences exist between humans and rodents. This calls for a need to look for animal models, which are anatomically, genetically and immunologically closer to humans (for instance, primates) and an infection strategy that mimics what is going on in humans.

Many primate species had been tested and are believed to be susceptible to *S. mansoni* infection. However, the level of permissiveness to *S. mansoni* varies considerably between different species of primates: baboons (Sadun *et al.*, 1966, Damian *et al.*, 1986 and Mola *et al.*, 1999), chimpanzees (Sadun *et al.*, 1970), different species of monkeys (Sadun *et al.*, 1966 and Portillo and Damian, 1986). Experiments on rhesus monkeys carrying an initial *S. mansoni* infection showed that after a challenge infection, those animals were able to eliminate the new infective cercariae without being able to destroy the adult worms resulting from the previous infection (Sadun *et al.*, 1966). When infected subcutaneously, tamarin monkeys met the criteria of permissiveness to *S. mansoni* (Sadun *et al.*, 1970). Baboons have been found to be a permissive host for *S. mansoni* and a good model for schistosomiasis (Sadun *et al.*, 1966). Besides being experimentally infected with *S. mansoni*,

baboons are also naturally infected with *S. mansoni*. However, the cost of using baboons has risen to almost prohibitive levels, even in countries where they can be obtained locally.

The possibility of a reliable and a continuous supply of wild grivet monkeys, which can be maintained easily, prompted us to re-examine the suitability of the grivet monkey (*Cercopithecus aethiops aethiops*) for studying experimental *S. mansoni* infections. Grivet monkey was chosen because previous studies confirmed that it is a permissive host for infection by *S. mansoni* (Cheever and Duvall, 1974; Sadun *et al.*, 1966). The present study, however, was designed to see the effect of low doses of cercariae on the overall clinical manifestations of infection by the parasite and immunological changes particularly, the level of cytokines produced at different stages of infection.

2. Objectives

2.1. General Objective

To describe the immunological and clinical consequences of infection and assess the possible use of grivet monkey (*Cercopithecus aethiops aethiops*) as animal model of *S. mansoni* infection so that future drugs and vaccine trials would be facilitated.

2.2. Specific Objectives

1. To describe the natural course of the infection process in chronological order of disease manifestation both at micro and macroscopic levels
2. To measure plasma cytokine levels of *S. mansoni* before and after infection
3. To evaluate the role of cercarial dose in the immunology of infection

3. Hypothesis

S. mansoni is infective to grivet monkey (*Cercopithecus aethiops aethiops*) and the infection processes and immune response mimic that take place in humans

4. Materials and Methods

4.1. The animals

Thirteen males and five females, a total of eighteen *Grivet monkeys* (*Cercopithecus aethiops aethiops*) of different age groups were captured from Sodere, 106Kms Southeast from Addis Ababa. Four monkeys died before the commencement of the actual infection process. Before the beginning of the experimental work, the monkeys were quarantined for 2 months, and checked for intestinal and retroviral wild infections. Some were found to be *Trichuris trichuria* infected, and thus treated with Ketrax (25mg/Kg body weight). In order to screen schistosome infections, the stool of the monkeys was checked by Kato smear and concentration methods (Idris & AL-Jabri 2001). The animals were finally housed in individual cages of 90cm by 100cm; Maize, carrots, chickpeas, banana as well as water were provided.

4.2. The parasite

S. mansoni infected snail hosts, *Biomphalaria pfeifferi*, were collected from *S. mansoni* endemic areas around Kemisse (Wollo district) 320Kms from Addis Ababa, northeastern part of Ethiopia. All suitable aquatic conditions were maintained at the snail laboratory of the Aklilu Lemma Institute of Pathobiology (AL-IPB), Addis Ababa University. The infected snails were screened and used as the source of cercariae.

For recovery of cercariae, each snail was washed in fresh water and put in individual vials with water and then exposed to sunlight or electric light for about 10-30 minutes. More than 10 infected *Biomphalaria pfeifferi* snails were used to induce the shedding. The number of cercariae was counted by having few drops of iodine in 1ml of water with cercariae, which was observed under 40x binocular microscope.

4.3. Infection of the animals with *S. mansoni* Cercariae

All the monkeys were infected by skin exposure through unshaved abdomen, to mimic the natural infection, and using pouch method (Sturrock *et al.*, 1976). The cercarial concentration was adjusted from 100 -300 cercariae/ml of saline solution depending on the monkey group. The saline solution was added to individual vials of equal size and poured over the skin of the animals and kept there for 20 minutes. To decrease the likely hood of failure to penetrate the skin, on the part of the cercariae, a fresh batch of cercariae i.e. cercariae which were shed from the snails an hour or less before the commencement of the actual infection process was used. Confirmation of infection was done by taking a drop of the water solution with a syringe and by checking the presence of the cercariae in it. In cases where cercariae are found in the solution, the pouring is repeated until no cercariae remains in the vial. So, in this way, a total of 14 monkeys, Group I (4 monkeys) with 100 cercariae; Group II (4 monkeys) with 200 cercariae; Group III (4 monkeys) with 300 cercariae were infected and two monkeys (control) kept uninfected. Below (Table 1) is a list of monkeys with their corresponding number of cercariae infected.

Table 1. Group of grivet monkeys used and the cercarial dose they were exposed

Monkey Code	Sex	Number of cercariae applied	Group
M01	M	100	I
M02	M	100	I
M03	M	100	I
M04	F	100	I
M05	M	200	II
M08	M	200	II
M09	M	200	II
M10	F	200	II
M13	M	300	III
M14	M	300	III
M15	M	300	III
M16	F	300	III
M17	M	None	Control
M18	F	None	Control

4.4. Estimation of EPG

Three kinds of stool tests were conducted using a recent protocol (Idris & AL-Jabri, 2001). The tests conducted are the iodine mount or wet preparations, formalin-ether concentration and Kato smear method. The first two are used only for checking eggs to determine positivity for *S. mansoni* infection, but the Kato-Katz test was used for this purpose and for estimation of EPG of stool.

In iodine mount, 50mg of stool was mixed with one or two drops of saline or iodine on slides and its thin suspension covered with 22mm cover slip to screen for presence of *S. mansoni* ova. In formalin-ether concentration technique, about 1g of stool was placed in 15ml conical centrifuge tube of 7ml formalin saline for filtration. The filtrate was transferred to tubes and

3ml of diethyl ether added and centrifuged at 3,200 rpm for 3min. After decanting the supernatant, and iodine staining the material was examined with 10 x objectives. The third method was the Kato-Katz (Cellophane faecal thick smear) in which the stool of the animals was sieved on nylon screen, collected by spatula and filled in holes of templates. The holes form approximately equal cylinders of 41.7mg stool samples. The samples were placed on slides and covered with pre-soaked cellophane for microscopic counting of ova. The number of eggs in the given milligram is converted to eggs per gram of stool (EPG).

4.5. Enzyme Linked Immunosorbent Assay (ELISA) for Cytokines

Sera of monkeys were collected before infection and weekly after infection until the end of sixteen week *pi*. Serum cytokines (IL-4, IL-10, IL-12, IFN- γ and TNF- α) of the monkeys in each group were quantified using Sandwich ELISA. Flat bottom high ELISA-binding plates (One Alewife Center, Cambridge MA 02140, USA) were coated with 1 μ g/well of the monoclonal antibodies (mAbIL-4-I, mAbIL-10-I, mAbIL-12-I, mAbIFN- γ -I, and mAbTNF- α -I; MABTECH, National Institute of Allergy and Infectious Disease, USA and National Institute of Biological Standards and Controls, UK). The plates were incubated over night at 4-8 $^{\circ}$ C. On the following day, the plates were washed twice with PBS and then blocked by blocking solution (PBS with 1% BSA) and incubated for 1 hour at room temperature. After an hour, the plates were washed five times with PBS containing 0.05% Tween (PBS-Tween), and 100 μ l/well of serum samples or standards were added to be followed by incubation for 2 hours at room temperature. After having the plates washed five times with PBS-Tween, 1 μ g/well of the second monoclonal antibodies (mAb IL-4-II-Biotin, mAb IL-10-II -Biotin, mAb IFN- γ -II-Biotin and mAbTNF- α -II-Biotin) were added and the plates were incubated for an hour at room temperature. At the end of the hour, the plates were washed with PBS-Tween and 100 μ l/well of Streptovadin-ALP was added and the plates were incubated for an additional one hour at room temperature which is followed by washing (5x) with PBS-Tween. Finally 100 μ l/well of appropriate substrate solution, Para nitrophenylphosphate (pNPP), was added and the optical density was measured at 405nm (BIOLINX, MRX ELISA reader) after 30 minutes.

Cytokine production was measured in all the three group of monkeys and the control. Analysis was carried out by dividing the time periods into four based on previous knowledge of seroconversion. The first period (T1) covers the cytokine level before infection. The second period (T2) was assigned to represent the time that runs from 1st to 5th week *pi* because studies indicated that after infection an initial pro-inflammatory CD4 +T-helper-1 (Th1)-type polarized response dominates until the period of oviposition at around 5 weeks *pi* (Stadecker and Hernandez, 1998). A change in the cytokine environment is believed to occur the following 1-2 weeks, which under normal circumstances becomes dominated by anti-inflammatory Th2-type cytokines (Stadecker and Hernandez, 1998; Pearce *et al.*, 1991; Wynn *et al.*, 1993). Thus, the third period (T3) was assigned to represent the time that runs from 6th to 8th week *pi*. After about 3 months (nearly 12 weeks) of mixed-sex infection, a significant diminution of the Th2 response is noticeable, and this persists for the remainder of the infection (Grzych *et al.*, 1991). And, therefore, the fourth period (T4) was assigned to cover the time between 12th to 16th week *pi*.

4.6. Statistical Analysis

For all analysis of the parasitological and immunological results SPSS version 11.5 statistical package was used. Non-parametric statistical tests were used throughout. The Kruskal-Wallis test and the Mann–Whitney U-test were used to assess differences in the cytokine production between groups. Significance was tested at the $P < 0.05$ levels.

5. Results

5.1 Clinical observations

During the acute phase of the disease most monkeys became ill 7 to 9 weeks after infection with soft, bloody stools, failure to eat well, decreased activity and poor general appearance. One animal in group I and another animal in group II exhibited all the aforementioned signs; the later died lately at week 27 *pi*. Both animals are relatively younger compared to others in their respective groups. Most of the remaining animals, however, exhibited one or more of the said clinical signs at this phase of the disease. After the end of 20 weeks, most animals were in apparent good health except for the intermittent disease; diarrhea was seldom noticed and blood was seen in stools only occasionally. One monkey in group II (M09) became sick and died in week 4 *pi* perhaps for some reason other than *S. mansoni* infection. In group II again a monkey (M08) died in week 27. Its death is mostly likely due to infection by the parasite for it is the youngest of all and has shown many of the clinical symptoms of an infection including hepaticomegaly. It is difficult to say the clinical symptoms observed were dose related. Instead, it looks they are more age-related for the severity of the clinical signs is more apparent in younger monkeys than old ones.

5.2 Fecal Egg Count

Fecal eggs were detected 7 weeks after exposure in all three groups. To avoid some limitations typical of the Kato-Katz method (i.e. the daily output of eggs) stools were taken twice a week and a minimum of two slides per monkey were prepared for observations under the microscope. The mean weekly egg counts, expressed as egg per gram of stool (EPG) for the three groups are illustrated in Fig 4.

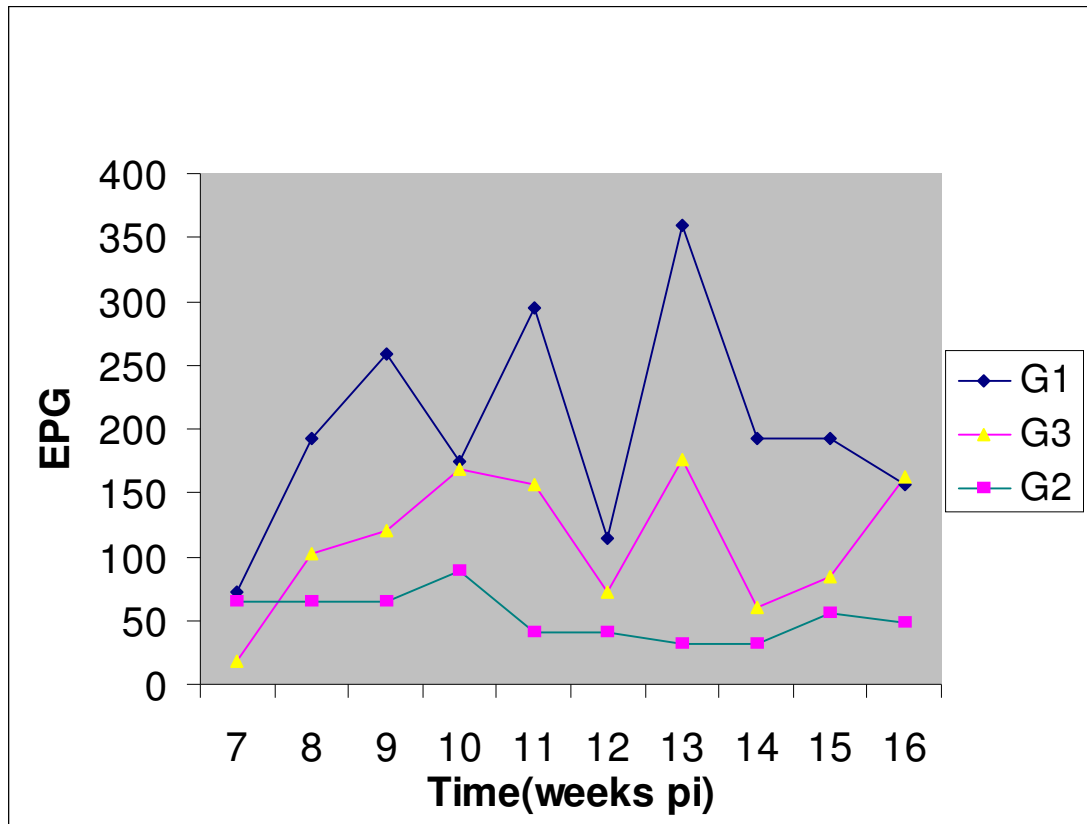


Fig.4. Group mean weekly egg output for grivet monkeys each exposed to a single dose of 100 (G1), 200 (G2) and 300(G3) *S .mansoni* cercariae.

There exists a difference in the time at which the highest and the lowest egg outputs were recorded among the groups. Group I monkeys have shown the lowest egg out put at week 7(70 EPG) and the highest at week 13 (380EPG); Likewise, group III monkeys have shown their lowest at week 7 pi (20 EPG) and their highest at week 13 pi (170 EPG). Group II monkeys, however, have shown their lowest (70 EPG) and highest (90 EPG) at week 7 and 10 pi, respectively. Some degree of dose-dependence is also observed with counts in group one monkeys rising as the highest.

5.3. Cytokine Measurement

In general, both Th1 like- IL-12 (Fig. 5), IFN- γ (Fig.6) and TNF- α (Fig. 7)-and Th2 like cytokines, IL-10 (Fig. 8) and IL-4 (Fig.9) were detected in all monkeys. Differences in the median OD values were exhibited among the groups although statistical significant variation was noted only in a few of the cases.

The median IL-12 OD values started to rise at T2, in all groups of monkeys and reached their highest at T3. And their levels began to decline at T4. The cytokine levels were slightly higher in Group III monkeys than the other two groups. However, the differences were not statistically significant in the two time points analyzed-T1 and T4.

The level of IFN- γ appears to be higher than what was observed in IL-12 production. OD values were higher in all groups of monkeys at all times analyzed except at T1 (Fig.6). Statistically significant variations were recorded between each group and control at all time points except T4. Unlike the case in IL-12, group III monkeys appear to have shown the highest median OD values only at T3. The picture looks the same in TNF- α OD values as well where group III monkeys again have recorded their highest at T3. There were, however, less statistically significant variations between groups and control than what we saw in IFN- γ production (Fig.7).

All monkeys, irrespective of the amount of dose inoculated, exhibited detectable level of Th2 cytokines (IL-4 and IL-10) at all time points analyzed. The picture, however, looks slightly different than what we saw in the aforementioned Th1 cytokines measured. In here, the highest median OD values were recorded at T4 unlike the case in Th1 cytokines where the highest median OD values were observed at T3 (Fig.8 and Fig.9).

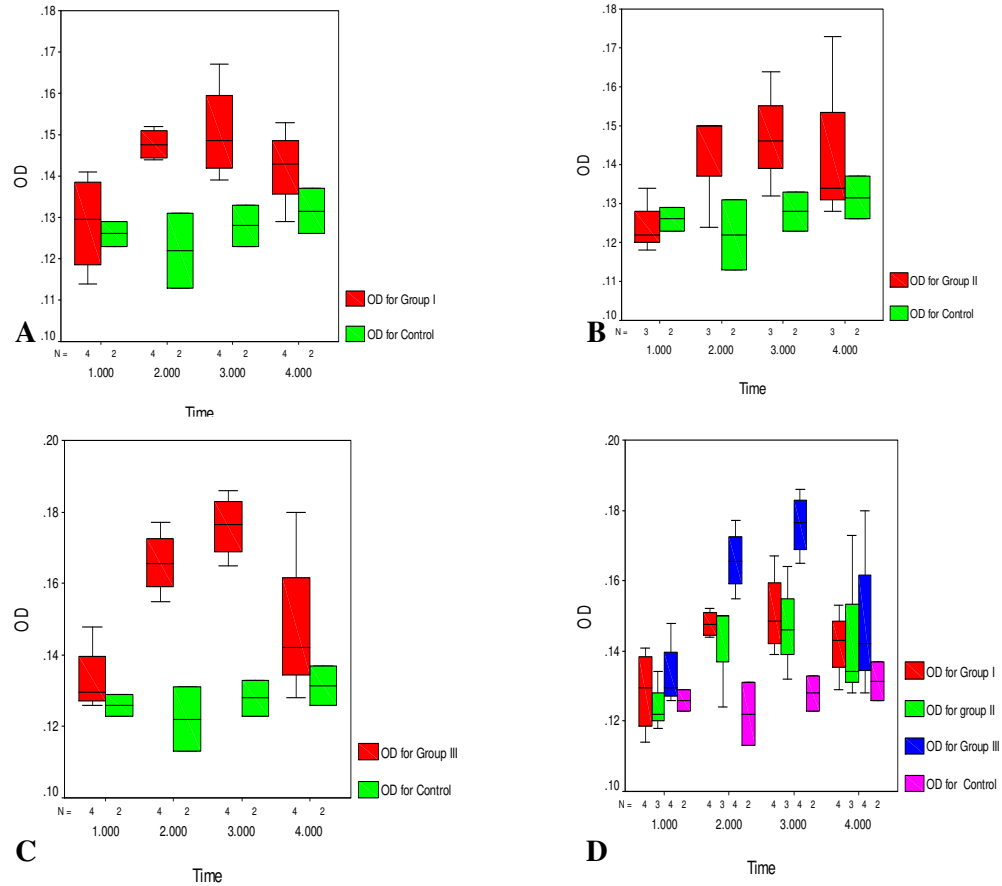
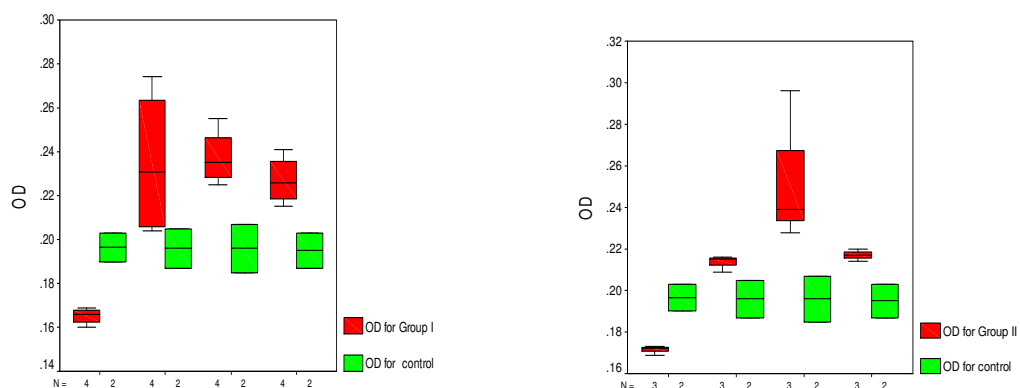


Fig.5. IL-12 OD values of monkeys at 405nm before infection and at different time periods after infection. Each group was compared with control (A-C) and with one another (D). Significant difference in OD values was observed between groups I and control (A) at T2 ($p=0.0180$), between group III and control (C) at T2 ($p=0.001$) and T3 ($p=0.001$). Comparing each group with the other (D) gave a statistically significant difference at T2 ($p=0.005$) and at T3 ($p=0.006$).



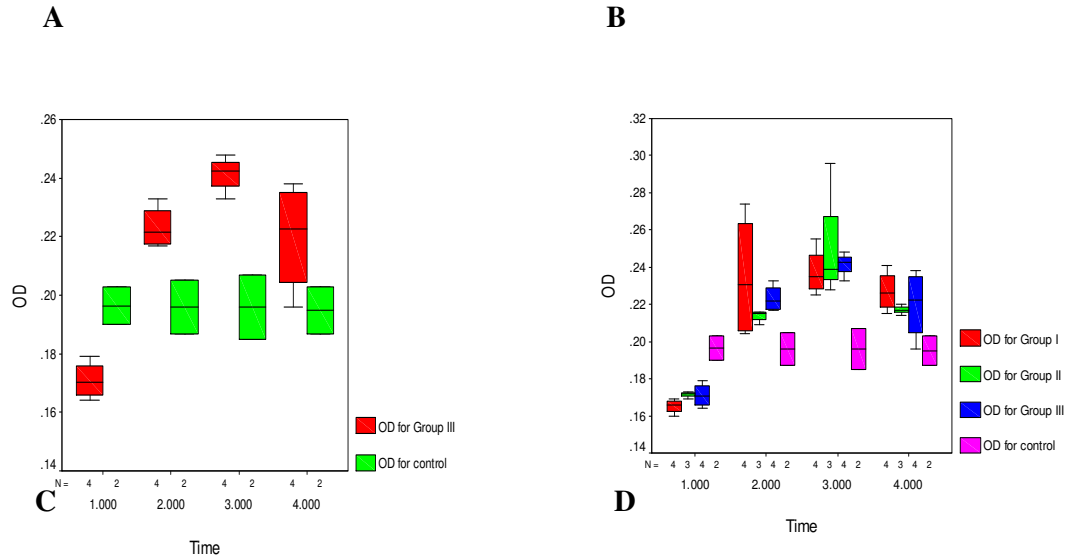
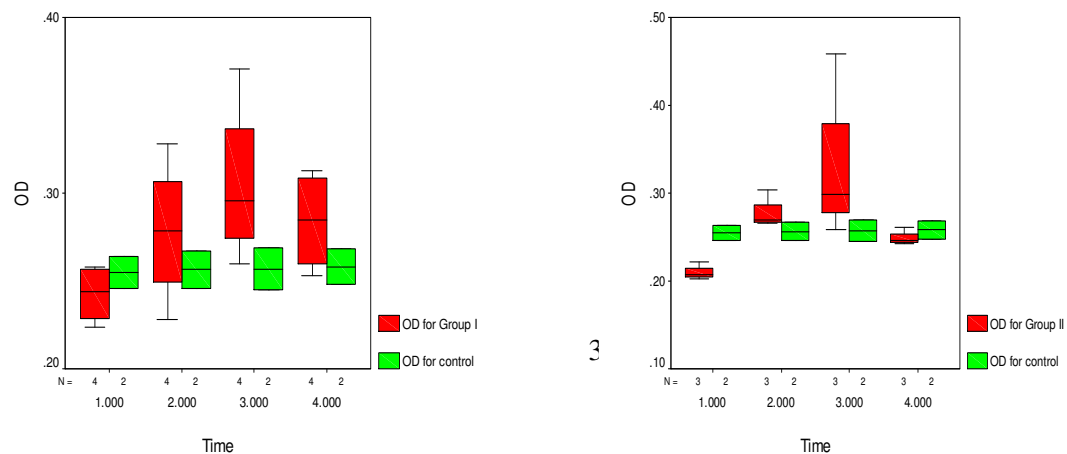


Fig.6. IFN- γ OD values of monkeys at 405nm before infection and at different time periods after infection. Each group was compared with control (**A-C**) and with one another (**D**). OD values showed a significant ($p < 0.05$) difference between group I monkeys and control (**A**) at T1 ($p < 0.001$), T3 ($p = 0.039$) and T4 ($p = 0.022$); between group II and control (**B**) at T1 ($p = 0.001$) and T3 ($p = 0.010$); and between group III and control (**C**) at T1 ($p < 0.001$) and T3 ($p = 0.026$). Comparing each group with the other (**D**) gave a statistically significant difference at T1 ($p = 0.001$).



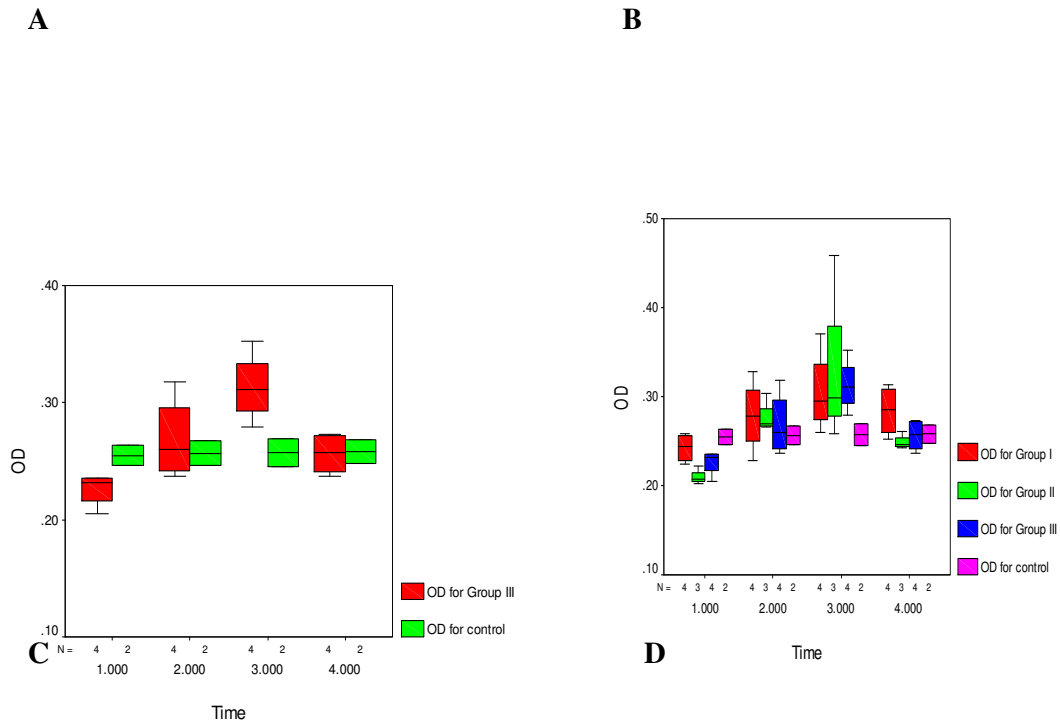
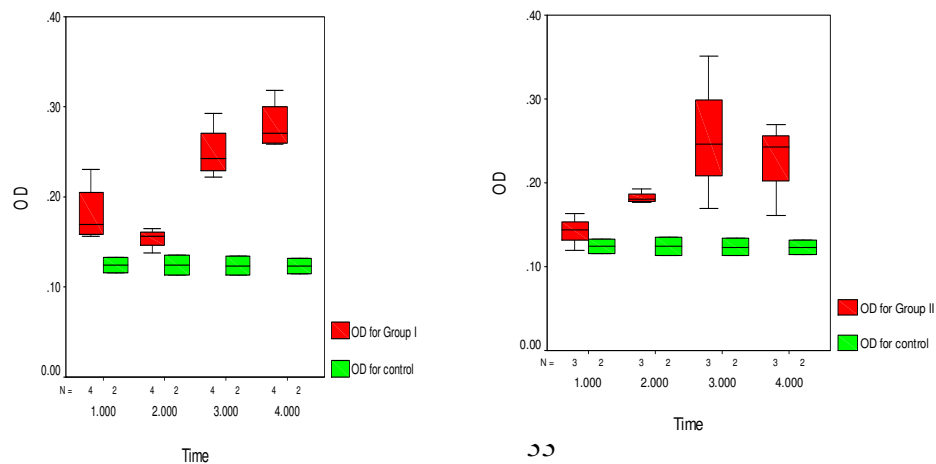


Fig.7. TNF- α OD values of monkeys at 405 nm before infection and at different time periods after infection. Comparing each group with control (**A-C**) gave a statistically significant difference in OD values between group II and control (**B**) at T1 ($p=0.007$) and between group III and control (**C**) at T1 ($p=0.041$). Comparing each group with the other (**D**) gave a statistically significant difference only at T1 ($p=0.026$).



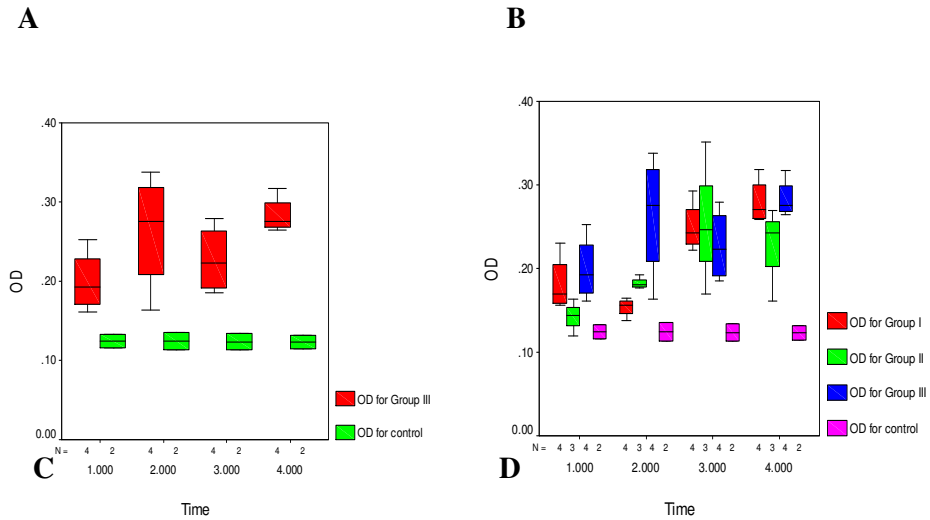
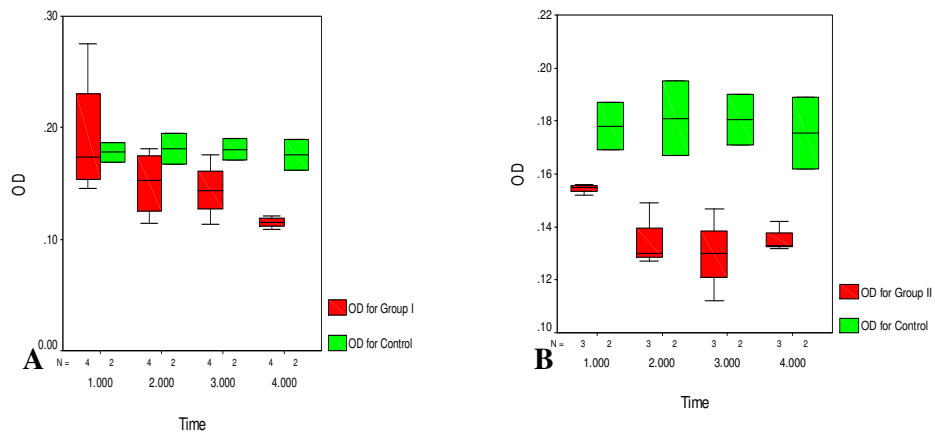


Fig.8. IL-4 OD values of monkeys at 405nm before infection and at different time periods after infection. Comparing each group with the control (**A-C**) gave a statistically significant difference in OD values between group I monkeys and control (**A**) at T3 ($p=0.023$) and T4 ($p<0.001$); between group II monkeys and control (**B**) at T3 ($p=0.024$) and T4 ($p=0.010$), and between group III monkeys and control (**C**) at T1 ($p=0.025$), T2 ($p=0.005$), T3 ($p=0.050$) and T4 ($p<0.001$). Comparing each group with the other (**D**) gave a statistically significant difference in OD values at T2 ($p=0.015$) and at T4 ($p=0.002$).



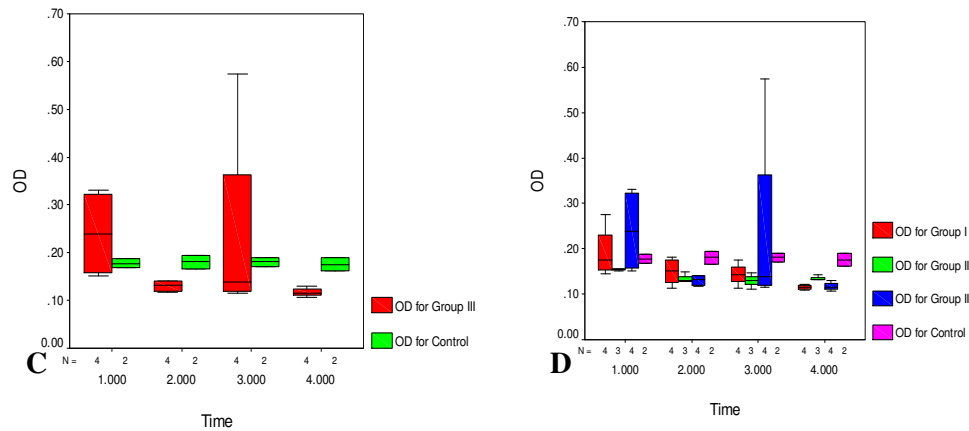


Fig. 9. IL-10 OD values of monkeys at 405 nm before infection and at different time periods after infection. Comparing each group with control (A-C) gave a significant difference in OD values between group I monkeys and control (A) at T4 ($p < 0.001$); between group II monkeys and control (B) at T2 ($p = 0.040$) and T4 ($p = 0.001$), and between group III monkeys and control (C) at T2 ($p = 0.021$) and T4 ($p < 0.001$). Comparing each group (D) with the other gave a very significant difference in OD values at T4 ($p < 0.001$).

6. Discussion

The study has demonstrated that all the 12 monkeys have been successfully infected using the pouch method (Sturrock *et al.*, 1976) as shown by the release of eggs by animals, 7 weeks after infection followed by clinical manifestations. To effect a more probable penetration on the part of the parasite, shaving the skin around the pouch might help, but that would come at the expense of the normal course of infection.

Almost all of the monkeys exhibited the clinical manifestations; especially those typical of the acute phase of the disease. These include the appearance of bloody stool, failure to eat well, and poor general appearance. A more or less similar finding was reported in humans (Marguerite *et al.*, 1999) and other experimental animals such as mice (Fallon *et al.*, 2000),

baboons (Sturrock *et al.*, 1976; Mola *et al.*, 1999), vervet monkeys (Sturrock *et al.*, 1984), and grivet monkeys infected with as high as 600 cercariae per monkey (cpm) (Cheever and Duvall, 1974). This, perhaps, indicates grivet monkey could serve as a good animal model for the study of this stage of infection by *S. mansoni*. The severity of the clinical manifestation, however, does not seem to be dose-dependent unlike the case in vervet monkeys (Sturrock *et al.*, 1984). The difference might lie in the number of cercariae used in this study in which the three groups of monkeys infected differ one from the other by only 100 cercariae. On the contrary, in the study by Sturrock *et al.* (1984), groups differ by more than 600 cpm. The severity of clinical manifestations in this study, however, seems to look age dependent. Older (in this case larger) monkeys seem to exhibit less of the symptoms than their younger (or smaller) counterparts. Similar trend exists in humans (Stelma *et al.*, 1993) and primates (Fallon *et al.*, 2003) but those studies refer age and sex as points of difference than age alone. In this study, since four female monkeys were present, one in each group, it was difficult to take sex as a factor and make comparisons. There are multiples of other factors that affect severity of infection such as genetic variation (Incani *et al.*, 2001), re-infection to the same parasite (Mola *et al.*, 1999) and co-infection with other parasites (Farid *et al.*, 2005), just to mention a few. Thus it is still difficult to determine which factor the severity of infection is attributed to.

Fecal eggs were detected 7 weeks after exposure in all three groups. The pattern of fecal egg excretion mirrored that seen in other primates: baboons (Sturrock *et al.*, 1976) and vervet monkeys (Sturrock *et al.*, 1984). In this study, there is a significant ($P < 0.05$) variation in EPG of stool among the three groups of monkeys with group one monkeys exhibiting the highest and group II monkeys the lowest EPG of stool. This stands as a contrast to what could be seen in the study by Sturrock *et al.* (1984) where three groups of vervet monkeys-group I, II and III-infected with 150, 600 and 1500 cercariae per monkey (cpm), respectively. The study demonstrated a variation in EPG between groups; group III monkeys (monkeys that receive the highest cpm) exhibiting the highest average EPG of stool. Since Sturrock *et al.* (1984) is the only study I could come across, it is difficult to speculate as to the main factors behind the variation in egg output between groups that are exposed to different number of cercariae. Future studies drawn along the same line and on other animal models are expected to shed some light on it. Even then, too much emphasis should not be placed on absolute quantitative

difference in egg quantification using the Kato smear or concentration methods for they are prone to errors that come through various factors such as observer variation , the daily fecal egg production in experimental animals, and genetic variability in experimental animals (Incani *et al.*, 2001).

Earlier studies on experimental murine models indicated that the immune response leading to immunopathology is orchestrated by CD4 T-lymphocytes specific for schistosome egg antigens (SEA) (Mathew and Boros, 1986; Hernandez *et al.*, 1997; Pearce *et al.*, 2004). Thus, following infection until the onset of oviposition, the immune response is dominated by an initial pro-inflammatory Th1 type polarized response, which is characterized by an elevated production of cytokines such as interferon- γ (IFN- γ) and interleukin- 2 (IL-2) (Stadecker and Hernandez, 1998; Stadecker *et al.*, 2004). The Th1 polarized response is believed to be due to an early production of IL-12, which varies considerably among mouse strains. Following oviposition (which is believed to occur 5-6 weeks after exposure), however, there is a prompt transition to a Th2-type response, which is characterized by the increase in the level of cytokines such as IL-4, IL-5, IL-10, and IL-13 (Pearce and Mac Donald, 2002; Stadecker and Hernandez, 1998; Pearce *et al.*, 1991; Wynn *et al.*, 1993).

As far as the order of cytokine production (i.e. Th1 vis-à-vis Th2 cytokines) is concerned, a more or less similar picture, as those found in murine models, was shown in this study. In all groups of monkeys, Th1 cytokines reached their highest median OD values earlier than their Th2 counterparts as can be seen from the box plots (Fig. 5-9). Th1 cytokines (IL-12, IFN- γ , and TNF- α) reach their peaks at T3 (the time which covers 6-8 weeks after exposure) (Fig.5-7) compared to a Th2 cytokine, IL-4, which reaches its highest at T4 (the time which covers 12-16 weeks after exposure) in the majority of cases. The delay in timing of the production of the cytokines may partly be explained by some biological factors characteristic of the animal model used in this study, the grivet monkey (*Cercopithecus aethiops aethiops*). Different animal models react to *S. mansoni* infection differently. Rats, for instance, fail to exhibit the slightest pathologies because they launch a strong immune response and eliminate the parasite as soon as it gets into their system (Philips *et al.*, 1987). The primate model, common marmoset (*Callitrix jacchus*), does not pass eggs in its feces after infection (Oliveira *et al.*, 2004) .The rhesus monkeys kill worms, inhibit fecundity, destroy eggs and repair damage

quickly. The baboons and the grivet monkeys destroy eggs rapidly and repair tissue damage (or perhaps never synthesize much collagen) but kill worms slowly and inhibit oviposition slightly (Cheever and Duvall 1974). Thus, the rapidity in egg destruction and the sluggishness exhibited in killing the worms could have implications in the type of cytokines produced and their timing. But it will be too presumptuous to assume all the discrepancies observed could boil down to this factor alone for other factors of greater importance might exist. One factor worth considering, at this juncture, could be the type of *Schistosoma* strain used. Results from mice infected with one Brazilian (BH) and two Venezuelan (YT and SM) laboratory strains of the parasite showed that the parasite appeared to determine the infectivity, the sex ratio, the onset and timing of oviposition, the number of eggs produced, initial egg laying towards the liver, and the ability to cross the intestinal wall (Incani *et al.*, 2001). Most of the findings in primate models came from studies conducted using the N.I.H. Puerto Rican strain of *S. mansoni* where as the one used in this study might be a different one. For lack of appropriate lab facility, the type of cercariae strain used was not determined.

IL-10 seems to stand as a paradox to what is normally observed in other cytokines. i.e., in almost all groups of monkeys the highest IL-10 OD values were recorded at T1 (before exposure) and most monkeys exhibit a statistically significant ($P < 0.05$) decline in the production of IL-10 after exposure. For lack of other evidences, little can be said on this discrepancy but what might perhaps has contributed to this could be the nature of the animal model used and due to failure not to measure the cytokine at a far later time after exposure. IL-10 has been indicated to play the role of suppressing immune polarization to either Th1 or Th2 direction and its level tends to rise in a later stage of infection (Hoffmann *et al.*, 2000). Considering this case where there seems to exist a delay in the over all cytokine production in this particular animal model used, IL-10 level is expected to rise in far later time than the other cytokines (IL-12, INF- γ , IL-4) measured. That is a time that may go beyond 16 weeks *pi*. For shortage of the cytokine kits, only cytokine levels from T1 (the time before infection) up until 16 weeks *pi* were measured.

Sturrock *et al.* (1984), in their studies on vervet monkeys, have noticed dose-dependence in many of the subsequent parasitological, hematological and pathological responses following infection of groups of monkeys with different numbers of cercariae. Taking this study into

account, this experiment was designed in a way that gives different number of low dose cercariae to three groups of monkeys. What anticipated was a significant difference in the level of the cytokines produced between groups after infection, but a significant difference after infection was observed only in the minority of the cases. Kruskal Wallis test for the three groups of monkeys with the control group gives rise to a statistically significant difference ($P < 0.05$) in the level of cytokines at T2 ($p = 0.005$) and T3 ($P = 0.006$) for IL-12; at T2 ($P = 0.015$) and at T4 ($p = 0.002$) for IL-4; and at T4 ($P < 0.001$) for IL-10. Comparing each group with every other group using the Mann Whitney U test confirmed a statistically significant variation only in few of the cases in which the difference is distributed almost equally between Groups I and II, Groups I and III and Groups II and III. Thus, it seems the number of cercariae plays a minor role in bringing a significant variation in the level of cytokines produced by this group of monkeys (grivet monkeys). But one should bear in mind the fact that the three groups of monkeys infected differ by only 100 cercariae and this might not be enough to bring a significant difference in the overall parasitological, pathological and immunological response of this particular species of monkey. Future investigations drawn along the same line are expected to tell us the optimal number of cercariae that brings a noticeable difference between groups of experimental animals that receive different doses of cercariae.

7. Conclusion and Recommendations

7.1 Conclusion

The grivet monkeys (*Cercopithecus aethiops aethiops*) exhibit most of the clinical manifestations typical of *S. mansoni* infection in man: The appearance of bloody stool, failure to eat well, and poor general appearance are just some of the symptoms observed. Considering these features and the fact that the monkeys remain positive for *S. mansoni* infection for a very long time, it can be said grivet monkey could serve as suitable experimental model for the study of the acute stage of infection by *S. mansoni* and for studies in which prolonged, stable infections are desired.

Grivet monkeys exhibit a more or less similar trend in their production of Th1 vs Th2 cytokines with that of other experimental models. Thus, they may serve as good models for the study of the immune response of mansonian infection. Besides, as was shown in other studies and partly in this study, the grivet monkeys exhibit a closely similar immunopathology with that of baboons, labs favorite primate models. Since grivet monkeys are easy to handle and less costly to keep them in the lab, they may prove good substitutes for baboons for the study of infection by *S. mansoni*.

A 100 cercarial dose difference between groups of grivet monkeys fail to bring a significant difference in both clinical manifestations and cytokine profile. Thus, future trials on the same line should be tried with cercarial doses greater/less than this in order to determine the optimal cercarial number that can bring a significant difference in the overall immunopathogenesis of mansonian infection between groups of animals.

7.2 Recommendations

- Further studies on whether cytokine levels could be affected by different cercarial doses need to be investigated using kits designed to measure cytokines levels in primates other than humans
- Other protocols which are designed to measure cytokines produced by PBMCs upon activation by SEA or SWAP are recommended to increase the probability of detection of cytokines in sera of experimental animals.
- In order to have a better picture of the overall immune response, determination of the humoral response of the infection by *S. mansoni* is strongly recommended.

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