

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

**Preliminary studies on Immunopathogenesis of *S. mansoni* in
Grivet monkeys (Cercopithecus aethiops) Vaccinated with 20Krad
Irradiated *S. mansoni* cercariae**

By
Workineh Torben

January 2005

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Grivet monkeys (Cercopithecus aethiops) vaccinated with 20Krad
Irradiated *S. mansoni* cercariae**

M. Sc THESIS

**A Thesis Submitted to the School of Graduate Studies, Addis Ababa
University in Partial Fulfillment of the Requirements for Masters of Science
Degree in Biomedical Sciences (Parasitology)**

**Under the supervision of Asrat Hailu, Professor of Immunoparasitology, in
Department of Microbiology, Immunology and Parasitology (DMIP), Faculty
of Medicine, Addis Ababa University.**

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

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

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ACKNOWLEDGEMENT

I am greatly thankful to acknowledge Asrat Hailu (Professor of Immunoparasitology, AAU), my principal adviser, for his close supervision, brotherly and very kind assistance. In addition to his day-to-day supervisions, he provided most of the costs for immunochemical and animal feed. Therefore the successes in the work are all due to the concerns and very encouraging assistance of Asrat Hailu. Eduardo Mourne Jose do Nascimento, from Brazil has also tried to have collaborative supports for the study. Thus I owe hearty thanks to him, especially for his helpful comments on my work. He has also tried to provide some immunochemicals, but due to logistic problems we couldn't make use of the reagents from Eduardo.

My special thanks and appreciation also goes to the Leshmania lab technicians of IPB, especially W/o Woineshet Mekonnen, W/o Bayiasu G/Medihin, and W/t Addis Yifiru for their very consistent help in all my work. My friends Dr. Jofe Abu, Daniel Elias, Jiksa Mulissa, Abebe Muche, Gezahagn, Tariku Demelash, Tadesse Kebede, and especially Tamrat Abebe for his kind help during trapping monkeys from field and having his comments on my work.

I also appreciate my instructors; especially Dr Beyene Petros, Dr Mekuria Lakew and Birhanu Erko for enabling me understand Immunology and Biology of schistosomes.

I am very thankful to the Ethiopian Science and Technology Commission; especially the Manager of STEP (Sterile Tsetse Eradication Program) at Kaliti, Dr Solomon Mokonnen and W/t Bethelehem Bizuneh for their kind cooperation during irradiating *Schistosoma mansoni* cercariae.

I am also thankful to Dr. Gobena Ameni and Mr. Mengistu Leggesse from IPB, for their personal encouragement and sharing some helpful instruments. I specially thank Nega Niguse from IPB, for his assistance during trapping monkeys and collection of snails from field, IPB Administration and animal attendants who were with me throughout my work especially during blood collection, and others related to handling monkeys.

I appreciate and thank the cooperation of the department of Pathology Medical Faculty (AAU), especially Dr. Senait Ashenafi for her kind help in the histopathological work; sectioning and preparation of the slides of liver histology.

I would also like to thank School of Graduate Studies for partly funding the research, Department of Biology, especially Dr Kifle Dagne for facilitating my work.

Finally my special appreciation is also extended to my mother W/o Mulatua Gamachu; my brothers Mekonnon Torben, Sori Torben and Birhanu Torben and Dr. Zergabachew Asfaw for their encouragement and financial support for my study and living costs.

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LIST OF ABBREVIATIONS

Sm28GST	- <i>Schistosoma mansoni</i> 28 Glutathione-S-transferase
Sj28GST	- <i>Schistosoma japonicum</i> 28 Glutathione-S-transferase
Sm97	- Myofibrilar protein Paramyosin
Irv5	-Irradiated vaccine antigens five
TBI	-Trios-phosphate isomerase
Sm14	- <i>Schistosoma mansoni</i> fatty acid binding protein fourteen
EPG	-Egg per gram stool
IFN- γ	-Interferon gamma
TNF- α	-Tumor necrosis factor alpha
SPSS	-Statistical Package for Social Sciences
IL-4	-Interleukin 4
IL-7	-Interleukin 7
sTNF-RII	-Soluble tumor necrosis factor receptor two
IL-10	-Interleukin 10
Sm-p80	- <i>Schistosoma mansoni</i> protein eighty
IL-6	-Interleukin 6
BSA	-Bovine serum albumin
PBS	-Phosphate buffered saline
IL-12	-Interleukin 12
ADCC	-Antibody dependent cellular cytotoxicity
Th1	-T helper 1
Th 2	- T helper 2
mAb	-Monoclonal antibody

SEA	-Soluble egg antigens
SWAP	-Soluble adult worm antigen preparations
HSC	- Hepatic satellite cells
IL-2	-Interleukin two
UV	-Ultra violet
pNPP	- Para-nitro phenyl Phosphate
ELISA	-Enzyme linked Immunosorbent assay
IgM	-Immunoglobulin M
IgA	-Immunoglobulin A
IgG1	-Immunoglobulin G 1
IgG2	- Immunoglobulin G 2
IgG3	-Immunoglobulin G 3
SmEA	- <i>Schistosoma mansoni</i> egg antigen
IgG4	-Immunoglobulin G 4
IgE	-Immunoglobulin E
PBMC	-Peripheral blood mononuclear cells
p38	- <i>Schistosoma mansoni</i> egg antigen thirty eight
PIII	-Fraction protein three of SWAP
CTB	-Cholera toxin B subunit
FGS	- Female genital schistosomiasis
BCG	-Bacilli Calmette-Gue´rin
MAPs	-Multiple antigen peptides

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ABSTRACT

In this study, we established *S. mansoni* infection in the primates, *Cercopithecus aethiops aethiops* (*Grivet monkeys*). Most of the clinical manifestations in human schistosomiasis like fever, bloody diarrhea, loss of appetite, and swollen lymph nodes were noted in the experimentally infected monkeys. Due to the absence of the appropriate animal models, there is little understanding of immune-response development in schistosomiasis, which has created a challenge for designing an effective anti-schistosome vaccine. Repeated intraperitoneal immunization of the monkeys with 20Krad irradiated *S. mansoni* cercariae resulted in a relatively less granulomatous reaction and EPG ($P < 0.05$) than in the controls. The difference in the total adult worms is statistically not significant ($P > 0.05$) between both groups of monkeys. In primary infection, IL-4 was significantly ($p = 0.03$) raised in the immunized monkeys, and for the same group an insignificant ($P > 0.05$) increases in IL-10. However, ova excretion did not have an influence on cytokines, except for the controls both IL-4 and IL-10 are significantly increased ($p < 0.05$). Both IL-12 and INF- γ levels were lower after ova excretion in the controls, but the inflammatory TNF- α has increased ($P = 0.049$) and these can be associated with more liver pathogenesis in the group. The immunization has minimized egg production and the development of granuloma in the liver of *Grivet monkeys*. Thus, this work has underlined the potential importance of the monkeys as the helpful models and provided a direction for research studies of drugs or anti-schistosome vaccines.

1. INTRODUCTION

1.1. Background

Schistosomiasis (or Bilharziasis) is one of the most prevalent tropical diseases, and a leading cause of severe morbidity in large areas of the world (TDR, 1997). It is the oldest trematode infection of humans, which belongs to the family *Schistosomatoidae* and genus *Schistosoma*. About eighteen species of the genus are known, out of which human schistosomiasis is mainly caused by five species (WHO, 2004). These are *Schistosoma haematobium*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma mekongi* and *Schistosoma intercalatum*. These are the group of flat worms that deposit eggs in blood vessels surrounding the gut or bladder of the infected hosts. There are also other minor species affecting man, as well as some mammals that may cause accidental infections, or cercarial dermatitis. The first three species, *S. mansoni*, *S. japonicum* and *S. haematobium* are the major blood flukes that are commonly pathogenic to humans. They are the most important ones in terms of prevalence and public health importance; *S. intercalatum* and *S. mekongi* have a limited distribution in the world.

1.2. Epidemiology and Global distribution of Schistosomes

Among parasitic diseases, schistosomiasis ranks globally second to malaria. The disease is estimated to affect millions of people living in 76 tropical and subtropical countries with more than 200 million being infected and 600 million people at a risk of infection. About 80% of the infected people are living in Africa (WHO, 1997). It globally claims an estimated 500,000 deaths

per year (Bergquist *et al.*, 1994), and the latest estimate for sub-Saharan Africa is more than 200,000 (Utzinger & Keiser, 2004).

The transmission of the disease is favoured by the tropical climate, presence of appropriate snail hosts, poor sanitary conditions, fresh water resource development or irrigation and lack of health facilities in most of the developing countries. It is well spread in areas where agricultural and fresh water development is occurring in Africa, South America and Asia. The global distribution of *S. japonicum*, *S. haematobium*, and *S. mekongi* is shown on (**Figure 1**). *S. japonicum* is found in the Far East, particularly in China and the Philippines. In Indonesia it is found in a few isolated valleys in central Sulawesi. Means of transmission is by amphibious *Oncomelania* snails, and water buffalos serve as reservoir hosts. *S. haematobium* is found in large parts of Africa, parts of Arabia, the Middle East, and Khuzestan province in Iran, Madagascar and Mauritius. It is transmitted by *Bulinus* species. *S. mekongi* is found in limited areas of Laos and Cambodia.

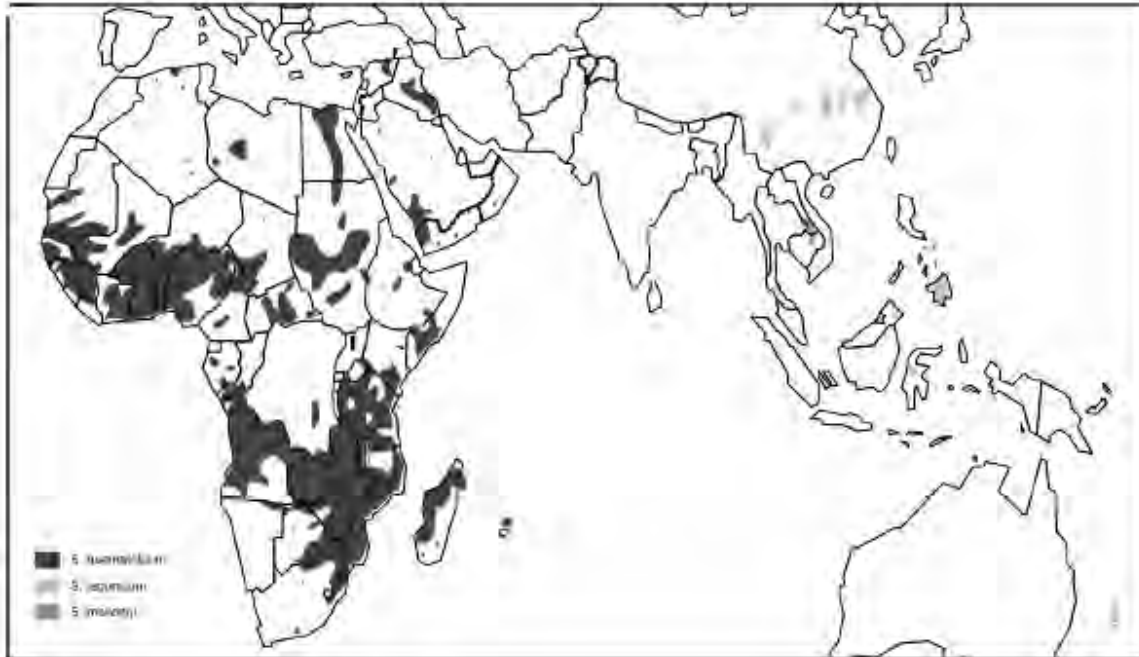


Figure 1 Global distribution of *S. haematobium*, *S. japonicum* and *S. mekongi*

The Control of Schistosomiasis, WHO Technical Report series 830; image WHO 92721 (cited in Blanchard, 2004)

S. mansoni is found in many countries in Africa, it is the only species in Latin America (Brazil, Surinam, and Venezuela), and the Caribbean including Puerto Rico, St Lucia, Guadeloupe, Martinique, Dominican Republic, Antigua and Montserrat and in parts of the Middle East. It is transmitted by *Biomphalaria* species of snails. *S. intercalatum* occurs in ten countries in the rain forest belt of Africa. The global distribution of both *S. mansoni* and *S. intercalatum* is also indicated on (Figure 2).

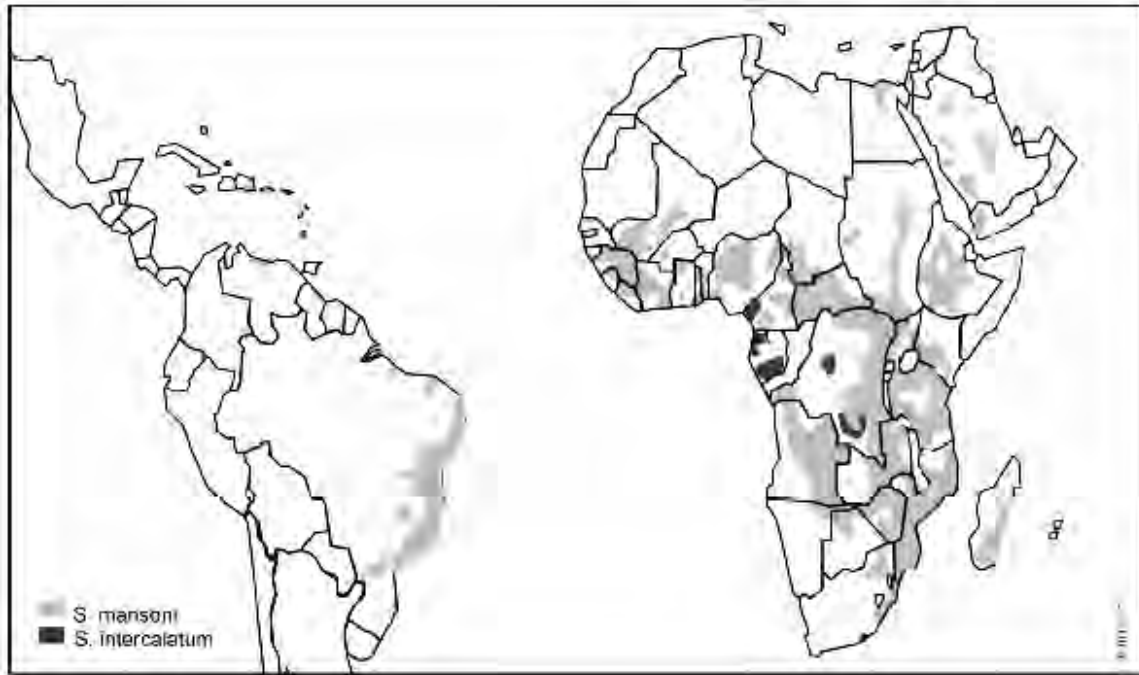


Figure 2 Global distributions of *S. mansoni* and *S. intercalatum*

The Control of Schistosomiasis, WHO Geneva 1993: image WHO 92720(cited in **Blanchard, 2004**)

In Ethiopia both intestinal and urinary schistosomiasis occur (Figure1 and Figure 2). *S. mansoni* is transmitted by *Biomphalaria pfeifferi* and *B. sudanica*; while *S. haematobium* is transmitted by *Bulinus abyssinicus* and *Bu. africanus* (Lo *et al.*, 1988). According to 1980s estimate, there were about 18 million people at risk of infection with *S. mansoni* and 2.5 million people infected and 4 million people at risk of infection with *S. haematobium* in the country (Lo *et al.*, 1988; Kloos *et al.*, 1988). The current status of the disease is unknown. The disease has continued to increase in magnitude and to spread to new localities especially in connection with water resource development and unlimited population movements (Lo *et al.*, 1988; Kloos *et al.*, 1988; Erko *et al.*, 1996). Thus in order to establish integrated and sustainable control mechanisms epidemiological studies have to be conducted in the country.

1.3. Schistosome life cycle and transmission

The life cycle of most human schistosomes is basically similar (Figure 3). It involves an intermediate snail host and a definitive mammalian host. Humans are the principal definitive hosts for the major five schistosome species. The parasitic and the free-living stages alternate and the infective forms, cercariae develop within freshwater snails. Eggs passed out in the urine or stool of infected hosts and in fresh water miracidia emerge from the eggs and infect snails. Inside the snails it undergoes asexual development to first stage (mother) sporocyst, and second stage (daughter) sporocyst and finally emerges as a forked-tailed infective cercariae.

After exiting from the specific vectors, the cercariae can actively swim for (8 - 12) hrs until they come in contact with human or any other susceptible host coming in contact to the water. They penetrate the skin and transform to the next other form of larvae, the schistosomulum which subsequently migrate to lungs, 5 - 7 days post penetration and then to hepatic portal circulation after about 15 days where the worms can develop to male and female adults after 28 days. The adults establish their residence in mesenteric circulation of the intestine or urinary bladder where they pair, mate, and produce large number of eggs after about 32 days. Some of the eggs are excreted in either stool or urine and end up in the water supply, where they hatch and complete the cycle by infecting new snail hosts.

But those eggs that are not excreted become trapped in the tissues of the liver, spleen, intestine and bladder, where they become calcified. Over time the accumulation of thousands of eggs causes severe and irreversible damage to the organs.

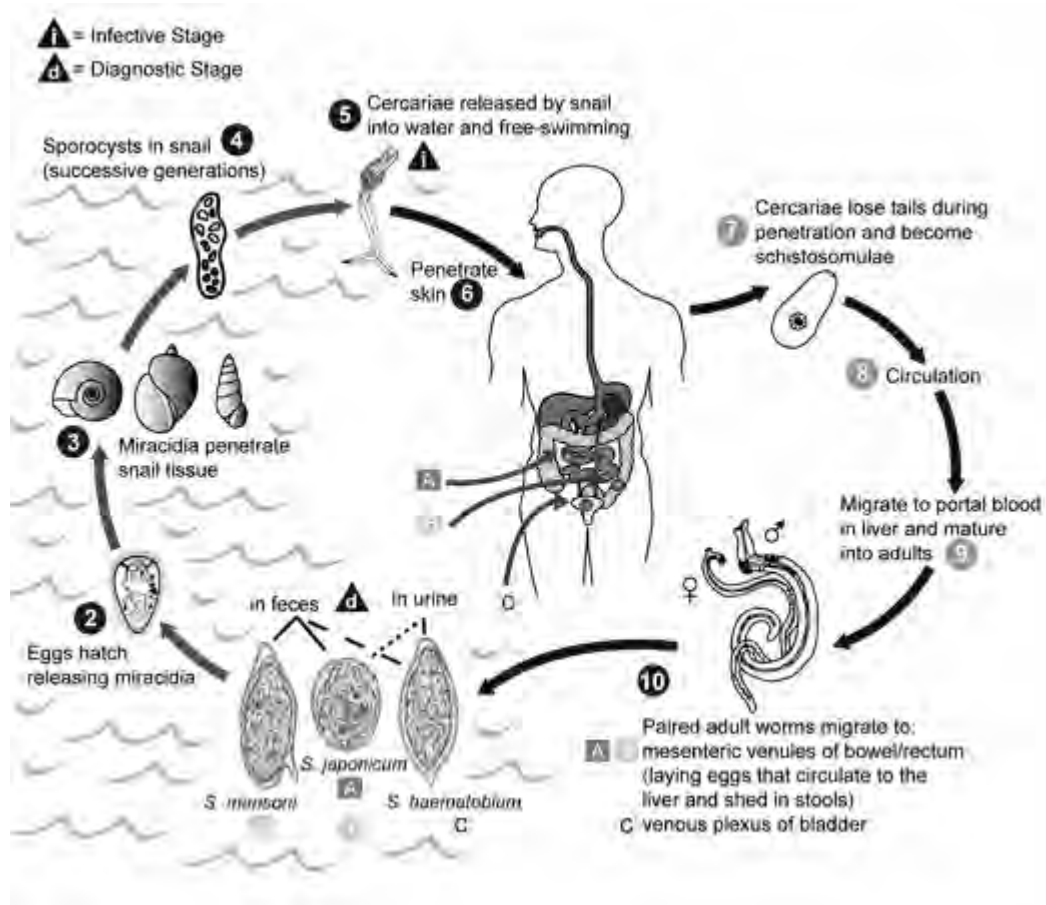


Figure 3 The life cycle of the three common human Schistosomes

CDC. <http://dpd.cdc.gov/dpdx>

1.4. Pathogenesis

Adult male and female schistosomes pair and live together in portal blood vessels. The females release eggs, some of which are passed out in the urine (*S. haematobium*) or stools (*S. mansoni* & *S. japonicum* or *S. mekongi*), the rest are trapped in body tissues. The accumulation of thousands of the eggs causes severe and irreversible damage to important organs. The major cause of diseases is due to immune reactions to eggs lodged in tissues of the hosts. Death is mostly associated to bladder cancer due to urinary schistosomiasis and to bleeding from varicose veins in the esophagus associated with intestinal schistosomiasis.

Three major disease syndromes occur in schistosomiasis, which are coinciding with different stages of development of the parasite within the host. These are acute form dermatitis, Katayama fever, and the chronic fibro obstructive sequelae. The penetrating cercariae have been associated to schistosome dermatitis or swimmers itch. When the worms have matured and egg deposition begins, acute schistosomiasis or Katayama fever will be observed. This is a serum sickness like and usually seen in heavy primary infections. Especially in *S.japonicum*, it may be due to immune complex formation initiated by the massive antigenic challenge produced by eggs (Bethlem *et al.*, 1997).

In chronic schistosomiasis mature worms produce large number of eggs and some of which remain in the body. Due to the habitat of *S. mansoni*, *S.japonicum*, and *S. mekongi*, intestine is primarily involved, and the releases of eggs result in the secondary involvement of the liver. In *S. haematobium* infection urinary tract is mainly affected. The host reaction to eggs retained in the tissues results in granuloma formation consisting mainly lymphocytes, macrophages, and eosinophils. In *S. mansoni* and *S. haematobium* infections the granulomatous response is

immunologic reaction of the delayed-hypersensitivity type (Warren *et al.*, 1978 & Kassis *et al.*, 1978). The host granulomatous response in schistosomiasis is regulated by several immunological mechanisms that involve the development of Th1 and Th2 responses and the production of several cytokines locally within the granulomas and in the system of hosts (Mwatha *et al.*, 1998), and the responses have shown to be regulated by multiple immune mechanisms.

In urinary schistosomiasis (due to *S. haematobium*), damage to the urinary tract is revealed by blood in the urine. Urination becomes painful and is accompanied by progressive damage to the bladder, ureters and then the kidneys. Bladder cancer is common in advanced cases.

In intestinal schistosomiasis (infection with *S. mansoni*, *S. japonicum*, *S. mekongi*), disease is slower to develop. There is progressive enlargement of the liver and spleen, intestinal damage due to fibrotic lesions around eggs lodged in these tissues, and hypertension of the abdominal blood vessels. Bleeding from these vessels leads to blood in stools, and can be fatal. The patients become seriously weakened by the disease and, in some cases, the functioning of organs such as spleen and kidneys become impaired.

The eggs trapped especially in the liver of an infected host cause the major pathological manifestations of schistosomiasis. Miracidia within the deposited eggs secrete soluble egg antigens (SEA) that induce periovular granuloma formation, which may lead to severe hepatic fibrosis. In general, most of the related diseases or pathology of schistosomiasis are summarized in the next subtopics.

1.4.1. Cercarial Dermatitis

Schistosome dermatitis usually occurs within twenty-four hours after penetration of the cercariae. It is a pruritic popular skin rash known as swimmer's itch. The eruption may arise at the site of cercarial penetration of the skin. This condition is not common among those living in endemic areas, but migrants or visitors who had previous history of infection may develop dermatitis within a few hours of re-exposure. The problem is similar to swimmer's itch, noted in persons sensitized and re-exposed to avian or other nonhuman schistosomes found in freshwater bodies all over the world (Warren, 1973). But for some schistosome species like *S. japonicum*, it is not known if re-exposure is a feature of cercarial dermatitis.

1.4.2. Role of Schistosome Eggs in Development of Disease

Acute schistosomiasis, first described in Japan as Katayama fever (Warren, 1973), is common throughout the high transmission risk areas of endemic countries. The disease appears an average of 41.5 days after individuals are exposed to a first infection or a large re-infection or super infection (Chen & Mott 1989; Kane & Most, 1948). The timing of disease onset appears to relate to initiation of egg production by young female worms, some individuals present with nocturnal fever peaks, coughing, generalized muscle pain, headache, and enlarged liver.

Worms are not thought to be responsible for disease. However, host responses to various secreted worm products and larval miracidia in eggs account for most pathological effects. The acute form of the disease Katayama fever coincides with the beginning of oviposition, four to eight weeks after infection. It has been estimated that each female worm of *S. mansoni* and *S. haematobium* produces about 200-2000 eggs, but in *S. japonicum* it can be up to 3,500 eggs per day. Large

proportions of eggs are trapped within intestinal and hepatic tissues and adaptive host immune responses results in fever.

The retained eggs induce cell-mediated granulomatous reaction that accumulates to produce the pathology of chronic disease (Asahi *et al.*, 1999; Van De Vijver *et al.*, 2004). The immunodominant T cell epitope characterization of the specific Th responses during murine *S. mansoni* of the major *S. mansoni* egg antigen p38 (Chen & Bros, 1998), and the generation of Th1 or Th2 type cytokines and granuloma in response to the single immunodominant epitope (Chen & Bros, 1999) also indicated that most of the pathology due to schistosomiasis is egg induced.

The colon, especially the recto sigmoid area, and the left lobe of the liver are usually the most affected, and the number of eggs accumulating can be huge. An autopsy report has indicated up to 1 billion eggs could be recovered from liver tissue of a schistosomiasis victim (Chen & Mott, 1989).

The miracidium larvae in the eggs matures over 5 days and remain alive for up to 20 days, secreting enzymes and other toxic products that elicit intense inflammatory responses. A characteristic perioval granuloma forms, with a necrotic center containing the egg or egg cluster surrounded by epithelia cells, giant cells, and lymphocytes and an outer layer of plasma cells, eosinophils, and fibroblasts. Single eggs are usually reabsorbed, but the tissue damage leads to fibrosis. Large egg clusters tend to calcify. Perioval granulomas have been found in many tissues, including skin, lung, brain, and muscle. They have also been noted in the adrenal glands and the urogenital system of both sexes. Most granulomas develop at the sites of maximum egg accumulation, the intestine and liver.

A number of reports (Cheever *et al.*, 1994; Dunn *et al.*, 1994; Jankovic *et al.*, 1998 and Wynn *et al.*, 1998) from animal experiments show that host responses to schistosome eggs are modified over time and vary according to host and schistosome species, tissue, and infection history. The phenomena of modulation or the decrease in the volume of granulomas near recently laid eggs containing viable mature miracidia have been reported. Modulation in humans is not yet well known; but animal models reveal a complex interplay of regulatory cytokines, tumor necrosis factor, various immune effector cells, and fibroblasts, all subject to genetic influence. The balance of cellular and humoral influences on modulation may vary with the chronicity of the infection, with humoral activity dominating in the later stages of disease.

1.4.3. Intestinal and Hepatosplenic Diseases

The worms migrate frequently within the mesenteric veins but are thought to favor certain locations to lay eggs, usually in clusters. Egg clusters aggregate and induce mucosal inflammation, hyperplasia, ulceration, micro abscess formation, blood loss, and pseudopolyposis (Chen, 1991; Cheever *et al.*, 1982; Chen *et al.*, 1978 & Faust & Meleney, 1924). Lower abdominal pain is frequent, often colicky, and usually referred to the left lower quadrant. Diarrhea is common, usually with occult blood. Sometimes blood is visible in the stools, and diarrhea may alternate with constipation. Diarrhea is particularly notable in children, and despite the well-known link with viral and bacterial infections, its presence remains a powerful predictor of chronic Schistosome infection (Zhou *et al.*, 1998). In severe cases of chronic disease, bowel fibrosis and stenosis result, most often in the lower colon and rectum, and the mesentery may thicken to form an abdominal mass. Some evidences suggest that the most serious consequence of intestinal schistosomiasis is colorectal cancer.

Schistosome eggs that do not pass through the mucosa to reach the intestinal lumen are either trapped or swept up in the portal blood flow. Eggs reaching the liver are too large to reach the sinusoidal plexus and accumulate in presinusoidal venules within the portal triads, especially in the left lobe. There they induce granulomatous inflammation, fibrosis, venous obstruction, portal hypertension, and splenomegaly. Liver enlargement initially tends to correspond in size to the intensity of concurrent or recent infections. Thus, hepatomegaly reflects granulomatous perioval inflammation rather than consequent fibrosis and occurs early in the evolution of chronic disease (Olds *et al.*, 1996). Hepatic fibrosis in human *S. mansoni* was suggested to be associated with type 2 cytokines; IL-5, IL-10, IL-13, IFN- γ , TNF- α , and transforming growth factor beta (de Jesus *et al.*, 2004).

Modulation of the inflammatory response that has been observed in murine models is expected to occur in humans. Thus, it may partly account for the decrease in liver size that is often observed after the initial enlargement. Spleen enlargement may appear early in the course of chronic infection but is usually minor and reflects cellular hyperplasia without granulomatous inflammation. Enlarged spleens rarely contain schistosome eggs. In some cases, severe hypersplenism is noted and is the usual indication for splenectomy.

Perioval granulomas in the liver lead to fibrosis. The links between granulomatous inflammation, subsequent fibrosis and its persistence are complex (Cheever, 1997). Collagen deposition, cross-linking, contraction, and re-absorption are in dynamic balance and each component is subject to immune regulation. Most of the information available is from murine models and indicates that the determinants of granuloma size may frequently dissociate from those of hepatic fibrosis.

The hepatosplenic end product of chronic schistosomal infection is the final result of the immunoregulated host response to the sustained intravascular eggs on the liver. Eventually, after years or decades, gross periportal fibrosis appears along with presinusoidal portal hypertension and secondary gastroesophageal varices. The liver surface resembles clay pipe stems due to the wide bands of fibrous tissue extending along the portal tracts.

The parenchymal form of hepatic fibrosis is common in *S. japonica*. And it may persist as a common sub clinical finding in populations treated so frequently that overt liver disease has resolved (Ross *et al.*, 1998). Most populations in affected communities had detectable hepatosplenic schistosomiasis. Hepatocellular function was usually well maintained, but patients with portal hypertension often develop ascites and are prone to gastroesophageal hemorrhage, which is the common cause of death. Hepatic coma is a frequent and usually fatal consequence of upper intestinal bleeding, and it also occurred in some post surgical patients (Chen & Mott, 1989). These forms of severe disease are now uncommon. However, weakness, diarrhea, growth retardation, hepatomegaly, and liver fibrosis are still common in endemic areas, especially in the lake and marshland zones (Li *et al.*, 2000; Olds *et al.*, 1996, and Ross *et al.*, 1998). All improve with chemotherapy but recur with re-infection.

1.4.4. Cerebral Schistosomiasis

Cerebral disease can be caused by the host reactions to schistosomes eggs. Some persons with eggs in the central nervous system develop no symptoms. The mechanism of egg deposition is unknown, but their presence suggests that eggs may cross the blood-brain barrier or that some worm pairs may reach the venous side of the cerebral circulation (Warren, 1973). However, there are no recent evidences to support both theories

Symptoms of cerebral schistosomiasis were found in American soldiers infected in the Philippines during World War II (Kane & Most, 1948). Among this group, epilepsy mostly appeared within the first year. In a few patients, cerebral schistosomiasis and cysticercosis may occur together, praziquantel therapy has indicated to cure seizures caused by neurocysticercosis (Vazquez & Sotelo, 1992).

1.4.5. Cancer and Schistosomiasis

Some studies which connect *S. japonica* infections to esophagogastric cancer or carcinoma of the liver was hardly accepted (Chen & Mott, 1989). The diseases can occur together but may not be related. Some studies are also relating colorectal cancer and schistosomiasis. On aggregated data, the incidence of colorectal cancer correlates with prevalence and intensity of schistosome infection. Affected individuals usually have a long history of inflammatory large bowel symptoms and schistosome infection. The cancers correspond in anatomic location to the large bowel area most affected by schistosomiasis, the age of onset is lower in schistosome endemic areas, and the length of survival is shorter. Correlation of schistosomiasis and colorectal cancer has also been reported from Japan (Polderman *et al.*, 1984), but later further studies did not support the association of the diseases (Abanilla, 1986). In patients with colonic schistosomiasis, epithelial dysplasia is common and may be severe, but the mechanism of relating schistosome infection to colorectal cancer is not well known.

1.4.6. Other Problems of Schistosome Infection

Female genital schistosomiasis (FGS) is a frequent complication in women with urinary or systemic schistosomiasis, particularly in geographic areas where the disease is endemic. *S.*

haematobium is the organism most frequently identified in these cases. Persistent, untreated infections may lead to increased susceptibility to sexually transmitted diseases, or even sterility. A case of FGS involving the uterine cervix has been reported (Kameh *et al.*, 2004), in which speculum examination of a patient revealed a friable cervical lesion. Both the cervical smear and biopsy contained intact, viable schistosome eggs consistent with those of *S. haematobium*.

Extragenital or ectopic lesions may again occur and are most frequently caused by *S. haematobium*. For example, mildly pruritic skin lesions with localized eruptions on a side of a patient's abdomen have been reported (Khalid *et al.*, 2004). A punch biopsy was taken from the main lesion and showed several ova with outer shells having apical spines in the dermis. The eggs were surrounded by a dense chronic inflammatory infiltrate with abundant eosinophil. But clinically, the ectopic skin lesions present as asymptomatic or pruritic firm papules, which was not associated with clinical findings or definite laboratory evidence of active visceral schistosomiasis. Therefore the cases indicate that ectopic skin disease may occur irrespective of the presence of other internal organ involvements.

The exact mechanism for the deposition of schistosomes' ova in the skin is not clearly known. But as it was suggested by (Faust, 1948), anastomoses between venous systems are associated with migration of ova or adult worms to ectopic sites.

1.5. Schistosomiasis Immunology

In their vertebrate hosts, schistosomes are non-replicating complex multi cellular pathogenic worms. The parasite flourishes in the human host despite the development of pronounced immune responses (Pearce & MacDonald, 2002). Understanding how the immune system deals with such pathogens is not clearly known. Thus, the pathogens are causing chronic disease globally in millions of people.

Anti-schistosomes resistance is not complete; and the partial protection is expressed as the percentage reduction in the number of adult worms that develop upon challenge infection. In mouse models, the Th1 and Th2 responses in protective immunity has been described, and in murine schistosomiasis the establishment of chronic infection has also shown to be associated with Th2 responses. In human beings, Th2 responses are associated with resistance to re-infection. However, most immunological studies, especially on naturally acquired resistance in endemic normal individuals have provided evidence that both Th1 and Th2 responses develop protection against *S. mansoni* infection (Correa_Oliveira *et al.*, 2000). Similarly recent studies on some cytokine deficient mice have provided the relevance of both type-1 and type-2 responses to resistant generated by the various schistosomiasis vaccine candidates (Wynn & Hoffmann, 2000).

In recently exposed communities, intensity of schistosomiasis infection increases as children age and then drops again in adulthood, indicating that host maturity is an important aspect of resistance to schistosomiasis. For example, in endemic areas, the infection intensity increases as children age, to a peak between 8 and 15 years and then drops in adulthood (Woolhouse, 1999). This pattern was also indicated in recently exposed communities where children and adults have

been exposed for the same number of years and have had similar levels of day-to-day exposure (Scott *et al.*, 2000). This indicates that host maturity is an important aspect of resistance to schistosomiasis, independent of resistance acquired through cumulative experience of infection, which was also supported by other reports (Scott *et al.*, 2002). A description of the cellular immune response of children and adults with *S. mansoni* infection (Scott *et al.*, 2004), indicates that children and adults do have different capacities to mount a response to schistosomes infection.

The condition of partial resistance or concomitant immunity by actively infected experimental hosts was reported to occur in schistosome challenge infections of mice. Based on epidemiological studies, concomitant immunity also occurs in human beings (Butterworth, 1987). The worms can persist for many years in the immunocompetent host. However, infected individuals can develop resistance to superinfection.

The parasite induce a dominant, distinct, polarized Th2 response that is intimately involved in the development of many of the pathological changes that accompany infection, but which also allows host survival while infected. The schistosomular surface glycoproteins themselves induce T cell dependent antibody isotypes that do mediate killing. So response induced by eggs, protects incoming migrating larvae.

Although schistosomes typically induce a pronounced T_H2 response, it is the development of balanced Th cell responses that is most important to prevent disease progression (Wynn & Hoffmann, 2000); both T_H1 and T_H2 components, if excessive, can lead to damaging pathology (Hoffmann *et al.*, 2000). The T_H2 response to Schistosomes is initiated by the egg stage of the

parasite, and carbohydrates on egg antigens. Dendritic cells that are exposed to schistosome egg antigens are not activated in a conventional manner, but they are able to potently induce T_H2 responses.

A glycoprotein from soluble extracts of *Schistosoma mansoni* eggs (smEA) triggered basophils of non-sensitized donor individuals to release IL-4 (Haisch *et al.*, 2001). This may result the concept that IL-4 inducing activity may be released from eggs, thus the development of Th2 immune responses could be observed after the release of eggs in hosts.

IgG4 is known to interfere with IgE-mediated mast cell degranulation and may therefore reduce the harmful consequences of schistosome induced allergic reactivity (Khalife *et al.*, 1989). Moreover, IgG4 was also indicated to block IgG1 and IgG3-mediated killing of schistosomula by human eosinophils in vitro.

Treatment has shown to increase parasite-specific immunoglobulin E (IgE) and other Th2 responses that have been associated with subsequent resistance to re-infection in the months following chemotherapy (Correa_Oliveira *et al.*, 2000). After praziquantel therapy a rapid increase in interleukin-5 levels in plasma but decreased levels of eosinophilia and worm-specific immunoglobulin E was reported (Fitzsimmons *et al.*, 2004). This indicates the different patterns of immune reactions to treatment.

Antibody-dependent cell-mediated cytotoxicity (ADCC) has been shown to efficiently kill newly transformed schistosomula in vitro (Capron & Capron, 1994) with immunoglobulin E (IgE) and subclasses of IgG or IgA acting in conjunction with effectors like macrophages, eosinophils and

platelets. This was also shown in primate and human schistosomiasis as well as in a semi permissive rat host. However, the evidence for efficiency of antibody dependent cell mediated cytotoxicity (ADCC) mechanisms in vivo is still limited.

Natural regulatory T cells and, to a lesser extent, Th2 cells have been shown to play roles in suppressing Th1 responses and ensuring Th2 polarization during schistosomiasis (McKee & Pearce, 2004). Mice infected with *S. mansoni* developed polarized Th2 responses in which Th1 responses were prevented by IL-10-mediated suppression of IL-12 production. IL-10 can reduce immunopathology in many persistent infections. Innate effectors and regulatory T cells producing IL-10 may cooperate to reduce morbidity and prolong survival in schistosomiasis (Hesse *et al.*, 2004).

The complexity of the host–parasite relationship and little understanding of both immune-response development and parasite biology make the designing of an effective anti-schistosome vaccine a problem. Therefore studying the immunology of schistosomiasis especially in the human equivalent primates like *Cercopithecus aethiops* (Monkeys) may provide an important guide for further appropriate vaccine and drug research.

1.6. Immune Evasion Mechanisms

Helminthes are large, multicellular parasitic worms that are extra cellular but cause in so many diseases. Protection has been thought to be both humoral and cell-mediated immunity (Jankovic *et al.*, 1999). However, schistosome species have evolved different protective or immune evasion mechanisms against the host.

The surface of larval and adult schistosome tegument that is in intimate contact with the host immune system is the unique immune evasion organ. The parasite exhibits a remarkable ability to avoid immune destruction while being consistently bathed in host antibodies and immune effector cells. The outer layer of the schistosomes' surface membrane has reduced surface antigenicity to escape immune recognition (El-Ansary, 2003), and the development of a tegument that is resistant to immune damages or it is replaced if damaged by immune attacks (Pearce and Sher, 1987). The surface antigens of schistosomes are also known to be stage specific (Simpson, 1990).

The tegument also adsorbs host serum and RBC antigens rapidly. Schistosomes can also induce ineffectual or blocking antibodies, for example surface of migrating or schistosomule larvae has glycoprotein antigens that share carbohydrate epitopes with large polysaccharide antigens in parasite egg. These polysaccharides induce T cell independent antibody responses that react with the schistosomule surface. In humans these are IgM & IgG2, but they cannot mediate eosinophil killing of the schistosomule, and 'block' the killing mediated by IgE & other IgG subclasses. During cercarial infections, the antibodies produced are also reported to be against the cercarial secretions (Bahgat *et al.*, 2001), thus the induced immune responses have no role to defend the host.

The other mechanisms of evasion are by anti-immune response in which the parasites can develop proteolytic cleavage of host immunoglobulin and immunosuppressive mechanisms. Recent evidences show that the worms are not only evading immune defenses, but also actively use molecules of the immune system to grow and reproduce.

1.7. Control methods

The aim of schistosome control method is usually to reduce transmission and infection in populations to levels at least low enough to minimize the risk of serious morbidity. As a result of the relatively high cost of praziquantel, screen-and-treat campaigns (selective population chemotherapy) were considered the most cost-effective strategy in most endemic countries (WHO, 1985). However, the long-term results were often disappointing due to rapid re-infection and the expensive nature of the programs. Even though, chemotherapy is the most preferred intervention methods, re-infections need repeated treatments.

Integrated regular Primary control systems like health education, diagnosis and treatment, promotion of safe water supply, sanitation and snail control were defined (WHO, 1993). It was, however, emphasized that the first essential component should be adequate clinical care for patients presenting at a health post or clinic with early signs and symptoms. This is indicated to be the only option in countries without any form of organized schistosomiasis control (Engles *et al.*, 2002). In Morocco case detection and treatment was implemented by snail control, community based actions and intersectoral collaborations and reduced the prevalence of schistosomiasis to a point that elimination is a possibility in most endemic areas (Laamrani *et al.*, 2000).

As pathology is strongly related to intensity and duration of infection, treating early cases may also prevent most of the severe morbidity (WHO, 2002). Moreover, the treatment that may meet the demands of populations can strengthen the health system as a whole. The recent resolution of the 54th World Health Assembly (WHA 54.19, 22 May 2001) and the WHO expert committee on

schistosomiasis and soil-transmitted parasites (WHO, 2002) recommended a comprehensive approach and ensured access to treatment in primary health care services associated with regular delivery of treatment to high-risk groups, particularly school-age children, and implementation of plans for basic sanitation and safe water supplies.

In Ethiopia a pilot control trial of intestinal schistosomiasis at Fincha Sugar Estate was tried by selective treatment with praziquantel, application of Endod to control the intermediate host *B. pfeifferi* in transmission sites especially along streams (Erko *et al.*, 2003). Other inputs like transplantation of Endod cuttings, training and health education were used as helping control methods. The result has indicated some reduction in the prevalence of schistosomiasis, and intensity of infection from 1995 up to 1998 in the area. The application of *Soapberry Endod* (*Phytolacca dodecandra*) molluscicide around *S. mansoni* endemic localities in Wollo also achieved some reduction in prevalence and intensity of schistosomiasis (Erko *et al.*, 2002). This has again indicated some supplementary effect to chemotherapy in control of the disease.

Despite the possibilities of the defined methods of control, due to problems of sustainability in relation to funding (Kumar & Gryseels, 1994), logistic and the related problems, schistosomiasis remains the problem in most of the endemic developing countries. However, mass drug treatment of school age children in endemic areas has shown as a promising method to control schistosomiasis (Pearce, 2003). As different studies have also been indicating partial development of immunity against schistosomes, combination of drug therapy with vaccination might be logical for the control of schistosomiasis (Bergquist, 1995). A protective vaccine could have provided a preferable long-term solution against the disease. However, although a number of vaccine candidates have been identified, no vaccination has yet a real option.

1.8. Prospects of Vaccine development

Adults of schistosomes cause little in the way of disease, but their eggs trapped in host tissues and elicit powerful and potentially damaging immune responses. The parasites are non-replicating organisms in their mammalian hosts. Thus, partial non-sterilizing naturally acquired or vaccine-induced immunity may potentially decrease human pathology and transmission in areas where schistosomiasis is endemic, and despite the very longer efforts of researchers there is still no effective vaccine against schistosomiasis.

A number of studies have been reporting several membrane or muscle proteins, tegumental antigens, enzymes and recombinant vaccine molecules (Doenhoff, 1998; McManus, 1999; McManus *et al.*, 1999; McManus, 2000; Waine *et al.*, 1997; Waine *et al.*, 1998). Varying degrees of successes were reported for different candidate vaccines, but the results of most are proved to be not promising. Although some multiple antigen peptides like construct of sm28GST and *S. mansoni* TPI were induced B cell T cell responses and protective IFN-gamma in mice (Ferrus *et al.*, 1997), multi-epitope schistosome vaccine candidate constructs of several other antigens were tested to be inappropriate or inadequate for parasite killing in vivo (Yang *et al.*, 2001).

Recent studies are underling the potential importance of DNA vaccine candidates. The DNA vaccines of sm32 with enzymes like endopeptidase and asparaginyl induced immune responses against schistosome cysteine proteinase in mice (Chilchila *et al.*, 2002). It has an anti-fecundity effect and thus minimizes most immunopathology related to egg production. *S. japonicum* 28GST and *S. japonicum* 23GST also showed promises in sheep and water buffalos (Shi *et al.*, 2002). Partial protection or reduction in parasite counts was observed in vaccinated groups of

both hosts. Cocktail DNA vaccines of Sj62, Sj28, Sj23 and Sj14-3-3 induced Th1-type cellular immune response and high level of IFN-gamma production by splenocytes (Zhang *et al.*, 2002). Partial protection was developed in vaccinated mice against *S.japonicum* infections. Recombinant DNA vaccines with plasmids encoding IL-2 and IL-12 also induced protective immunity against *S. mansoni* (Siddiqui *et al.*, 2003). Co-injection of plasmid DNA encoding IL-12 with Sm-P80 DNA produced a higher protection level in mice. Some other recombinants like *S. japonicum* cathepsin D aspartate protease reduced worm burdens in mice (Verty *et al.*, 2001), but increased egg output per female adults. However Recombinant paramyosin (rSj-97) induced specific antibodies that reduced worm burdens and liver eggs in mice and water buffaloes (McManus *et al.*, 2002)

S. mansoni glutathione-S-transferase (Sm28GST) reduced female worm fecundity and egg viability in immunized mice (Xu *et al.*, 1991),the reduced egg production was similarly noted in immunized baboons (Boulander, 1991). Recombinant fusion protein vaccine candidate was constructed by hybrid proteins in which two dominant T- and B-cell epitopes from *Schistosoma mansoni* 28 kDa glutathione-S-transferase (Sm28GST) antigens which were fused to Cholera Toxin B subunit (CTB) to treat *S. mansoni*-infected mice (Lebens *et al.*, 2003). It has significantly reduced total worm burden and liver egg counts due to the induction of Sm28GST-specific antibodies. In addition, it has suppressed immunopathologic granuloma formation in the liver and delayed-type hypersensitivity reactions to both Sm28GST and to total soluble egg antigen in infected animals. Thus, the hybrid protein was suggested as being important as a combined anti-immunopathology and anti-infection vaccine against schistosomiasis. Mucosal administration of a *S. mansoni* Glutathione S-Transferase-Cholera Toxoid Conjugate Vaccine was also suggested to evoke antiparasitic and antipathological immunity in mice (Sun *et al.*,

1999). The studies are indicating the possibility of designing a therapeutic vaccine that may limit infection and suppress parasite-induced pathology. Very recent TDR reports are indicating that several native or recombinant proteins, synthetic multiple antigen peptides (MAPs) and DNA constructs are entering clinical trials. The leading recombinant *S. japonicum* or worm enzyme glutathione-S-transferase (GST) has successfully passed Phase I, and now it is on Phase II clinical trials in France (TDR, 2004).

Several studies demonstrated the induction of protective immunity against a challenge infection with schistosomes by immunization and subsequent exposure of mice with cercariae attenuated with either UV or gamma irradiation (Moloney *et al.*, 1985; Rodrigues *et al.*, 1999). The feasibility of vaccines that has been provided by successful vaccination-challenge experiments using attenuated cercariae infection of animal models has been supported continually. The attenuated cercariae vaccine was especially well studied in mice. It induced resistance and the immunized mice were reported to minimize worm load after challenge infection (James *et al.*, 1981). Irradiation associated vaccine antigens also achieved a higher protection in mice (Bergquist, 1995). The 20Krad irradiated *S. mansoni* cercariae vaccine was shown to be effective to induce resistance in mice. However, in order to have more knowledge about the vaccine candidates, it could be advantageous to look further how the irradiated schistosome cercariae vaccine works in primate models.

In Ethiopia, *Colobus abyssinicus* and *Papio anubis* were reported to be naturally infected with *S. mansoni* (Fuller *et al.*, 1979 cited in Erko *et al.*, 2001). The potential relevance of 20Krad of gamma irradiated cercariae vaccine was similarly assessed in the *Papio anubis* (Yole *et al.*, 1995), eliciting some level of protection in the animals. Other reports also indicated comparable

induction of protection in vaccinated baboons with the same dose of irradiated cercariae vaccine (Amory-Soission *et al.*, 1993). Irradiated cercariae vaccination was also tried on vervet monkeys (*Cercopithecus aethiops*) in which three times immunization elicited some protection against challenge infections (Yale, 1993 cited in Yale *et al.*, 1995). Thus, this paper has focused on looking at how the 20krad irradiated *S. mansoni* cercariae vaccine performs and preliminary studies of schistosomiasis on *Cercopithecus aethiops aethiops* (*Grivet Monkeys*). The study has identified the appropriateness of the animals for vaccine studies against schistosomiasis. The effect of the immunization on the monkeys was compared with those of mice and hamsters as controls.

In spite of intensive and various studies about mechanisms of anti schistosome immunity in different model animals as well as population based epidemiological studies have been undertaken, no consistent or cohesive direction for schistosomiasis vaccine development has emerged. In light of the gaps in vaccine development against schistosomiasis, we conducted an experimental vaccine study using 20Krad-irradiated cercariae in the *Grivet monkeys*.

2. Objectives

2.1. General objectives:

To describe the immunopathogenesis of *S. mansoni* in *Grivet monkeys* (*Cercopithecus aethiops aethiops*) vaccinated with 20Krad irradiated *S. mansoni* cercariae

2.2. Specific objectives:

- ▶ To establish *S. mansoni* infection in *Grivet monkeys*.
- ▶ To see the effect of 20Krad irradiated *S. mansoni* cercariae vaccination on the level of worm loads and egg per gram of stool (EPG) in the monkeys, mice and hamsters.
- ▶ To see the effect of 20Krad irradiated *S. mansoni* cercariae vaccination on the development of hepatic granuloma in the monkeys.
- ▶ To describe immune responses of monkeys against the irradiated *S. mansoni* cercariae with respect to serum levels of cytokines IL-4, IL-10, IL-12, IFN- γ , and TNF- α
- ▶ To examine the relevance of monkeys in vaccine and drug research against *S. mansoni*

3. Materials and Methods

3.1. The animals

A total of twelve age matching nine male and three female *Grivet monkeys* (*Cercopithecus aethiops aethiops*), captured from Sodere, 106Kms Southeast from Addis Ababa were involved in the study. Eight monkeys were used for 20Krad irradiated *S. mansoni* cercariae vaccinations, but the rest were controls. The monkeys were quarantined for 3 months, and checked for parasitic infections by Kato smear and concentration methods (Idris & AL-Jabri 2001); and for retroviral wild infections, ELISA and Unigold confirmatory tests were used. The animals were then housed in individual cages of 90cm by 100cm; Maize, carrots, chickpeas and banana were provided.

Twelve male mice and twelve male hamsters which were all laboratory bred were also involved for comparison. The mice and hamsters were both grouped in to two and each were involving six animals; group one's were 20Krad irradiated *S. mansoni* cercariae vaccinated as the monkeys, and group two's in both species were again controls.

3.2. The parasite

S. mansoni infected snail hosts, *Biomphalaria pfeifferi* were collected from 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 Institute of Pathobiology, 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 then exposed to sunlight or electric light for about 10-30 minutes. At least five infected *Biomphalaria pfeifferi* snails were induced to shed cercariae.

The number of cercariae was counted by having few drops of iodine in 1ml of water with cercariae and observed under 40x, binocular microscope.

3.3. Immunization with 20Krad irradiated *S. mansoni* cercariae

S. mansoni cercariae collected from seven *Biomphalaria pfeifferi* snails and mixed in a glass bottle of normal saline and exposed to 20Krad of gamma radiation. The irradiation machine emits 13.5Krad/hour, thus the time of exposure was set to 106 seconds in order that the cercariae could receive the total of 20,000rad of UV rays.

All animals in the first groups, monkeys (n = 8), hamsters (n = 6) and mice (n = 6) were given intraperitoneal injections of 100, 200, and 200 irradiated *S. mansoni* cercariae in 1ml of buffered saline solution at days 0, 14 and 28 respectively. The control groups of all the animals; (monkeys (n = 4), hamsters (n = 6) and mice (n = 6) were given the same volume of buffered saline solution in similar procedure.

One week before and right after the first day of vaccination, sera had been collected weekly from all the monkeys, and after superinfection, from seven of the monkeys up to twenty-five weeks from the first vaccination.

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

All the immunized and control groups of monkeys, mice and hamsters were challenged at week eight by skin exposure through shaved abdomen using the methods used by others (Fallon *et al.*, 1996 & Kruatrachue *et al.*, 1982). The cercarial concentration was adjusted to 200 -250 cercariae/ml of water. Similarly in the case of superinfection or further exposure of some of the primarily infected monkeys from both groups, the concentration of cercariae was adjusted to 300 cercariae per ml of water. 1ml of the water was added to individual vials of equal size and poured over the skin of the animals and kept for 20 minutes. Finally, a simple hand lens was used to check the penetration the larva in to the skin of the animals.

3.5. Estimation of EPG

Stool tests were conducted according to the recent protocol (Idris & AL-Jabri, 2001), which involves direct saline and iodine mount or wet preparations, formalin-ether concentration and Kato smear methods.

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 22nm 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).

3.6. Estimation of worm load

In order to estimate the worm loads of the infected animals, perfusion was done by the previously described method (Farah *et al.*, 1997). After primary infection, five monkeys (four from group one and the other one from the controls) were randomly selected and perfused at 19th week from initial immunization. The remaining seven monkeys (four from group one, and three from the control group) were perfused at 25th week, which is at the end of the 6th week after superinfection. However, both the hamsters and mice were all perfused after 6 weeks from primary infection.

The animals were sacrificed by intraperitoneal injection of anesthetic anti-coagulant solution of heparin and sodium pentobarbital. The skin of the animals was teared off and peritoneal, thoracic cavities as well as diaphragm were cut open to expose the aorta. Perfusion fluid was prepared by dissolving 8.5gm of sodium chloride and 7.5gm of Na-citrate per litter of distilled water and pumped into the aorta. The body cavity was also rinsed thoroughly with the fluid. The perfused fluid was finally poured into Petri dish and sieved through a nylon filter to collect and count the worms.

3.7. Liver Histology (Histopathology)

Two-millimeter square biopsy samples were taken from the liver of monkeys and fixed with 4% formalin in 0.1 phosphate buffer (Ph 7.4) for 12 hours. Dehydration was performed with increasing concentration of graded alcohols of 70%, 80%, 95% and 100% for 45 minutes in each and then in 100% alcohol for an hour. The tissues were cleared in two glasses of xylene for one hour in each, and then subsequently impregnated in three paraffin glasses for one hour in each. Finally, the tissue specimens were embedded in paraffin wax and two blocks were prepared for each specimen.

The tissue blocks were sectioned on Zeiss Microtome (Carl Zeis Zunch AG, West Germany) with 5-micrometer thickness. After mounting the sections, the slides were stained with haematoxylin-eosin and examined with a Zeiss binocular microscope (Carll zeiss, Axiostar, Germany), 10x objective. The diameters of granulomas were measured according to the method used by (Farah *et al.*, 1997). Granulomatous tissue reactions around eggs were also identified from liver sections.

3.8. Enzyme Linked Immunosorbent Assay (ELISA) for Cytokines

Serum cytokines of the monkeys; IL-4, IL-10, IL-12, IFN- γ and TNF- α were quantified by 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 °C and on the next day washed twice with PBS, then blocked by blocking solution (PBS with 1% BSA) and incubated for 1 hour at room

temperature. Further washed five times with PBS containing 0.05% Tween (PBS-Tween), and 100µl/well of serum samples or the standards were added and incubated for 2 hours at room temperature. After additional five time washes with PBS-Tween, 1µg/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 incubated for 1 hour at room temperature. After further five time washes with PBS-Tween, 100µl/well of Streptovadin-ALP was added and the plates were incubated for one hour at room temperature, and again washed five times with PBS-Tween. Finally 100µl/well of appropriate substrate solution, Para nitrophenylphosphate (pNPP) was added and after 30minutes, the optical density was measured at 405nm (BIOLINX, MRX ELISA reader).

2.9. Analysis of Results

In order to analyze the parasitological and immunological results, normal distribution was assumed, and SPSS version 11.5 Statical package was used. The results of EPG, worm load and granuloma diameters were compared by one-way ANOVA followed by Scheffe. The OD values for selected periods or weeks were presented on box plots. The results between time groups of immunized as well as control monkeys were subjected to Independent-Samples T-test and compared both in primary and superinfection. 95% confidence interval and $p < 0.05$ significance level was considered as significant.

4. RESULTS

4.1. Parasitology

4.1.1. Establishment of infection in *Grivet monkeys*

Egg per gram of stool (EPG) was observed from the 5th week post primary infection. The stool of the animals was checked continuously using Kato smear and concentration methods, but both the vaccinated and the control monkeys did not excrete eggs up to the 19th week or 11th week from the primary infection.

Five monkeys were randomly selected from both groups and perfused at 11th week post primary infection. Despite the absence of eggs in stool specimens of the perfused monkeys, male adult worms were identified in their inferior portal veins. Female adult worms were absent in all the five. Egg related pathology, especially in the liver, intestine and spleen was again not observed, and which showed the absence of egg production in the animals. Therefore, although, the primary infection was successfully established, female adults and thus egg excretion was not identified after primary infection of the monkeys.

At the beginning of the 19th week, seven of the monkeys; four from the vaccinated and three from the control groups were superinfected with 300 *S. mansoni* cercariae. Egg production was again continuously checked using Kato smear and concentration methods. After the 5th week from superinfection or at 24th week of primary immunization, almost all the animals became egg positive in their stool. The daily egg production of the animals was checked using Kato smear method for seven days in 41.7mg stool per slide and then the egg load was calculated for one gram of stool (EPG). The mean EPG of the vaccinated group ranges from 144 to 216, but in the

controls the range is from 216 to 456 (Table 1), thus there was a significant reduction in the vaccinated monkeys ($P < 0.05$). At the end of 25th week, all the superinfected monkeys were also perfused. The mean worm load of the vaccinated monkeys became slightly lower than that of the super infected controls; however the difference is statically not significant (Table 1).

Although the effect of the infection looks similar in the monkeys, the release of bloody diarrhea was a rare phenomenon in the vaccinated ones. However especially after the onset of egg production, most of the disease manifestations like weight loss, bloody diarrhea and loss of appetite became common in all the controls than the vaccinated group. Excretion of egg was delayed in one of the control monkeys, but later the monkey excreted eggs and upon perfusion its total worm load was comparable with the other controls (Table 1). In spite of the delay of the egg excretion, its internal organs were severely affected due to egg related complications.

Table 1 Kato smear result or EPG (Mean \pm SD), worm load and pathology in four vaccinated and three Control monkeys six weeks post super infection (25th week).

Categories	EPG ^a	Worm Load after perfusion			Pathology (General)
		Male	Female	Total	
Vaccinated (G I)	168 \pm 96	200	31	231	Severe
	216 \pm 168	150	34	184	Moderate
	168 \pm 144	197	16	213	Moderate
	144 \pm 120	140	29	169	Moderate
Control (G II)	456 \pm 120	215	49	264	Severe
	216 \pm 192	242	54	296	Severe
	360 \pm 96	270	60	330	Severe

^a Decimal values rounded off to nearer whole number.

At 95% confidence intervals, the mean EPG difference is significant ($P < 0.05$) between Group I and II, but the worm load difference is not significant between the groups.

NOTE: *Mild-Few and small macroscopic hepatic granuloma, no bloody diarrhea, minimal mesenteric Lymphodenopathy & few granulomas on the colon*
Moderate-Multifocal macroscopic hepatic granuloma, some times bloody diarrhea, slight Mesenteric Lymphodenopathy & many granuloma of the colon
Severe-Diffuse/Multifocal macroscopic hepatic granuloma, frequently bloody diarrhea, very Marked Lymphodenopathy & several granulomas on the colon

4.1.2. Infection in mice and hamsters

The parasitological results of the experimental *Cercopithecus aethiops* were compared with the previously studied mice and hamsters. Thus, eggs per gram of stool (EPG) by Kato smear method and worm loads were similarly determined for both mice and hamsters.

In mice and hamsters, egg production was not delayed as it was in the case of monkeys. Thus both male and female adults established from the primary infection. All the non-vaccinated groups of both mice and hamsters started excreting eggs at the beginning of 6th week post primary infection. Excretion of ova was delayed in few of the vaccinated mice, and a few from both species produced fewer eggs. After perfusion the total worm load was also reduced especially in the vaccinated mice (Table 2). The means of both EPG and total worm loads of

group one in both species was reduced. The mean difference became significantly lower in the immunized mice and hamsters ($P < 0.05$). The status of pathology in all groups of both mice and hamsters is indicated as shown in Table 1.

Table 2 Kato smear result or EPG (Mean \pm SD), male, female and total worm loads six weeks post primary infection for mice and hamsters.

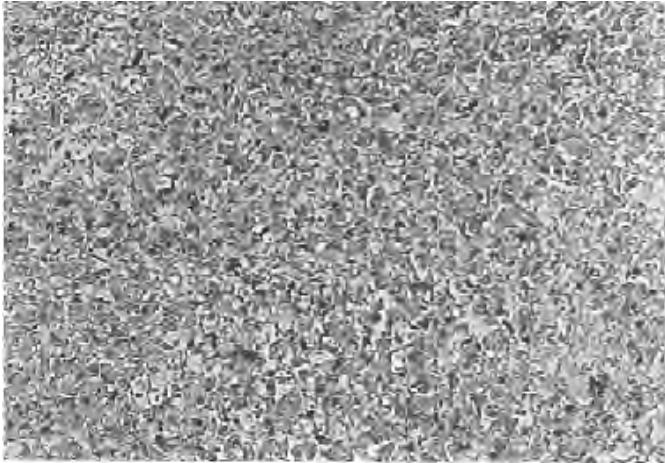
Animal Type	Group/ Number	Categories	EPG ^a	Worm Load			General Pathology
				Male ^a	Female ^a	Total ^a	
Mice	G I / 6	Vaccinated	70 \pm 42	16 \pm 6	4 \pm 2	20 \pm 6	Mild/Moderate
	G II / 6	Control	168 \pm 21	23 \pm 5	8 \pm 2	31 \pm 5	Severe
Hamsters	G I / 6	Vaccinated	108 \pm 39	24 \pm 7	8 \pm 5	32 \pm 10	Mild/Moderate
	G II / 6	Controls	200 \pm 20	35 \pm 7	13 \pm 4	48 \pm 6	Severe

^a Decimal values rounded off to whole number. At 95% confidence intervals, the mean EPG and total worm load is significantly different ($P < 0.05$) between group I and II in both mice and hamsters.

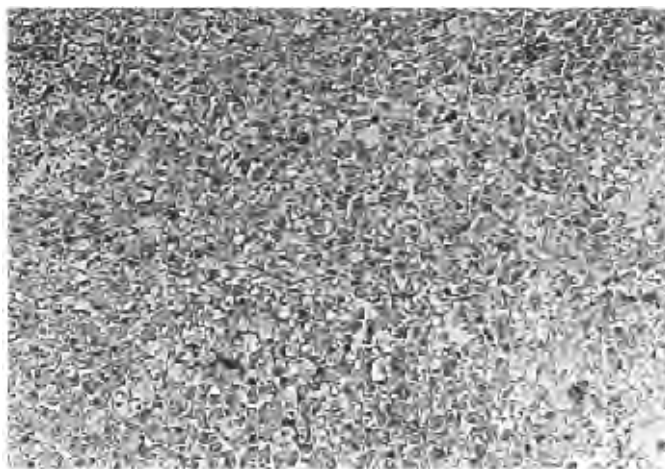
4.2. Histopathology

After both primary and superinfection, about 2mm square biopsies of liver tissues from both groups of monkeys were processed for studying the histopathological conditions and measurement of hepatic granuloma.

The triplicate slides of each of the monkeys, which were perfused after primary infection (11th week post primary infection), showed no granuloma or other indications of liver pathology (Fig 4; A & B). However, in the superinfected immunized and controls, which were perfused at 25th week (or 6th week post superinfection); small to diffuse and multifocal forms of macroscopic hepatic granulomas occurred (Figures 5 & 6).

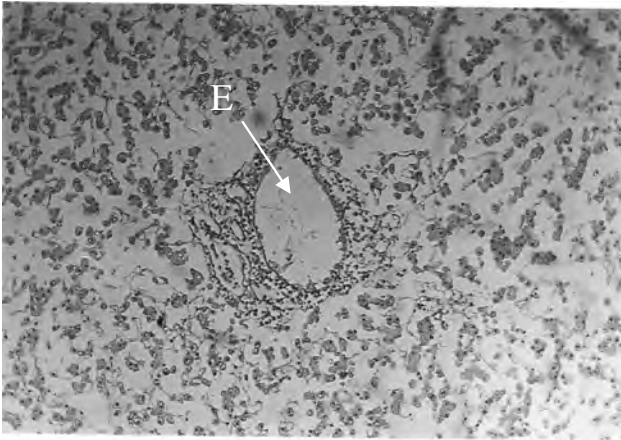


A. Irradiated *S. mansoni* Vaccinated

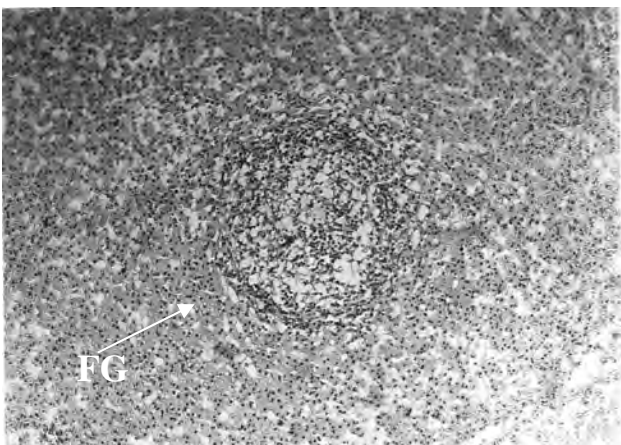


B. Control

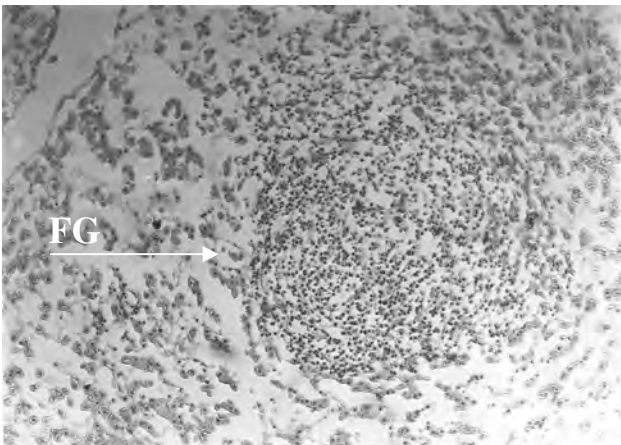
Figure 4: (H & E-stain, 10x) Liver histology of a vaccinated and a control monkey (11th week post primary infection). No clear difference was found in histological results of the groups (A & B). No egg related lesion or granuloma on the tissue sections of all the groups after 1^o infection



A. An egg (E) and surrounding cells

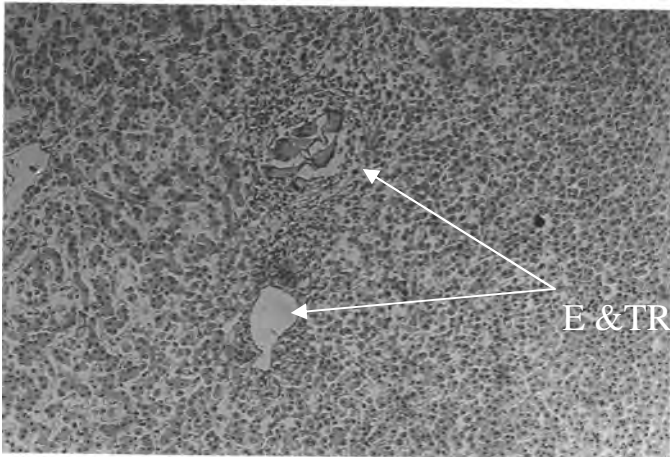


B. Florid type granulomas (FG)

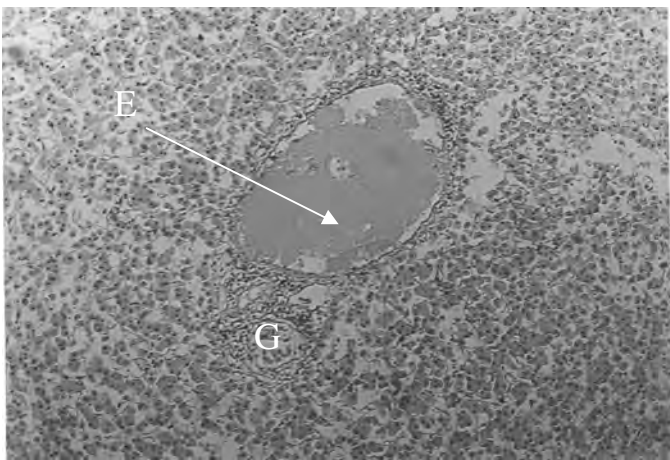


C. Florid type granulomas (FG)

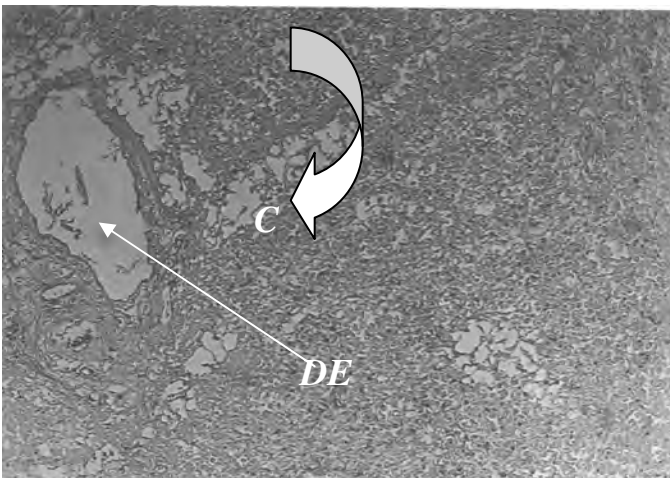
Figure 5 (*H & E-stain, 10x*) Liver histology of some immunized monkeys six weeks after super infection or at 25th week, Egg and minimum cell reactions with miracidial secretions (A) and Florid granuloma (B & C).



A. Eggs (E) and surrounding tubercle type reactions (TR)



B. An intact egg (E) and Potential granuloma (G) Developments



C. Infiltrating immune Cells(C) around a Decaying egg (DE)

Figure 6 (H & E-stain, 10x) Liver histology of some control monkeys six weeks after supra infection (25th week), tubercle type reactions around eggs (A), Intact egg and developing granuloma (B) & destruction of tissue around an egg(C).

Hepatic granulomas were seen with centrally placed ova or around the eggs which may not be included in the sections, but the lesions due to inappropriately responding immune cells around the secretions of miracidia in the eggs could be formed. Only the non-confluent granulomas containing central eggs or clear lesions due to eggs were measured for comparison (Table 4).

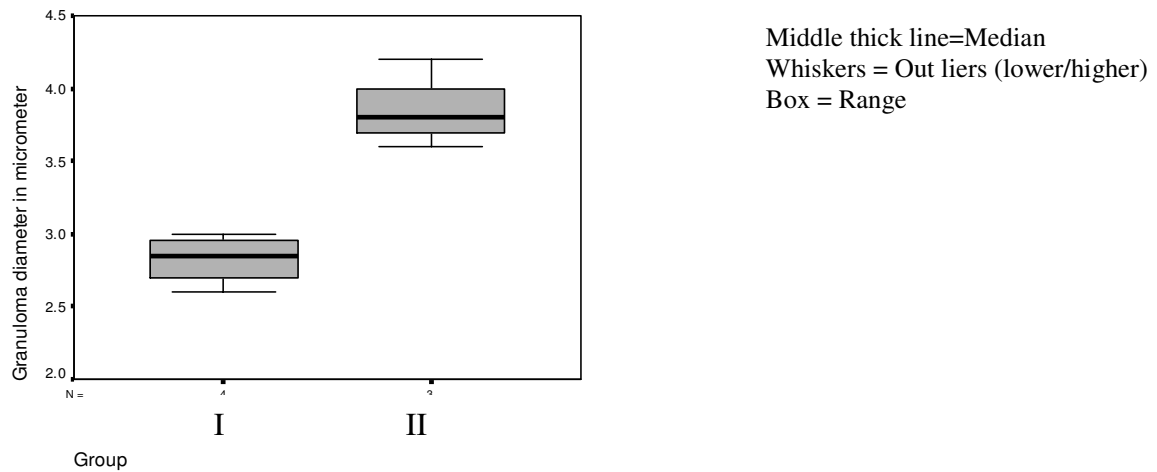
The tissues were sectioned serially in triplets, that is for each of the processed tissue around two hundred sections of five micrometer were assumed and after every sixty three sections a slide was prepared. Thus for each of the animals three slides were prepared and for comparison the pooled means of the groups were used.

Table 3: Diameter of liver granuloma (Mean \pm SD) in some vaccinated and control monkeys after super infection (25th week).

Categories	Mean \pm SD (Diameter in μ m)
Vaccinated G I (4 monkeys)	3.0 \pm 0.2 2.8 \pm 0.2 2.9 \pm 0.2 2.6 \pm 0.1
Control G II (3 monkeys)	3.8 \pm 0.1 3.6 \pm 0.3 4.2 \pm 0.1

At 95% confidence interval, the mean difference is significant (P<0.05) between the groups

The variation in the diameter of granulomas between the groups is shown on the following graph.



N = Number of animals per group, at 95% confidences, the size difference is significant ($P < 0.05$)

Figure 7 the mean diameter of granulomas in the superinfected seven monkeys of group I and II

The diameters of the granulomas showed variation between the immunized and the controls. The means of the granuloma ranges from 2.6 μm to 3.0 μm in the vaccinated monkeys, but the range is from 3.6 μm up to 4.2 μm in the controls (Table 4), and the difference is significant ($P < 0.05$) between the groups.

Although eggs are observed in the liver tissues of the vaccinated animals, the infiltrated immune cells caused less egg related liver pathology than the controls. The granuloma is also more of the florid type and no tubercle reaction is observed around the granulomas of the immunized group. The cells around the granulomas are few lymphocytes, eosinophils, epithelioid cells and many macrophages (Figures 5; A, B, C).

In the tissues of control monkeys, largely infiltrating immune cells, macrophages and eosinophils are observed around granulomas. The destructive potential of the cells can easily be predicted around the calcifying egg (Figure 6; B) and the pseudo tubercle type reactions around the granuloma in (Figure 6; A). Destruction of liver tissue is especially seen around a decaying egg in (Figure 6; C). The invasion of collagen and fibroblasts were also observed in the liver sections of the group, and which could result congestion of capsule in the liver.

4.3. Measurement of Cytokines by ELISA

In order to make some immunological associations with the parasitological and histological results of the monkeys, the serum cytokines; IL-4, IL-10, IL-12, INF- γ and TNF- α were quantified for fourteen selected weeks by enzyme linked immunosorbent assay (ELISA).

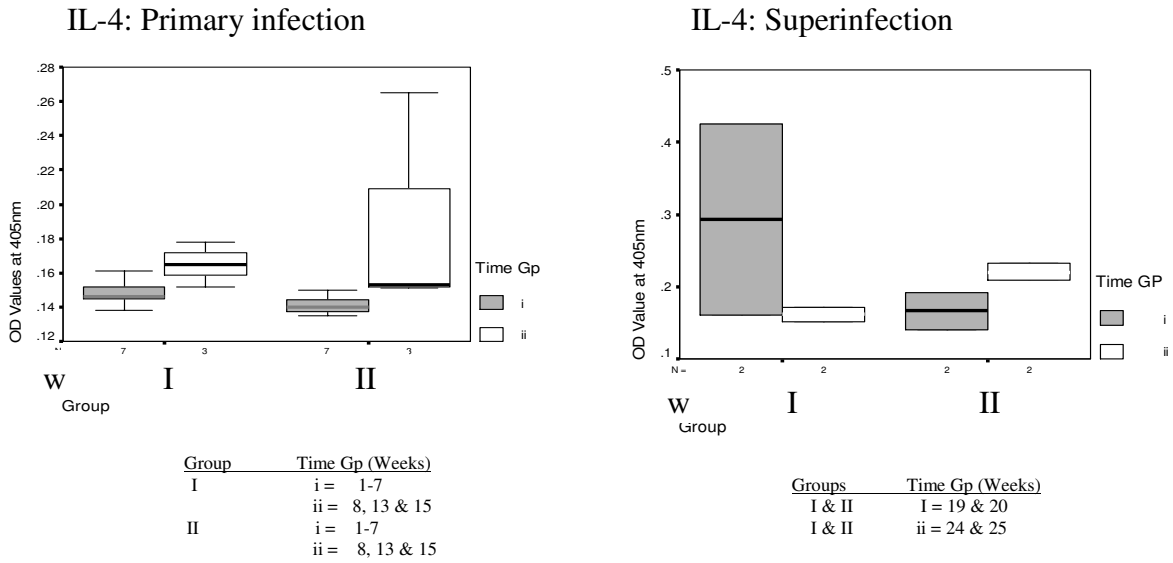


Figure 8 The level of IL-4 before & after primary infection and after superinfection. Significant difference ($p = 0.03$) between time groups of group I in primary infection, but no significance difference ($p > 0.05$) for group II, and for both groups after superinfection, w = weeks, Gp = Group.

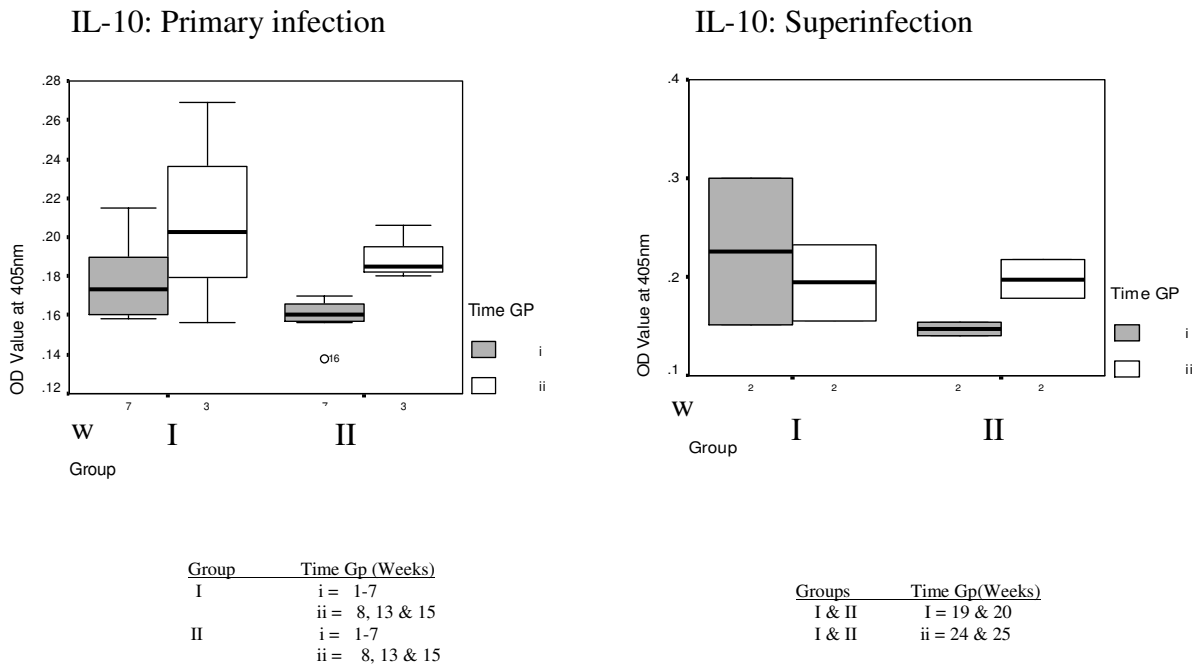


Figure 9 The level of IL-10 before & after primary infection and after superinfection. No significant difference ($p > 0.05$) between time groups of group I in primary infection, and for both groups after superinfection, significance difference ($p = 0.004$) for group II in primary infection, w = weeks, Gp = Group.

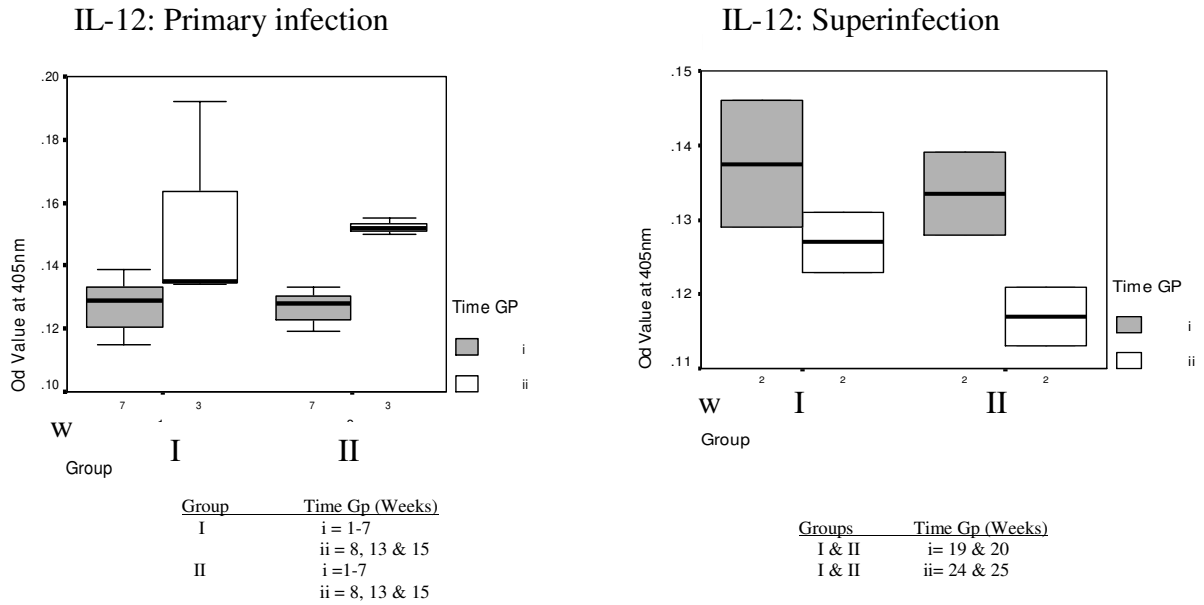


Figure 10 The level of IL-12 before & after primary infection and after superinfection. Significant difference ($p = 0.000$) between time groups of group II in both primary and superinfections, but no significance difference ($p > 0.05$) for group I both in primary and superinfections, w = weeks, Gp = Group.

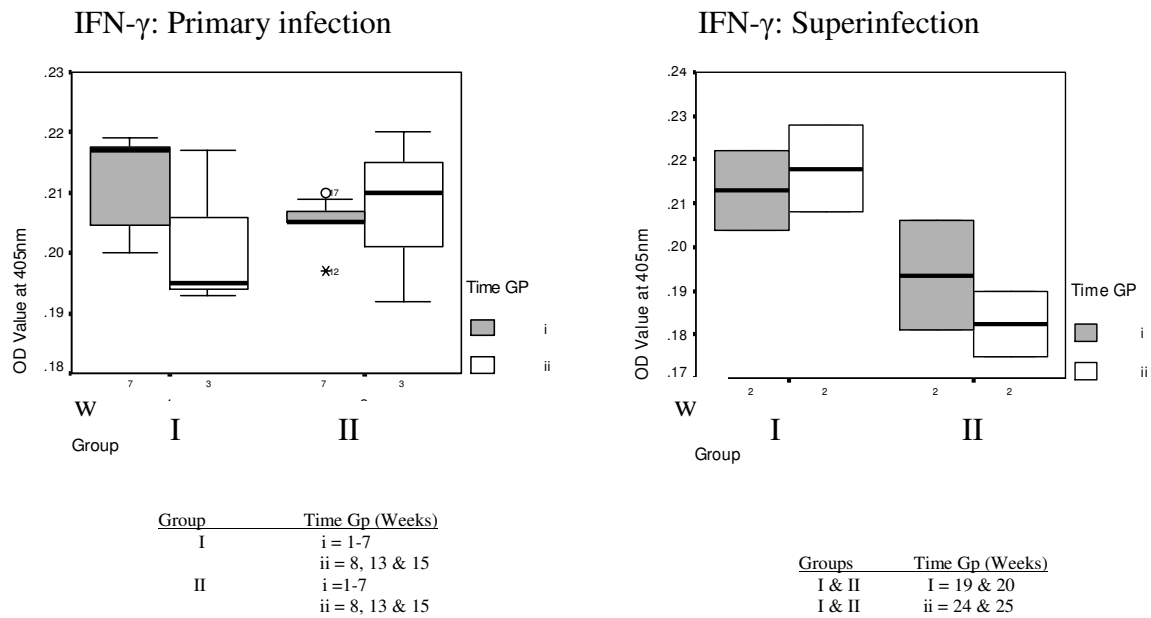


Figure 11 The level of INF-gamma before & after primary infection and after superinfection. No significant difference ($p > 0.05$) between time groups of both groups in primary and superinfections, w = weeks, Gp = Group.

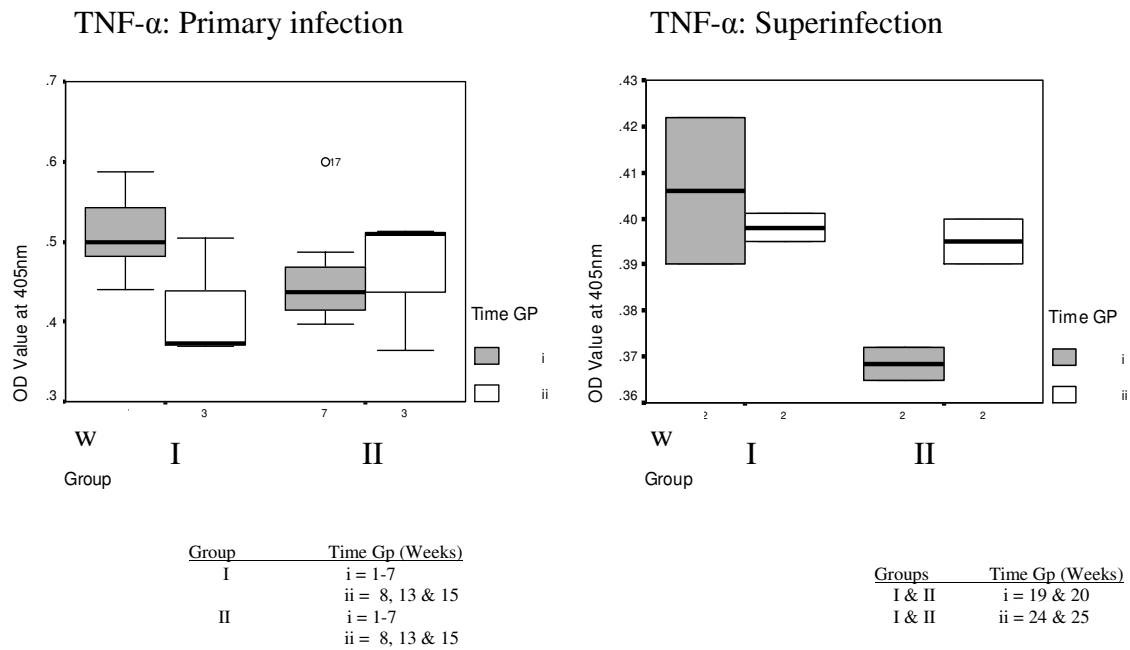


Figure 12 The level of TNF- α before & after primary infection and after superinfection. Significant difference ($p = 0.047$) between time groups of group I in primary infection and group II ($p = 0.049$) in superinfection, no significant difference ($p > 0.05$) between time groups of group II in primary infection and group I in superinfection, w = weeks, Gp = Group.

After primary infections, the level of IL-4 and IL-10 increase similarly in group I (Figures 8 & 9) than controls, and the difference was significant ($P < 0.05$). The cytokines were even higher until ova deposition at week 24. After egg excretion, the levels of the cytokines become lower in group I however in the controls, both IL-4 and IL-10 are significantly higher ($p < 0.05$) after egg excretion.

In both the immunized and control groups, the level of IL-12 has significantly increased ($P < 0.05$) after primary infection, and the difference is higher in the controls. However, the cytokine level after superinfection is in general lower in both groups; after egg excretion at week 24, the OD value has decreased in both groups, but more significantly in the controls (Figure 10).

Before primary infection, the level of INF- γ in group I monkeys was significantly higher than the after primary infection. However, after superinfection at week 19, the OD values were higher after egg excretion in group I monkeys. In general, the level of the cytokine is low in the superinfected controls, but the value is even lower after ova excretion (Figure 11).

For group I monkeys, TNF- α has significantly decreased after primary infection, but in the controls the increase is insignificant (Figure 12). In superinfection, the measure of the cytokine is in general low for both groups. For group I monkeys, TNF- α has decreased after ova excretion, but the difference was not statistically significant ($P > 0.05$). However, after ova excretion, there was a significantly increased TNF- α production ($p = 0.049$) in the controls (Figure 12).

5. Discussion

Despite the existence of pronounced immune responses, schistosomes can flourish or establish their residence in most definitive animals including human hosts (Pearce & MacDonald, 2002). Schistosomiasis has been well studied in lower definitive hosts, particularly in rodents and some domesticated hosts like pigs and sheep. Thus, most of the data on host immune reactions and pathogenic properties of the parasites are from the experimental studies on the lower animals. However, as the rodents and other domestic animals can fairly mimic the immune responses of humans, further studies on primates like *Cercopithecus aethiops* may have some alternative approaches for treatment and vaccine designs.

This study has demonstrated that the cercariae from intermediate hosts of *S. mansoni* snails (*Biomphalaria pfeifferi*) infected the monkeys within ten to twenty minutes by pouch method. The method assumes the ways the parasite is infecting human beings. Few milliliter of water with cercariae in a vial was held on the exposed abdominal skins of the animals. After the exposure, a simple hand lens helped see the successful penetration of cercariae in to the skin of the animals.

The protection level of the immunization was found to be low in *Grivet monkeys*. However, for the mice and hamsters a relatively good protection level as measured by worm and egg load was seen. The result especially in the mice is comparable with a number of similar reports (Bergquist, 1995).

After primary infection both the vaccinated and control *Grivet Monkeys* did not produce eggs. However the results from random perfusion of some of the animals indicated the development of

the adult male worms. This may show that the 20Krad irradiated *S. mansoni* vaccine might have inhibited the development of females after a single low dose infection. Because, the continuous Kato thick smear examination of all the vaccinated and control monkeys indicated no ova production up to the 19th week (11th week after primary infection). At the 19th week, random perfusion of five monkeys from both groups showed the nonexistence of the female adults. This has supported the stool examination results, the gross pathology examination of the perfused animals also showed the absence of egg related pathogenesis.

The result may also indicate single sex cercariae infection; however, later the same species of the intermediate snails were collected from the same endemic site for *S. mansoni* and other ten monkeys were infected. Although the experiment was for the other purpose, it was used to see if single sex infection of the animals might occur again. Six weeks post primary infection; similar Kato smear and concentration procedures were followed to check schistosome eggs. In this case most of the animals became ova positive. The adult females have therefore properly matured after six weeks from primary infection in almost all of the animals. Thus, in spite of the four ova negative control monkeys in the original experiment, it may be difficult for one to conclude that single sex cercariae infection has happened. But the absence of eggs for eleven weeks and female adults after perfusion, as well as the nonexistence of egg related pathology in the live sections (Figure 5) confirmed that the primary infection was most probably a single male cercariae infection for the monkeys. In the case of mice and hamsters the uncertainty of single sex infection did not happen.

The delay of the female worm establishment of schistosomes has been reported by some studies (Hernandez *et al.*, 2004). It was suggested that the development of the female schistosomes is not directly influenced by the adaptive immune system, whereas that of the male is. Thus, the

adaptive immune system signals might affect the development of mature male, which subsequently stimulate the development of mature females in some hosts. Therefore, the male schistosome may have a role both in introducing signals from the adaptive immune system and facilitate female establishments.

Therefore, it may be possible to think that the 20Krad irradiated *S. mansoni* cercariae vaccine might have complemented the delay of the female worms in the *Grivet monkeys*. Thus, in order to solve the uncertainty of the similar delay in the four controls of the experiment, further careful investigations may be recommended to see if the vaccination might have affected the development of female adults in the low dose primary infection of the monkeys.

After primary infection, as there was no egg production by the animals, little pathology has resulted during eleven weeks after infection. From the gross pathological observations, no defined difference was seen in randomly perfused animals in each of the groups. But some times loose stool and nausea was seen especially on the controls. Histopathological results also indicate that no egg related complications (Figure 4; A and B), lesions or granulomatous immune reactions could be demonstrated in hepatic sections of the animals.

A follow-up of the remaining seven monkeys, which were superinfected at 19th week after primary immunization, indicated the production of eggs at the beginning of 24th week. All the groups released eggs, but from the daily examination of egg per gram of stool (EPG), the mean EPG results (Table 1) of the immunized group became significantly lower than the controls ($P < 0.05$). Clinical manifestations like weight loss, fever, bloody diarrhea, loss of appetite and swollen lymph nodes were detected in association with the onset of egg production. This was

again a rare phenomenon in the vaccinated group. At the end of the experiment, perfusion and gross pathologic observations demonstrated related complications like lymphadenopathy along the gut, and lesions on the liver.

The histopathological results of the superinfected animals (Figures; 5 and 6) also indicated the immunopathologic consequences of miracidial antigens from eggs in the liver. Different types of granulomatous reactions including intact and decaying eggs were seen in serially prepared slides of the tissues. The triplicate slide preparation and measurement of the diameters of granulomas showed significantly lower mean values of the immunized animals ($P < 0.05$). Although there is a relatively reduced liver immunopathogenesis in the 20krad irradiated cercariae vaccinated group, the worm load and the significant reduction in egg production is low in *Grivet monkeys*.

From the quantitative measurements of serum cytokines of the monkeys (Figures 8-12), the reduction in the mean diameter of granulomas in the vaccinated group might be due to the counteracting Th1 and Th2 mediating cytokines.

After primary infection, IL-4 and IL-10 levels were increased for group I monkeys; the increase is significant ($p = 0.03$) for IL-4, and insignificant ($P > 0.05$) for IL-10. In the controls IL-10 was also significantly raised ($p = 0.004$). After primary infection the level of the cytokines is generally high, however after ova excretion, although the level of both IL-4 and IL-10 decreased, the variation was not statistically significant ($P > 0.05$). But, in the controls, the cytokines have increased significantly ($P < 0.05$). This may show early Th2 mediated immune inductions before ova excretion. The lower level of the cytokines or the minimal change after egg excretion in the group may indicate the counteracting effect of Th1 immune responses. However, in the controls

the value of both IL-4 and IL-10 was raised after ova production. This can be associated to the more granulomatous reactions in the liver of the control monkeys.

After egg excretion, the relatively low level of Th2 cytokines (IL-4 and IL-10) in the immunized monkeys may have associations with the minimal development of granulomas than in the controls. This can also be related with studies in which large granulomas were observed after increased levels of Th2 cytokines like IL-4, IL-10, and IL-5 (Lukacs & Boros, 1993). Other studies associated strong Th2 responses and minimal lesions with dominant Th1 cytokines in mice (Jankovic *et al.*, 1997).

In the controls monkeys, the sizes of granulomas were significantly higher than that of in group I (Figure 5 and Table 4). In the controls, both IL-12 and INF-gamma levels were lower after ova production (Figures; 10 and 11); which is the opposite of IL-4 and IL-10 responses. Therefore the result may suggest that generation of strong Th1 responses might be effective in the suppression of the development of the Th2 mediated granulomas in *Cercopithecus aethiops* (Grivet monkeys). The cross regulation of granulomas by both Th1 and Th2 type cytokines has been reported in mice. INF-gamma and IL-12 were shown to down regulate IL-4 mediated granuloma responses (Lukacs & Boros, 1992; Oswald *et al.*, 1994). However IL-4 and IL-10 can regulate INF-gamma and IL-2 production (Chensue *et al.*, 1994; Wynn *et al.*, 1997). Thus, in most hosts pre-exposure to the parasite antigens or as the infection progresses, *S. mansoni* egg granuloma might be diminished in size including those around newly deposited eggs.

Natural regulatory T cells and, to a lesser extent, Th2 cells have been shown to play roles in suppressing Th1 responses and ensuring Th2 responses during schistosomiasis (McKee & Pearce,

2004). *S. mansoni* infected mice developed Th2 responses in which Th1 responses were prevented by IL-10-mediated suppression of IL-12 production. IL-10 can reduce immunopathology in many persistent infections, and it has shown to be generated by both innate and adaptive immune responses following infection. Innate effectors and regulatory of T cells producing IL-10 may cooperate to reduce morbidity and prolong survival in schistosomiasis (Hesse *et al.*, 2004).

IFN- γ and IL-12, Th1 inducing cytokines were shown to dominate *S. mansoni* infected mice prior to egg laying (Mossman & Coffman, 1989). Similarly, both the vaccinated and control superinfected monkeys produced higher IL-12 and INF- γ before egg production (Figure 10 and 11). However, although the measure of INF- γ after superinfection is higher for group I monkeys, it was lower prior to ova excretion.

A strong regulatory role of IL-12 response in mice has reported to be mediated by IFN-gamma (Wynn *et al.*, 1994), and so the generation of strong Th1 responses can be effective in the suppression of the florid development of the Th2 type granuloma. Repeated injection of exogenous recombinant IL-12 (rIL-12) into egg primed and subsequently infected mice also showed moderate inhibition of liver granuloma development (Wynn *et al.*, 1995).

From the histological results of the controls, the higher granulomatous reactions (Figure 6) can be associated to the significantly ($p = 0.049$) increased level of the inflammatory TNF- α after egg excretion. TNF- α might have induced the cell-mediated immune responses that could enhance inflammatory reactions due to the activation effects on macrophages, eosinophils and lymphocytes. In the superinfection, the level of the cytokine was lower. Before ova production, it

is higher in the vaccinated monkeys; however, in both groups the level became almost equal after ova excretion, and yet there is a significantly increased difference of the cytokine ($p = 0.049$) in the controls. This may have a direct association with the higher liver pathology in the group.

In general, the complexity of the host–parasite relationship and little understanding of immune-response development due to the absence of the appropriate animal model created a challenge for designing an effective anti-schistosome vaccine. Thus this study has underlined the potential importance of *Grivet Monkeys* (*Cercopithecus aethiops*) as the helpful model animals and provided a good direction for research studies of drug or vaccine. Because the *Grivet monkeys* are almost human equivalent primates and the pathologic mechanisms of the parasite can more represent humans than the other models.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

- ▶ *S. mansoni* cercariae infection has established in *Grivet monkeys* (*Cercopithecus aethiops aethiops*).
- ▶ Clinical manifestations of the intestinal schistosomiasis due to *S. mansoni* like weight loss, nausea, hepatosplenomegally, lymphadenopathy, enlargement of abdomen and bloody diarrhea occurred in *Grivet monkeys*.
- ▶ *Grivet monkeys* can be the suitable models for further studies of immunopathologic problems of human schistosomiasis. They can therefore also help studying host-parasite interactions to evaluate effective vaccines or drugs.
- ▶ The 20Krad irradiated *S. mansoni* cercariae vaccination; which was optimally protecting mice from challenge infections provided some reduction of egg production and worm load, but reduced hepatic granuloma developments in the *Grivet monkeys*.
- ▶ After egg excretion of the monkeys; the lower level of Th1 mediating cytokines (IFN- γ and IL-12), and the significantly increased Th2 mediating cytokines (IL-4 and IL-10) in the controls; but the insignificant variations of the response and the minimal granulomatous reactions in the vaccinated group can be associated to the existence of immune modulation in the immunized group.
- ▶ The balanced responses of Th1 and Th2 mediating cytokines may have an effect on egg induced pathology in the liver and rate of egg excretion in the monkeys.

6.2. Recommendations

- ▶ Further studies on the effect of the 20Krad irradiated *S. mansoni* cercariae vaccination on the maturation of female worms after primary infections are recommended.

- ▶ Irradiation doses other than the 20Krad need to be tried.

- ▶ Determination of immune response at the level of immunoglobulin (IgM, IgE, IgA, IgG1, IgG2, IgG3, and IgG4) of the monkeys against the 20Krad irradiated *S. mansoni* cercariae vaccine and other doses of irradiation, are suggested.

- ▶ Measurement of the cytokines by stimulation of peripheral blood mononuclear cells (PBMC) with parasite antigens will add more information to the understandings of immune responses in *Cercopithecus aethiops*.

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