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IMMUNO-EPIDEMIOLOGY OF *Schistosoma haematobium* INFECTION IN ZIMBABWEAN COMMUNITIES WITH DIFFERENT INFECTION PATTERNS.

A thesis submitted to the Faculty of Science University of Glasgow

For the Degree of Doctor of Philosophy

by

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IMMUNO-EPIDEMIOLOGY OF *Schistosoma haematobium* INFECTION IN ZIMBABWEAN COMMUNITIES WITH DIFFERENT INFECTION PATTERNS.

To my wife Shiela, daughter Tariro and son Takada:-

"I will always be with you after this work."

Kuna amai nababa: VaJ. Bangomwe Mduluza na VaJ. Mduluza.

Makandiudza ndikanzwisisa kuti, "Ashaya shungu ashaya makumbo."

Ndinotenda nezvese zvamakandiitira.

Chaitika Takafira

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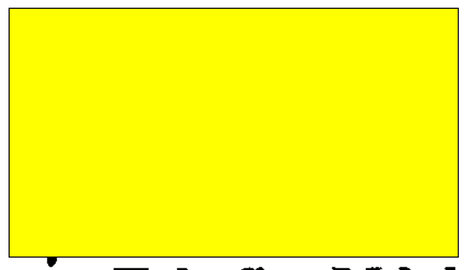
Finally, knowledge, like protective immunity to schistosomiasis, comes with age and experience of infection, which I think has began in my life through this work with Drs Paul Hagan and Patricia Ndhlovu. I dedicate the thesis to Paul and Patricia to whom I am so much indebted.

DECLARATION

I declare that the work presented in this thesis was carried out by me personally unless where it is stated to the contrary.



Takafira Mduluza

SUMMARY

This study addressed the immuno-epidemiology of *Schistosoma haematobium* in people living in communities in Zimbabwe with distinct patterns of prevalence and intensity of infection. The studies were located at three different areas within known endemic regions of the country. The two areas located in The Burma Valley were within 10 km of one another. One was designated a Low Transmission (LT) area and the other a High Transmission (HT) area. In these two localities, the immunological studies were centred on the cellular immune responses. Levels of cellular proliferation of peripheral blood mononuclear cells (PBMC) in response to specific (schistosome) antigen stimulation were measured from both school children and adults (age range 5 - 64) in each area (Chapter 2), while cytokine production by PBMC was investigated on a smaller cohort of school children aged 7-16 years from each area (Chapter 2). T cell clones were derived from PBMC collected from two infected and two uninfected children from the High Transmission area (Chapter 3). This aspect of the study attempted to establish if any T helper cell dichotomy existed during human *S.haematobium* infection by defining the cytokine phenotypes of the clones derived from the PBMC. The longer term aim was to assess the potential predictive value of T cell clones in determining resistance or susceptibility to infection, and thus contribute towards a rationale strategy for vaccine development. The third area of the study was in an endemic region with very high *S.haematobium* and low *S.mansoni* infection. This study focused on pre-school children two to six years old (Chapter 4) and examined the impact of repeated chemotherapy with praziquantel. The children were followed up for 14 months, with praziquantel treatment at each examination point, every eight weeks. The main aim was to prevent the development of any schistosome egg laying stages, thereby providing the opportunity for the protective immunity to develop in the absence of any pathological consequences arising from the eggs. The study reports on the antibody isotype profiles in sera collected at each follow-up point during the 14 months of the study.

The cellular immune responses of the PBMC collected from residents in the two areas of Low and High Transmission in the Burma Valley showed contrasting features before treatment. After stimulation with *S.haematobium* worm and egg antigens, the PBMC from infected individuals from the Low Transmission area gave high levels of

proliferation indicating possible experience of the parasite while the uninfected individuals gave very low levels of responses indicating lack of exposure, a consequence of the low transmission in this area. Similar patterns were observed from the PBMC collected from the same individuals after treatment, with those who were reinfected giving high levels of proliferation to specific antigen stimulation. The levels of proliferation correlated positively with infection for the individuals living in the Low Transmission area, suggesting the possible existence of an acute phase of *S.haematobium* infection in people in this area as uninfected children begin to have their first exposures to infection.

The PBMC from individuals living in the High Transmission area showed different patterns of responses from the individuals in the Low Transmission area. Before treatment, the PBMC from uninfected individuals gave high levels of response to specific *S.haematobium* worm and egg antigen stimulation, and in the infected individuals, who had very high infection intensities, the cellular responses showed unresponsiveness to antigenic stimulation. However, after treatment, the responses were reversed with the PBMC from infected individuals giving higher levels of proliferation than the PBMC from uninfected individuals. There was an overall increase in response levels of the PBMC collected after treatment when compared to PBMC collected before treatment. The high levels of response of the PBMC collected from uninfected individuals indicated the role played by experience of infection.

The study on cytokine profiles produced by the PBMC after five days of incubation with *S.haematobium* antigens showed that the production of cytokines is in some way a function of transmission, and could be related to experience of infection, exposure over a long duration and the development of full immune responses. Individuals from the High Transmission area produced high levels of all the cytokines measured: IL-4, IL-5 , IL-10 and IFN-γ. Before treatment, the PBMC from infected individuals from the High Transmission area gave low levels of proliferation but high levels of cytokine production. After treatment, the PBMC from infected individuals gave high levels of proliferation and low levels of IL-4, IL-5 and IL-10 cytokine production. The uninfected individuals produced high levels of IL-4, IL-5 and IL-10 when compared with the infected counterparts in that area, after treatment. The cytokine profiles showed that IFN-γ is associated with infection, and its levels were high in infected individuals regardless of

area. However IFN-γ levels could not be related to susceptibility and it was concluded that IFN-γ may be produced rapidly and abundantly during the early stages of infection. The other cytokines (IL-4, IL-5 and IL-10) had different patterns when compared to IFN-γ. High production of IL-4, IL-5 and IL-10 by PBMC from infected individuals was observed at the start of the study, i.e. before treatment but the levels were later observed to be high in the individuals who were uninfected after treatment. Before treatment, the infected individuals had high levels of infection and cytokine production (IFN-γ, IL-4, IL-5 and IL-10). When considering the ratios of the cytokines in relation to their purported Th1 and Th2 classification; before treatment the ratios of the cytokines IL-4:IFN-γ and IL-5:IFN-γ were similar in the infected and uninfected individuals from the High Transmission area. The IL-10:IFN-γ ratio indicated a high production of IL-10 by the PBMC from uninfected individuals from the High Transmission area. After treatment the ratios indicated that there was high production of IL-4, IL-5 and IL-10 relative to IFN-γ by the PBMC from the uninfected individuals.

The 'signature' cytokines for Th1/Th2 subsets were not as polarized as the classification of cytokine profiles reported in murine models. The clones derived from both infected and uninfected children from the High Transmission area produced a mixture of the 'signature' cytokines believed to mark distinct murine T cell subsets. As a consequence, an alternative classification different to that reported for murine models of schistosomiaisis was constructed. Most of the clones could be classified as either Th0/1 and Th0/2 depending on the ratios of the 'signature' cytokines, IL-4:IFN-γ and IL-5:IFN-γ. The results from this aspect of the study support the inference that none of the cytokines could be related to susceptibility to infection but that they play different roles during infection and may possibly be produced at different stages of infection with some even persisting after elimination of infection. However, the study also showed that a cytokine environment that is dominated by IL-4 and IL-5 has the ability to protect the individuals from infection possibly due to involvement of these cytokines in driving the ADCC and the humoral arm of the immune response which may serve a key role in eliminating invading infection. The raised IFN-γ levels could be inferred to be useful possibly in driving the cellular mobilisation of the responses during early infection with the failure to develop a Th0/2 cytokine response exposing the individuals to continued infection.

Young children below six years old have been found to be at high risk of becoming infected with *S.haematobium* in the Madziwa area of Zimbabwe. Provision of treatment every eight weeks played an essential role in preventing any further infection that could have contributed to the development of pathology. But frequent treatment could also influence the development of protective immune responses to infection. The antibody isotypes measured (IgA, IgE, IgG1, IgG2, IgG3, IgG4 and IgM) showed little change within the 14 months follow-up period. This could be due to lack of maturation in the immune responses in these children. However, frequent treatment could be extremely helpful in areas of high transmission where children could be infected even at a very young age. Since vaccines for schistosomiasis are not yet available chemotherapy remains the key method of controlling the damaging effects of the infection in children whose specific immune defence has yet to develop and mature.

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Abbreviations

°C	Degrees centigrade
µCi	micro Curie
µl	micro litre
µg	micro gram
ADCC	Antibody dependent cell-mediated cytotoxicity
ag	antigen
ANOVA	Analysis of Variance
APC	antigen presenting cell
BrdU	5-Bromo-2'-deoxyUridine
BCA	Bicinchoninic Acid
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CAP	cercarial antigen preparation
$CD4^+$	Cluster determinant type 4 positive cell
CO_2	carbon dioxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DTH	delayed type hypersensitivity
ELISA	Enzyme-Linked Immuno-sorbent Assay
ep10ml	eggs per 10 ml of urine
et al.	*et alia*
FABP	Fatty acid binding protein
Fc	constant fragment
FCS	Feotal calf serum
fig.	Figure
g	gravitation
GAPDH	glyceraldehyde phosphate dehydrogenase
GM-CSF	granulocyte macrophage-colony stimulating factor
GST	glutathione S-transferase
hAB	human AB serum
hIL	human interleukin
HRP	horse radish peroxidase
H_2SO_4	Sulphuric acid
HT	High Transmission area
Ig	Immunoglobulin
IFN-γ	Inteferon gamma
IrV	Irradiation associated vaccine antigen
IU	international units
kDa	kilo dalton
LT	Low Transmissiom area
Mab	monoclonal antobody
MHC	major histocompartibility
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	nano metre
PBS	Phosphate buffered saline
PBMC	Peripheral bood mononuclear cell
PEI	Pulmonary eosinophilia infiltrate
PHA	Phytohaemagglutinin
PMA	Phorbol-12-Myristate 13-Acetate

POD	Peroxidase dismutase
PPD	Purified protein derivative
RPMI 1640	Rosewell Park Memorial Institute medium 1640
rIL	recombinant interleukin
rpm	revolution per minute
S.D	Standard deviation
SDIF	Schistosome-derived inhibitory factor
SEA	Soluble egg antigen
S.E. mean	Standard error of mean
SI	Stimulation index
sm	Schistosoma mansoni
SWAP	Soluble worm antigen preparation
TCC	T cell clones
TCL	T cell lines
TCR	T cell receptor
Th1	T helper cell type 1
TM	Trade mark
TNF-α ,β	Tumer necrosis factor alpha, beta
TPI	triose phosphate isomerase
WHO	World Health Organisation
w/v	weight per volume
WWH	Whole adult worm homogenate

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Schistosomiasis or bilharzia is an important parasitic disease and a major health problem for many tropical and sub-tropical countries where hyperendemic areas are frequently documented (Iarotski and Davis, 1981; Doumenge *et al.*, 1987). The Egyptians were aware of the disease in ancient times as evidenced from hieroglyphic depiction, but it was not until 1851 that Theodor Bilharz, documented the disease in Egypt in the residents along the Nile river. The first schistosome to be described was *Schistosoma haematobium*, discovered in the veins of a man at autopsy in Cairo by the German surgeon Theodor Bilharz in 1851 and described in 1852 (Rollinson and Southgate, 1987). Elucidation of the schistosome life cycle was not made until 1913 when Mayairi and Suzuki showed that *Schistosoma japonicum* developed in the hydrobid snail *Oncomelonia hupensis nosophora* (Rollinson and Southgate, 1987).

Over 300 million people are infected with schistosomiasis world-wide and an estimated 500,000 deaths per year are reported (Rollinson and Simpson, 1987; Bergquist, 1995). Currently it is thought that about 600 million people are at risk of contracting the infection (Doumenge *et al.*, 1987). The disease ranks fifth on the list of the most important diseases in developing countries in terms of morbidity and mortality (Stephenson, 1987). Signs and symptoms include anaemia, diarrhoea, abdominal pain and haematuria. In Zimbabwe schistosomiasis is predominant in the agricultural and rural areas with about 3 million infected and another 3 million suspected of harbouring or being exposed to the disease. In most agricultural and rural areas of Zimbabwe, schistosomiasis ranks, after malaria, the second most important cause of hospital attendance (Ndhlovu, 1994), which is an indication of its public health importance.

Transmission of schistosomiasis has always been associated with poor socio-economic conditions. Co-ordinated integrated control strategies often make little impact due to causes such as escalating social unrest caused by war, droughts, floods and the effects of man-made water resources development projects (Hunter *et al.*, 1982). Increasing population density has led to wide-spread development of large- and small-scale irrigation schemes that increase the potential for transmission. In most cases these irrigation schemes are not designed to combat the proliferation of the snail intermediate hosts.

Five schistosome species commonly infect humans, *Schistosoma haematobium*, *S.mansoni*, *S.japonicum*, *S.intercalatum* and *S.mekongi*, while *S.mattheei* is known to occasionally infect man. In Africa, *S.haematobium* and *S.mansoni* are widely distributed, while *S.intercalatum* is confined to North East Zaire, Gabon, Cameroon and Congo, with some isolated reports indicating the possible existence of endemic foci in Central Africa Republic, Chad, Nigeria, Upper Volta and other parts of Central and West Africa (Iarotski and Davis, 1981; Doumenge *et al.*, 1987). In Zimbabwe *S.haematobium* and *S.mansoni* are both present, the former being widely distributed (Taylor and Makura, 1985; Ndhlovu *et al.*, 1996). Snail vectors of *S.haematobium* and *S.mansoni* in Zimbabwe are *Bulinus globosus* and *Biomphalaria pfeifferi* respectively. *B.globosus* is known to have high rates of natural increase, survival and reproduction in both stable and unstable water bodies. *B.pfeifferi* on the other hand has a slower rate of natural increase and is only found in stable water bodies. *S.haematobium* and *S.mansoni* are most prevalent in the north of Zimbabwe which has the highest rainfall, and where most streams and rivers are perennial.

1.2 TAXONOMY AND LIFE CYCLE

The life cycle of Schistosoma is illustrated in Figure 1.1. Human schistosomes are digenetic trematodes (commonly called flukes), are parasites which live within the blood vessels of humans and belong to the family *Schistosomatoidea*. Female and male schistosome worms live in permanent copula in the mesenteric veins around the intestine for *S.mansoni* and *S.intercalatum*, and the bladder for *S.haematobium*. The female worms produce about 300 - 3000 eggs each day. Some of the eggs become lodged in different tissues of the host and induce cellular granulomatous reactions, but the majority of the eggs pass into the lumen of the intestines or bladder and may leave the human host either in urine for *S.haematobium* or in faeces for *S.mansoni* and *S.intercalatum*. When deposited in fresh water, the eggs hatch releasing free-swimming larval stages, miracidia, which may infect an appropriate snail intermediate host, in the case of *S.mansoni* snails belonging to genus *Biomphalaria* and in the case of *S.haematobium* snails belonging to genus *Bulinus*. Asexual multiplication takes place in the snail intermediate host, resulting in the production and release of a large number of cercariae, which infect the human host by direct penetration of the skin. Cercariae transform into schistosomula as they penetrate the skin. The schistosomula then migrate to the peripheral lymphatic or circulatory system from where they are carried passively to the lungs. After spending some time in the lungs, the schistosomula migrate to the liver where growth and further development takes place. After pairing, each worm pair actively migrates towards either the mesenteric veins draining the intestine (*S.mansoni*, *S.intercalatum* and *S.japonicum*) or the bladder and ureters (*S.haematobium*). The whole life cycle takes 6 - 12 weeks. In their definitive sites, adult worms generally live for 3 - 8 years but have been reported to survive up to 30 years (Vermund, Bradley and Ruiz-Tiben, 1983; Harris, Russel and Charters, 1984; Wilkins *et al.*, 1984b).

1.3 PATHOLOGY

1.3.1 INVASION

Schistosomiasis begins with penetration of the skin by cercariae. Awareness of the event is usually minimal on initial exposure. Pruritis and local erythema may occur within 15 minutes to 2 - 3 hours of penetration in the case of multiple exposure. Sometimes in sensitised individuals within 5 - 15 hours post-entry a local dermatitis occurs. A wide variety of clinical manifestations, even exfoliation may also occur. Tissue oedema with mononuclear cells are observable on histological examination (Feldmeier *et al.*, 1985a, 1986). People with experimental acute dermatological reactions have been described as having high titres of IgE and IgG antibodies (Capron *et al.*, 1977; Joseph *et al.*, 1978; Anwar *et al.*, 1979 and Santoro *et al.*, 1979).

1.3.2 MIGRATION

When the parasites are migrating through the body, fever and mild wheezing are sometimes observed. These events usually follow extremely heavy re-exposures to schistosomes. Chest X-rays at this time have shown pulmonary infiltrates with eosinophilia (PEI) syndrome, 7 - 10 days after initial exposure. Patients with PEI usually manifest a peripheral blood eosinophilia. Studies in primates and mice have indicated that schistosomes migrating through the lung are associated with accumulations of significant numbers of mononuclear cells. These cells include macrophages that are involved in production of high levels of IFN-γ associated with early infections during the lung stage of migration. A number of parasites are trapped in the lungs where they are destroyed. Some evidence suggests that much of this destructive process is mediated through antibody-dependent cell-mediated cytotoxicity (ADCC) mechanisms involving a variety of immunoglobulin isotypes and effector cell subpopulations of macrophages and

eosinophils (Butterworth *et al.*, 1974; Dean, 1977; Ellner and Mahmoud, 1979). The cells involved are eosinophils, mononuclear macrophages and platelets, and can cause damage to the migrating schistosomula (Capron *et al.*, 1977; Joseph *et al.*, 1978, 1983; Sturrock *et al.*, 1981). During the same migratory period, it has been reported that there is an elevated amount of IFN-γ cytokines production in the lungs (Wilson *et al.*, 1996).

1.3.3 ESTABLISHED INFECTIONS

As far as is known live adult worms have little or no pathogenic effect on the definitive host (Butterworth *et al.*, 1990) and chronic disease is largely attributed to the deposition of eggs in the tissues, with granuloma formation and subsequent fibrosis. The adult worms do not replicate within the mammalian host and the extent of the disease depends in part on the number of eggs that are deposited in the tissues, which in turn depends on the number of adult worms that are present, their egg-laying capacity and their longevity within the host (Hagan, 1987). Host genetic and immunological factors also play a role in determining the level of pathology ensuing from infection.

Several hundred to several thousand ova are produced daily. Of these approximately half migrate through either the intestine or the bladder viscera depending upon species. The rest settle in the liver and other organs, where they induce inflammation and organ damage (Weinstock, 1992). When the eggs are deposited in organs or tissue, the host mounts a characteristic focal inflammatory reaction that surrounds the eggs; the granuloma. The granuloma eliminates toxic and antigenic materials released from the eggs and may kill the eggs and eliminate the egg debris (Feldmeier *et al.*, 1985a, 1986; Wyler, 1992; Weinstock, 1992). Since the worms continuously produce more eggs, the

recurrent cycle of organ injury and repair leads to organ damage (Weinstock, 1992; Wyler, 1992).

The major disease manifestations of schistosomiasis are similar for *S.mansoni* and *S.japonicum* with regard to liver damage. Ova deposited in the portal areas result in portal fibrosis, phlebitis and periphlebitis (De Cock, 1987). Sometimes the deposited eggs can lead to portal hypertension, which causes splenomegaly, ascites and oesophageal and gastric varices. The liver then becomes hard and nodular on palpation (Feldmeier *et al.*, 1985a, 1986). Weinstock (1992) also reported that bleeding oesophageal varices are the major cause of death. Other symptoms common to intestinal schistosomiasis include intermittent diarrhoea, which can contain mucous and blood. This results from egg-induced inflammation of the intestine. Sometimes when the disease is in an advanced state, there is portal systemic shunting which allows eggs to embolize to the lungs and other organs.

In contrast to intestinal schistosomiasis, the symptomatic stage of chronic *S.haematobium* manifests as haematuria, frequent micturation and dysuria. These symptoms are indicative of egg deposition which results in inflammation and ulceration in the bladder mucosa. An injured bladder usually has permanent fibrosis, pseudopolyps and sometimes neurogenic dysfunction. The ureters are often involved, leading to ureteral obstruction, hydronephrosis and renal failure (Al-Shukri and Olwan, 1983). Sometimes the brain, lungs and spinal cord are involved which in these severe cases leads to considerable morbidity. When eggs are deposited in the skin, symptoms of perineal itching and lesions occur (Feldmeier *et al.*, 1985a,b, 1986).

1.4 CURRENT CONTROL MEASURES

Schistosomiasis is one of the most widespread parasitic diseases of man and is currently endemic in 75 countries of the world (WHO report, 1985). Over the past few years the disease has invaded some new areas such as Richard Toll in Senegal as a result of economic developments; in this case the building of the Manantali Dam on the Senegal river. With the development of man made water projects the disease may increase in other areas. Theoretically; control seems possible but in practical terms it is impossible without the participation of the people in the affected areas and their governments. In most developing countries where this disease is a problem the continued existence of the diseases has been attributed to the availability and permanence of surface fresh water and warm climate. Both are crucial to the development of large intermediate host snail populations. Also important is human water contact behaviour. The best control strategies have been documented as those which involve a co-ordinated approach that includes control of the intermediate snail host by environmental methods and molluscicides, chemotherapy, improved sanitation and ultimately health education. But in my view the lack of a vaccine is a major drawback to any plausible control strategy. Since with the availability of vaccines, control can easily be instituted by a single or a few doses, eliminating the problems faced with other methods of schistosomiasis control.

1.4.1 DRUGS

Safe and effective drugs for treatment - oxamniquine, metrifonate and praziquantel - have been available for more than two decades. The mainstay of control, at present, is repeated mass or targeted chemotherapy. This is effective, but is expensive in both drug costs and the need for skilled manpower in the diagnosis and delivery of treatment. This has been attempted in Kenya by Butterworth *et al.*, (1984, 1985); The Gambia by

Wilkins *et al.*, (1987b) and in Brazil by Machado (1982). Repeated mass treatment is extremely expensive where high rates of reinfection of young children occur after chemotherapeutic cure. Such reinfection requires treatment at frequent intervals and long term funding is therefore needed but is usually unavailable. Alternative approaches are desirable in the long term, among which, the development of a vaccine must be considered as an essential goal.

1.4.2 INTERMEDIATE HOST CONTROL

Intermediate host control by mollusciciding has on many occasions had limited effects and has been found to be extremely expensive where large water bodies are concerned and unorganised water contact points are involved (Liese, 1986; Fenwick, 1987; Klumpp and Chu, 1987). In Zimbabwe, a number of control programmes have been in operation for about 10 years. The strategy which has been adopted is a community based approach integrated in the primary health care system (Taylor, 1986), where treatment is free to all those who are infected and present themselves at clinics i.e. passive case detection. Also, some biological control has been implemented with extracts from plant berries from *Phytolacca dodencandra* (Ndamba *et al.*, 1994). Although communities are involved in growing the plants and application of the berries at the water contact points offers a potential cost reduction on other approaches, some financial inputs are essential for such programmes to successfully take off, and trained personnel are required for initial training of the community, as over-application of the plant molluscicide into water bodies can result in killing all water fauna. The use of other chemicals like Bayliscide and copper sulphate are deterrently expensive for the developing countries. In most epidemiological settings control of the intermediate host alone cannot be successful in regulating infection

and disease without involving other aspects of parasite transmission stages, including the deposition of eggs into water bodies (Evans, 1983).

1.4.3 HEALTH EDUCATION AND SANITATION

Health education has been considered to be an essential component in controlling diseases of public health importance including schistosomiasis (Teesdale, 1986). Since the life-cycle of the schistosome involves an intermediate host which survives in fresh water, and the introduction of eggs into the water involves inadequate control of sewage pollution or insanitary habits, interventions can be designed to tackle these aspects of the life-cycle. In some areas where schistosomiasis is still endemic, little or nothing has been done in domestic water and sanitation improvement which is considered as the main source of maintaining the continuation of the cycle. Water for domestic use has not been decontaminated, and water pipe systems have not been introduced, although in some rural areas safe drinking water has been provided by construction of deep protected wells and boreholes. Such control systems are possibly the most effective measures, but implementation of such expensive programs suffers from a lack of adequate resources and not infrequently from insufficient long term government commitment (Liese, 1986; Fenwick, 1987; Klumpp and Chu, 1987).

The availability of surface water with temperatures favourable to intermediate snail survival is compounded by the behaviour of young children with heavy infections. They contribute most to contamination of the environment by indiscriminate defaecation and micturation in and around the water bodies. Even after education on the benefits of sanitary facilities and the provision of some clean water supplies, communities show some reluctance to stop the tradition of using natural water bodies. While children, and

some adults, continue contaminating these water bodies by indiscriminate excretion, such activities make it virtually impossible to combat schistosomiasis through sanitation, public health awareness and education. Efforts are thus being made to develop control methods that can combat the diseases even though continued contact with water occurs. Perhaps the most significant would be a vaccine.

1.5 SCHISTOSOMIASIS EPIDEMIOLOGY

1.5.1 AGE-PREVALENCE/INTENSITY CURVE

Age-specific intensity curves for communities in schistosomiasis endemic areas show a sharp rise in mean intensity of infection, from the time of first exposure during early childhood, until early in the second decade of life (Butterworth *et al.*, 1984, 1985). Intensity of infection normally then declines progressively with age (Hagan, 1987; Hagan *et al.*, 1987), although there is some evidence for a rapid worm elimination in people in some foci. Wilkins and Scott, (1978) showed from studies in The Gambia apparent rapid declines in infection. There is no firm evidence of rapid worm elimination being accompanied by a solid resistance to reinfection, as seen in many other infectious diseases. The slow decline in intensity of infection during adult life might be attributed to a slow spontaneous death of adult worms, together with a slowly acquired immunity to super-infection. However, the decline might also be attributed to the slow death of adult worms, a reduced exposure to contaminated water in the older age groups, alterations in the fecundity of adult worms and to the capacity of eggs to escape into the faeces and urine (Butterworth *et al.*, 1984, 1985; Hagan, 1987; Hagan *et al.*, 1987).

There is evidence that some patients resist reinfection after treatment in spite of high levels of observed contact with water known to contain infected snails (Wilkins *et al.*, 1984a,b, 1987; Hagan *et al.*, 1987; Butterworth *et al.*, 1987, 1992; Dessein *et al.*, 1992). In such patients the resistance to reinfection is attributed to immunological factors of the host (Hagan *et al.*, 1991; Butterworth *et al.*, 1992; Roberts *et al.*, 1993). Such resistance appears to be an acquired, age-dependent event, which appears to be greater in children over 10 years old and increases thereafter (Hagan *et al.*, 1991, 1994).

The convex age-prevalence curve of schistosomiasis in human populations may in part be the result of exposure patterns, but age-dependent acquired resistance to infection is now considered an important factor. Correlation studies have indicated a role for IgG4, IgM and IgG2 blocking antibodies in maintaining the continued susceptibility of young children and of IgE, IgG1 and IgA antibodies in mediating protection in older individuals. These studies were undertaken by Hagan and colleagues (1987) on *S.haematobium* in The Gambia and by Dessein *et al.*, (1992) on *S.mansoni* in Brazil with similar conclusions. Following treatment, the extent to which reinfection subsequently occurs is strongly dependent upon age. In conditions of continued transmission, young children become reinfected rapidly. In some cases intensities of infection as reflected by egg excretion in urine or faeces, may reach 50 % of pre-treatment levels within one year. In contrast, older children and adults become reinfected only at very low intensities. Interestingly, although reinfection intensities were reported to reach a peak in children aged 8 - 12 years, peak water contact is at 16 to 24 years. Butterworth *et al.*, (1985, 1987) attributed the lack of reinfection in adults to previous experience of infections which resulted in an acquired immunity. Some workers do not exclude age-dependent physiological changes but, generally, the development of resistance appears to be age-

dependent in that a long period of exposure to repeated challenges seem to be necessary for the full development of resistance. Two main factors that influenced infection intensities in reinfection studies have been reported as water contact levels and the age of the exposed individuals (Wilkins *et al.*, 1984a,b; Hagan, 1987; Chandiwana, Woolhouse and Bradley, 1991).

1.5.2 REINFECTION AND MODELLING STUDIES

Butterworth *et al.*, (1984) were the first to use a reinfection study design where chemotherapy was used to remove pre-existing infections. With this design it is possible to examine levels of newly acquired infection and to relate this to levels of observed exposure. If levels of reinfection cannot be related to exposure, then some other factors, such as immunological resistance may be functioning. A reinfection study design, involving all age groups, was carried out in The Gambia where the relationship between infection intensities and exposure was studied. An index of exposure was determined which made assumptions about the effects of different types of contact on likelihood of acquiring infection. The principle assumption was that the number of cercariae which penetrate during water contact is proportional to the product of exposure time, cercarial density and cumulative exposure of infection of each individual and is proportional to the sum of these products (Wilkins *et al.*, 1984a,b; Hagan, 1987; Butterworth *et al.*, 1987; Chandiwana, 1987b). The study design used in The Gambia added to an understanding of the re-infection intensity patterns at the population level and established a potential role for protective immunity, leading to the hypothesis that protective immunity gradually develops in most endemic communities (Wilkins *et al.*, 1987; Hagan *et al.*, 1987 and Butterworth *et al.*, 1987). Confirmation of the validity of this approach came from a study of school children in Kenya, where some children with low exposure and

high levels of reinfection were identified as susceptibles and those with high exposure but low levels of reinfection were classified as resistant (Sturrock *et al.*, 1983; Butterworth *et al.*, 1984, 1985, 1992).

Given that protective immunity appears to develop slowly with time in communities in endemic areas, it has been hypothesised that the development of immunity is in part dependent on repeated exposure to infection and therefore that in areas of intense transmission immunity may develop more quickly than in areas of lower levels of transmission (Hagan *et al.*, 1994). Woolhouse *et al.*, (1991) in a Zimbabwean study of school children analysed 17 data sets for *S.haematobium* infection. A significant negative correlation between the peak prevalence of infection and the age of peak prevalence was shown. The results were interpreted in the light of The Gambian, Kenyan, and Brazilian findings on the epidemiological effects of acquired immunity. It is now considered that in areas of intense transmission the peak intensity of infection is reached earlier and consequently the decline in infection resulting from the development of acquired immunity begins earlier, this has been referred to as 'peak shift' by Woolhouse and colleagues (1991) in Zimbabwean pupils living in an endemic areas of *S.haematobium*. The peak shift has been interpreted as giving indirect evidence for development of an acquired immunity in different areas with different levels of *S.haematobium* infection.

Mathematical modelling of the epidemiology and control of infection, transmission and morbidity could play an important role in evaluation of the immunological, genetic, water exposure and other factors which may determine resistance and susceptibility to infection and also in allowing those responsible for schistosomiasis control programmes to investigate the likely outcome of alternative intervention strategies (Woolhouse, 1991,

1992). One variable that has been little explored in modelling studies, to date, is the possible importance of variability amongst human in their genetic predisposition to infection (Dessein *et al.*, 1992). The importance of this possibility has been highlighted by a recent family study of schistosomiasis in Brazilians by Marquet *et al.*, (1996). The group identified the host genetic region (5q31-q33) that may determine an individual's resistance or susceptibility to infection. This genetic region contains loci that encode the cytokines IL-3, IL-4, IL-5, GM-CSF and IL-13 (Boulay and Paul, 1992a,b, 1993; Marquet *et al.*, 1996).

1.6 SCHISTOSOMIASIS DISTRIBUTION IN ZIMBABWE

Studies carried out by Taylor and Makura (1985) as part of a national schistosomiasis survey categorised the country into regions of different prevalence and intensities. The data were collected from 14, 619 school children in 157 schools throughout the country for *S.haematobium* and *S.mansoni*.

The major factors affecting the distribution and prevalence of schistosomiasis in Zimbabwe were the availability and permanence of surface fresh water which is essential for the development of high snail population densities (Clarke and Shiff, 1968; Shiff, 1975, 1979; Hustings, 1983). Also important was the poorly developed safe water supplies for domestic use in some areas which results in an increase in human water contact which in turn influences the amount of contamination and incidence of infection. Schistosomiasis distribution was also partly determined by environmental factors; temperature and rainfall, which vary according to local geographical area and in turn affect the snail population and amount of human water contact (Chandiwana and Christensen, 1988). From the studies cited above and that of Chandiwana (1987a,b),

endemicity of schistosomiasis was found to be closely related to availability of surface water. In the north-east of Zimbabwe the high rainfall and soil type result in most streams and rivers being perennial even in drought years, hence highest prevalences of *S.haematobium* and *S.mansoni* were found there. In the south-east, rainfall is generally lower and only the major rivers are permanent and perennial. In the west and south a combination of low rainfall and sandy soil leads to even the major rivers drying up, with water remaining only in some reservoirs. Since schistosomiasis is a disease which has a very focal transmission, within any endemic zone some specific areas may have a higher or lower prevalence than might be expected due to some local factors. Transmission is highly seasonal over most of Zimbabwe, with two peak transmission seasons March to May and September to November, with the exceptions of the Zambezi valley in the north and the south-east where, due to warmer conditions and perennial water, seasonality may be less marked (Shiff *et al.*, 1975, Chandiwana, Christensen and Frandsen, 1987). Zimbabwe has an active schistosomiasis control programme in the irrigation schemes and in some recreational areas and over the past few years it has adopted a national schistosomiasis control strategy based on chemotherapy and community-based action programmes on sanitation, water supplies, snail control and health education.

1.7 THE NEED FOR VACCINES AND POSSIBLE VACCINE CANDIDATES

Reports on the development of partial immunity make it logical to attempt vaccination but, apart from experimental vaccines for animal use, vaccine development has yet to be successful (Capron *et al.*, 1987; Butterworth *et al.*, 1992; Dessein *et al.*, 1992; Hagan, 1992; Wilson 1993). There is now a range of promising vaccine candidates which are on trial awaiting confirmation of their efficacy in human subjects (Table 1.1). Most of the vaccine trials in animal models have reported significant degrees of protection expressed

as the reduction of the number of adult worms in comparison to non-vaccinated controls after a standard cercarial challenge. In some studies, injection into experimental animals of live schistosomula attenuated by sub-lethal doses of radiation resulted in high levels of resistance to a subsequent challenge. This might depend on the exposure of particular epitopes, or might reflect an improved antigen presentation due to slow passage of the irradiated parasites through the lymph node and lungs. While the potential for undesirable pathological effects makes an attenuated vaccine unlikely, it provides a useful model system for examining mechanisms of resistance. Nevertheless most current interest lies in the development of recombinant "peptide" vaccines and DNA vaccines (Butterworth, 1990; Capron, 1994; Bergquist, 1995; Dunne, Abath and Hagan, 1995).

In developing schistosome vaccines, *S.mansoni* has been the easiest species to be maintained in the laboratory away from endemic areas and thus most progress has been made with this parasite. Several antigens identified in *S.mansoni* have been found to have homologues in other schistosome species, for example glutathione S-transferase (GST) (Capron *et al.*, 1987, 1994). Two larval surface molecules are glyceraldehyde phosphate dehydrogenase (GAPDH), and a 22kDa antigen recognised by IgE antibodies in adult worm preparations (Goudot-Crozel *et al.*, 1989 and Dunne *et al.*, 1992). Immunological findings by Dunne *et al.*, (1992) on Kenyan patients attributed the lack of subsequent reinfection to the 22 kDa molecule.

Several biologically active molecules including the GST isoenzymes (Capron *et al.*, 1992; Bushara *et al.*, 1994), GAPDH (Goudot-Crozel *et al.*, 1989) and the glycolytic enzyme, triose phosphate isomerase (TPI) (Reynolds, Shoemaker and Harn, 1992; Reynolds *et al.*, 1994) have been shown to induce partial protection in several

experimental animals. *S.mansoni* 28kDa GST confers partial protection in several animal species including patas monkeys (Boulager *et al.*, (1995), while the 26 kDa isoenzyme has been used in studies on *S.japonicum* (Henkle *et al.*, 1990; Bashir *et al.*, 1994). Much effort has been applied to circumvent the problem of undesirable side effects by use of synthetic peptides with isolated epitopes (Reynolds, Shoemaker and Harn, 1992; Reynolds *et al.*, 1994; Harn *et al.*, 1995). Some myofibrillar proteins, such as paramyosin are also being investigated as potential vaccine candidates (Bergquist, 1995).

As the adult worms evade immune attack, studies have focused on the apparently more vulnerable larval stage. Schistosomula membrane epitopes are also expressed on the adult worm tegument in which an array of antigens ranging from 8 kDa to more than 200 kDa have been identified (Grzych *et al.*, 1984; Soisson *et al.*, 1992). Important schistosomula antigens include Sm23 (Reynolds, Shoemaker and Harn, 1992) and Sm38 (Grzych *et al.*, 1984, 1985), but the simultaneous elicitation of blocking antibodies has ruled out the use of Sm38. Sm10 has been reported to be of great interest due to production of strong T cell responses in subjects already sensitised by natural infection (Couissinier-Paris and Dessein, 1995).

Table 1.1: Summary of Schistosome vaccine candidates:

Identity of antigen	Size (kDa)	Stage	Function	Claimed protection (%)		
				Mouse	Rat	Other
Glutathione-S-transferase (Sm28GST)	28	Schistosomula Adult worms	Enzyme	30-60	40-60	40 (Baboon)
Paramyosin (Sm97)	97	Schistosomula Adult worms	Muscle protein	30		
Irradiation-associated vaccine antigen(IrV-5)	62	All stages	Muscle protein	50-70	95	25 (Baboon)
Triose-phosphate isomerase (TPI)	28	All stages	Enzyme	30-60		
Sm23	23	All stages	Membrane antigen	40-50		
Fatty acid binding protein (FABP)-14 (Sm14)	14	Schistosomula	Membrane antigen	65 (outbred)		90-100 Rabbit)

Adapted from: **Bergquist, N.R.** (1995). Controlling Schistosomiasis by Vaccination: A Realistic Option? *Parasitology Today*, 11 (5), 191-194.

1.7.1 SCHISTOSOME ANTIGENS AND IMMUNO-DIAGNOSIS

The search for possible vaccine candidates and an easier methodology for immunodiagnosis has aided in the identification of antigens which preferentially stimulate particular arms of the immune response. The egg antigens are known to play a major role in pathogenesis of schistosomiasis (Dunne *et al.*, 1987, 1988; Dunne and Doenhoff, 1983; Doenhoff *et al.*, 1993). Several groups have prepared and characterised *S.mansoni* antigens which have allowed the differentiation between acute and chronic infections (McLaren *et al.*, 1978, 1981, Hillyer and Pacheco, 1979). Hillyer and Pacheco (1986) managed to isolate and characterise specific *S.haematobium* egg antigens which share epitopes with an adult worm antigen. Some workers, Simpson *et al.*, (1985) and Kelly, (1987), were able to characterise *S.haematobium* schistosomula antigens. Hayunga *et al.*, (1986) and Trottein *et al.*, (1992) also studied adult *S.haematobium* antigens and managed to clone glutathione S-transferase which has been suggested as a potential vaccine candidate. Most of the antigens so far identified are known to cross-react with schistosomula antigens of other *Schistosoma* species. Some major circulating

glycoprotein antigens associated with the schistosome gut have been identified as circulating cathodic antigen (CCA) and circulating anodic antigen (CAA). They are now used in sero-epidemiological diagnosis (Deelder *et al.*, 1989; De Jonge *et al.*, 1989a,b, 1990; Gryseels *et al.*, 1994; Ndhlovu *et al.*, 1995). The CCA and CAA antigens are genus-specific and are extremely useful in distinguishing present and past infections and in estimating worm burdens (Gryseels *et al.*; 1995), although the method cannot substitute for conventional egg counts which has always been used to estimate the worm burdens.

1.8 EVASION OF THE IMMUNE RESPONSE BY ADULT WORMS

The adult worms of a primary infection are capable of surviving for long periods, some are believed to survive for up to 30 years or more, yet the host develops immunity to super-infection, thereby allowing the opportunity for prolonged transmission of the parasite (Butterworth and Hagan, 1987). There are many mechanisms whereby the adult worm and the schistosomulum evade the immune response in immune competent individuals. Uptake by the developing schistosomulum of a range of host molecules parallels the loss of susceptibility of the worm to a variety of immune effector mechanisms (McLaren, Clegg and Smithers, 1975; McLaren and Terry, 1982; Smithers, McLaren and Clegg, 1985). Host molecules that may be on the worms' surface include blood group substances, such as glycolipids, that are expressed on the red blood cell surface (Butterworth, 1990). Studies by Butterworth *et al.*, (1982), McLaren and Incani (1982), McLaren and Terry (1982) and Smithers, McLaren and Clegg (1985) indicated that absorbed host molecules can effectively mask at least some parasite determinants. It was demonstrated that schistosomula recovered from the lungs of mice failed to bind, after 3 hours exposure, most monoclonal antibodies raised against skin-phase

schistosomulum surface antigens as these had been masked. However, if such schistosomula are subsequently cultured *in vitro*, binding of at least one monoclonal antibody can be demonstrated, and this is associated with a loss of host-derived molecules (McLaren and Terry, 1982; McLaren and Incani, 1982).

Some experiments suggested that there is an actual loss of expression of parasite antigens from the outer of the two bilayers, so that the membrane becomes immunologically inert (Mazza *et al.*, 1990). Experiments with monoclonal antibodies further suggest that different antigens are lost from the outer membrane at different rates. For some major antigens, a rapid loss of expression occurs within 24 to 48 hours after transformation of the cercaria into the schistosomulum. Other antigens show a more prolonged expression, being detectable on the surface, with the appropriate monoclonal antibody, as late as five days after skin penetration (Simpson *et al.*, 1986). New antigens not detected on the skin-derived schistosomula may also be expressed at this late stage (Clegg, Smithers and Terry, 1971; McLaren, Ramalho-Pinto and Smithers, 1978; McLaren and James, 1985; Butterworth *et al.*, 1979a,b,c; Butterworth, 1990, Agnew *et al.*, 1992). In addition to showing a reduced expression of some surface antigens, the tegument of the developing schistosomulum may become progressively refractory to immune attack (Kusel *et al.*, 1975, Payares *et al.*, 1985 and Butterworth 1990). This phase of reduced intrinsic susceptibility to attack appears to be limited to the lung-stage migration.

Another mechanism of evasion includes the production by the parasite of substances that actively inhibit host effector mechanisms. Capron *et al.*, (1982), Capron and Capron (1986) and Butterworth (1990), have shown that the developing schistosumula produce

and release at least two proteases, one with trypsin-like activity. This protease production results in the cleavage of surface bound immunologlobulins, with a consequent failure of ADCC effector mechanisms that depend on an intact Fc region. The action of the protease also results in the release of small peptides, such as the tripeptide threonine-lysine-proline, that actively inhibits macrophage cell function. Adult worms can be demonstrated to release soluble factors such as schistosome-derived inhibitory factor (SDIF), that inhibit lymphocyte function and mast cell degranulation (Phillips *et al.*, 1978, 1992; Dessein *et al.*, 1981; Auriault *et al.*, 1981; Phillips and Lammie, 1986).

1.9 IMMUNE MECHANISM IN ANIMAL MODELS

Much of the present knowledge about the nature and mechanisms of acquired immunity to schistosomiasis has come from studies in a range of experimental animal models (Smithers and Terry, 1967a,b; Colley *et al.*, 1977a,b; Capron *et al.*, 1977, 1982; McLaren *et al.*, 1978, 1982; Smithers and Gammage, 1980; Smithers *et al.*, 1985; Mazza *et al.*, 1990). It was observed that following low level primary infection of mice, rats or rhesus monkeys, adult worms of the first infection continued to survive, while the host was capable of resisting challenge schistosomula of a secondary infection. This phenomenon of acquired resistance to superinfection in the presence of continued primary infection was termed concomitant immunity by Smithers and Terry (1967a,b).

Antibody- and cell-mediated immunity both play a critical role in the protection of animals against schistosome infection (Capron *et al.*, 1975, 1977, 1982; Capron and Capron 1986). Eosinophils and monocytes are cytotoxic for opsonised larvae, and this is enhanced by cytokines released by a variety of cells including monocytes and T

lymphocytes. Most studies have identified the 1-4 day larvae as one of the major targets for protective immune mechanisms. One day old larvae exhibit high antigenicity and vulnerability to immune attack (Clegg and Smithers, 1971; Smithers and Gammage 1980; Dessein *et al.*, 1981).

1.9.1 HUMORAL RESPONSES IN SCHISTOSOME IMMUNITY

Capron and colleagues have provided useful information on humoral responses in resistance and susceptibility to *S.mansoni* infection, particularly in rats, in which antibody-dependent-IgE killing of the larval schistosomes by platelets, eosinophils and macrophages seem to play an important role (Capron *et al.*, 1975, 1977, 1982, 1989; Capron and Capron 1986; Capron *et al.*, 1977, 1978). Direct *in vivo* evidence for a major role for IgE in protection has been provided by passive transfer of IgE from immune to naive animals, (Ogilvie *et al.*, 1966; Capron *et al.*, 1984) and the demonstration that IgE-suppressed rats have an enhanced susceptibility to *S.mansoni* compared with normal rats (Capron and Capron 1986). The experiments with IgE suppressed rats confirmed that IgE, in addition to its role in ADCC was also critical for the rapid recruitment of effector cells and molecules to the site of penetration of the parasite before the invading schistosomula becomes refractory to attack by effector mechanisms. This function depends on the specific stimulation by parasite antigen of IgE-sensitised mast cells, basophils and monocytes to release mediators that amplify and prolong the immediate reactions into a later phase. Capron and Dessaint (1992), Capron *et al.*, (1987) suggested that immediate hypersensitivity reactions may have evolved as a specific immune defence against helminth infections.

1.9.2 CELLULAR AND EFFECTOR RESPONSES

In vitro studies have suggested a number of potential effector killing mechanisms involving both cells and antibodies directed against the schistosomulum stage parasite (Boursum *et al.*, 1982; James and Sher, 1990). Experimental models of infection and vaccination have underlined the importance of $CD4^+$ T cells in the induction of host responses to *S.mansoni* infection Capron and Capron, 1986; Sher and Coffman, 1992; Cheever *et al.*, 1992). Both T helper 1(Th1)- and Th2-like cytokine responses have been identified in murine models of *S.mansoni* infection (Mosmann *et al.*, 1986; Pemberton *et al.*, 1993, 1994, 1995; Romagnani, 1992; Wynn, *et al.*, 1994, 1995, 1996). In these models Th1 mediators have been linked to protective responses in vaccination studies, whereas Th2 responses induced following infection and the onset of the egg laying, are thought to contribute to the development of pathology. The importance of these functionally distinct T cell subsets and their contribution to protective immunity have yet to be established in humans. It is currently argued that in mice Th1 related events are associated with immunity (James and Sher, 1990). Some argue that Th2-dependent events which include eosinophil and IgE production may also be important, but it is not clear whether or not immunity in humans is dependent on similar responses as reported for the murine models.

At the site of parasite penetration, there is passive sensitisation similar to immediate hypersensitivity reaction combined with IgE-mediated effector responses involving eosinophils, macrophages and platelets. Schistosome cercariae have a protease enzyme which they secrete aiding their penetration of the host skin and this protease induces a dominant Th1 type response in murine hosts. At the onset of egg deposition after worm maturation, there is a shift to a Th2 profile. The switching was reported to be triggered

by injection of schistosome eggs alone (Sher, 1992; Sher and Coffman, 1992). After egg deposition responses are pre-dominantly of a Th2-type and are associated with eosinophilia and IgE production. Much focus has been concentrated on the immunoregulatory mechanisms involved in the events which the schistosome egg elicits and which result in formation of the hepatic granulomatous response which is the major cause of morbidity of schistosomiasis. Most reports have shown that the primary immune response to schistosome eggs is IFN-γ production by cells from draining lymph nodes followed by a Th0 (mixed) profile of cytokines (Vella and Pearce, 1992). It was reported that by day 7-10, IFN-γ production is severely depressed, while IL-2 and most of the Th2 cytokines continued to be produced. Several groups have reported that the downregulation of Th1 cell responses is effected by IL-10 released from the Th2 cells (Sher *et al.*, 1991; Vella and Pearce, 1992).

Granuloma formation may not be totally dominated by Th2 cytokines and the immune modulation that occurs in chronically-infected mice has been attributed to continued IFN-γ production. Granuloma modulation is the result of a generalised suppression of cytokine synthesis. Th1 cell clones reactive with egg antigens are capable of stimulating granuloma formation *in vivo* (Chikunguwo *et al.*, 1991). TNF-α appears to act as a reproductive or developmental stimulus for the adult female worms (Amiri *et al.*, 1992).

1.9.3 T HELPER CELL SUBSETS:

T helper cells regulate most aspects of the antigen-dependent response of B cells. T helper cells are required for activation, clonal expansion, differentiation and isotype regulation of B cell responses to most antigens. The subsets were first reported by Mosmann *et al.*, (1986). The lymphokine secretion phenotype of Th1 and Th2 clones is

stable in murine models, suggesting that individually stimulated Th cells may express only one phenotype. The Th2 supernatants enhanced IgE, IgG1 and IgA, while Th1 supernatants inhibited IL-4 mediated effects. IFN-γ at relatively low concentration completely inhibited IL-4-enhanced IgE production and reduced IgG1 production. IgE was regulated by opposing effects of two lymphokines: IL-4 and IFN-γ made by two different subsets of Th cells. These studies proposed that the level of IgE production during an immune response reflects the relative levels of IL-4 and IFN-γ to which the activated B cells are exposed (Coffman *et al.*, 1988; Mosmann and Coffman,1989), which in turn depends on the relative numbers or states of activation of Th1 and Th2 cells of the appropriate specificity. Their model predicts that Th2 cells will be helpers for an IgE response and that Th1 cells will act as isotype-specific suppresser cells for IgE and at the same time may be acting as helper cells for responses of other isotypes.

The best evidence for existence of the subsets comes from the rat, in which expression of the OX-22 marker splits Th cells into a fraction containing cells that produce IL-2 (OX-22+) and another fraction that produces little IL-2 but transfers most of the T helper cell activity for B cells (Arthur and Mason 1986). The lymphokines which appear to have greatest influence on B cells i.e. IL-2, IL-4, IL-5, IL-6 and IFN-γ are each expressed by only one of the Th subsets; this implies that there are at least two distinct pathways of B cell activation. It is important to note that Coffman *et al.*, (1988) found no cell surface antigen differences between the two groups. Given appropriate conditions, the majority of both Th1 and Th2 clones can provide help to B cells but the resultant immunoglobulin isotypes produced by these B cells varies in accordance with the different subsets. Evidence supporting the requirement for Th2 was obtained in animal models in which IgE, eosinophils and mast cells during helminth parasite infection of mice and rats

resulted from elevated levels of Th2 cytokines, yet non of these response occurred in nude or thymectomised animals.

1.10 IMMUNITY IN HUMANS

1.10.1 HUMORAL IMMUNITY

It is now known that all stages of the schistosome found in the definitive host are recognised by antibodies (Butterworth *et al.*, 1985, 1987, 1992; Hagan *et al.*, 1987, 1991). These studies have shown a reciprocal relationship between IgE and IgG4 levels for *S.haematobium* and *S.mansoni* infection. Specific IgE antibodies have been suggested as mediators of protective immunity in man. Hagan *et al.*, (1991) working on *S.haematobium* in The Gambia, provided the first convincing evidence that human resistance to reinfection is associated with high levels of parasite-specific IgE. Some age-related trends in specific IgE levels have also been reported from other studies: Rihet *et al.*, (1991, 1992), Dessein *et al.*, (1992), Demuere *et al.*, (1993) for *S.mansoni* infected Brazilians; Hagi *et al.*, (1990) on *S.haematobium* infected Somalis; Butterworth *et al.*, (1982, 1987, 1988, 1992) on *S.mansoni* infected Kenyans and Ndhlovu *et al.*, (1996) on *S.haematobium* infected Zimbabweans. The general consensus from these reports is that although total IgE levels were high in all age groups, anti-schistosome IgE levels were low in those under 10 years old and higher in 10 - 19 years old and above, which gives a pattern consistent with the acquisition of resistance to infections. Dunne *et al.*, (1992) demonstrated that IgE antibodies against a *S.mansoni* adult worm preparation correlated positively with age and negatively with reinfection, whereas IgE antibodies against other life cycle stages showed either no relationship or the reverse correlation. IgE does not work in isolation and hence there is evidence from diverse studies highlighting the roles of other antibodies which may be involved in the immune defence against helminth

infection (Capron and Capron, 1986). Some non-specific IgE and parasite-specific IgG4 are also found in the infected individuals. The non-specific IgE possibly competes with specific IgE to reduce the risk of anaphylaxis and protect the host from the potentially lethal consequences of hyper-reactivity to parasite antigens. IgG4 also plays a similar role (Hagan *et al.*, 1991). With developments of vaccines, it is anticipated that the protective antigens may stimulate protective IgE responses, although there is a risk of inducing harmful anaphylactic reactions in the process.

Some antibodies of IgM and IgG subclasses were reported as not having any protective role in schistosomiasis infections (Khalife *et al.*, 1986; Butterworth *et al.*, 1987; Dunne *et al.*, 1987; Hagan, 1987; Demuere *et al.*, 1993), but in another study IgM has been associated with protection against a 68kDa antigen (King *et al.*, 1988). These antibodies have been shown to compete with IgE and IgA for attachment to the schistosomulum. These antibodies have been collectively termed blocking antibodies. The blocking antibodies appear to be triggered by polysaccharide antigens released from the eggs (as reviewed by Hagan, 1987). During early infection blocking antibodies of IgM or ineffective IgG isotypes predominate. These bind to carbohydrate epitopes on schistosomulum surface glycoproteins (Dunne *et al.*, 1987, 1992; Dunne 1990; Butterworth *et al.*, 1988, 1992) thereby blocking the binding of effective IgG1 and IgE antibodies elicited in response to those glycoproteins and thus preventing effective immunity.

Epidemiological evidence from studies by Hagan *et al.*, (1991), Rihet *et al.*, (1991) and Demuere *et al.*, (1993) also suggest that high levels of IgG4 are associated with high levels of infection and reinfection. In a study on *S.haematobium* in Zimbabwe, Ndhlovu

et al., (1996) found a positive correlation of intensity of infection with IgG and IgM responses to soluble egg antigen (SEA) and with IgG4 to soluble worm antigen (SWA). This study indicated that IgG4 and IgM may be associated with infection. This was also reported in filarial infections where competing antibody of the IgG4 subclass was also produced, with levels particularly high in the least resistant individuals (Maizels *et al.*, 1993). Thus, IgG4 is suggested to block immune effector mechanisms especially in children living in endemic areas (Auriault *et al.*, 1990). As the children get older, evidence from epidemiological studies cited above suggests that there is switching of the response from susceptibility to protective response in which there is a marked increase in the ratio of protective IgE to blocking IgG4 levels and the children become immune to infection.

1.10.2 CELL MEDIATED IMMUNITY AND EFFECTOR CELLS

Some myeloid cells can interact with immunoglobulin-coated foreign cells to produce contact lysis rather than phagocytosis. This process is termed antibody-dependent cell-mediated cytotoxicity (ADCC), and the effector cells are termed killer cells. Antibodies lethal to schistosomula of *S.mansoni* have been described by several workers including those stated in the above sections on murine models. *In vitro* experiments have shown that ADCC might be a primary mechanism of defence against schistosome infection. Several ADCC killing systems *in vitro*, involving basophils, eosinophils, neutrophils, macrophages and mast cells have been described by different workers (Butterworth *et al.*, 1975, 1979a,b,c, Butterworth, 1984; Butterworth and Richardson, 1985; Colley *et al.*, 1977a,b; Capron *et al.*, 1975, 1982, 1989; Capron *et al.*, 1984; Anwar, Smithers and Kay, 1979; Ellner *et al.*, 1982; Sturrock *et al.*, 1983; Jarret and Haig, 1984; Hagan *et al.*, 1985a,b; McLaren and James, 1985; Pearce and James, 1986 and James and Sher, 1990).

Furthermore, human epidemiological studies have postulated that ADCC is involved in conferring protection (Hagan *et al.*, 1987).

1.10.3 T HELPER CELL SUBSETS AND CYTOKINES

T lymphocytes are important immunoregulatory cells, being involved in the activation of B cells and macrophages, in immune inhibition and in cytotoxicity against certain targets. T lymphocytes play an indirect role in protective immunity by recruitment of effector cells such as eosinophils, basophils, mast cells, neutrophils or macrophages, an activity which is mediated through lymphokines or cytokines. T lymphocytes have not been shown to directly damage schistosomula in culture (Butterworth *et al.*, 1974), although the lymphocytes are stimulated to proliferate and produce lymphokines. Depending on the cytokine producing T cells and the type of cytokines, the immune response can be activated to combat both microbes and multicellular pathogens.

The diversity of $CD4^+$ T cell dependent immune responses can now be explained after the development of immunotechniques in analysing T lymphocytes by the methods of propagating clones from T cell lines and assays of cytokines. Activated Th1 clones appear to induce a complex set of responses different from Th2 clones (reviewed by Abbas, Murphy and Sher, 1996; Romagnani, 1991; Coffman *et al.*, 1991). A major focus of Th1 responses seem to be cytotoxicity, both cell-mediated and complement-mediated. Th1 mediate DTH responses (Cher and Mosmann, 1987) and the combination of lymphotoxin and IFN-γ secreted by these clones is cytotoxic to certain types of cells and can stimulate optimum production of major opsonising and complement-fixing isotypes. IFN-γ activation of macrophages stimulates the ability of macrophages to mediate ADCC both by enhancing the expression of IgG-Fc receptors and by enhancing the production

of IgG1 and IgG3, the ligand for macrophage receptors, leading to phagocytosis, tumouricidal activity and killing of intracellular parasites (Warren and Vogel, 1985).

It has been reported that each T cell subset produces cytokines that serve as its own autocrine growth factor by promoting the development of naive cells into that subset. Subsets also produce cytokines that are cross inhibitory to the development of other subsets. IFN-γ produced by Th1 cells, for example, amplifies Th1 development and inhibits proliferation of Th2. As a result, once the T cell response begins to develop along one pathway it tends to be polarised in that direction (Pearce *et al.*, 1991; Pemberton *et al.*, 1993; Scott, 1993). The degree of polarisation of the T cells may reflect the state of antigen stimulation and the environmental stimulation involved in the cell responding. The degree of Th1/Th2 polarisation may also reflect the chronicity of the disease stimulating that response, especially in conditions where the antigen persists and cannot be eliminated easily, as reported by Murphy *et al.*, (1996) who employed *in vitro* repeated T cell antigenic stimulation to produce T cells, that expressed single transgenic antigen receptors, that led to increasingly polarised and irreversibly committed Th1 and Th2 subsets. The cytokine patterns dominating in a response have yet to be fully understood, especially when they have been produced from mixtures of cells in a population, such as from human PBMC samples, rather than from T cell clones. Because of these difficulties, some reports have settled on defining responses as Th1-like and Th2-like.

The functions of $CD4^+$ T cells are mediated through intimate interaction with other cells in which the T cell recognises cell surface molecules on the APCs. The ligand for the T cell receptor (TCR) is MHC molecule with an antigen peptide bound into a specialised

groove. Interaction of B7 on the cell surface of the APC with CD28 on the T cell provides a costimulatory signal that has received considerable attention (Linsley *et al.*, 1991). Activated $CD4^+$ T cells can stimulate both macrophages and B cells. To achieve B cell activation, the T cell provides two types of stimuli, induced cell surface ligands and soluble cytokines. The interaction of the T cell associated ligand with B cells results in activation of the B cell and endows it with the capacity to proliferate in response to soluble cytokines also produced by the T cells (Banchereau *et al.*, 1991; Banchereau and Rousset, 1991). These signals are also important in the process of immunoglobulin class switching, the mechanism through which antibodies of classes other than IgM are produced. According to current available information, only Th2 clones enhance secretion of IgG1 and IgE, either by causing selective proliferation or maturation of B cells already committed to IgG1 or IgE production or increasing the frequency with which B cells switch to the expression of those isotypes (Coffman *et al.*,1988; King and Nutman, 1993; King, Low and Nutman, 1993; Cheever *et al.*, 1994)

A few studies have investigated cytokine secretion during human schistosomiasis, the majority of the reports are on *S.mansoni* infections (Viana *et al.*, 1994; Williams *et al.*, 1994; Couissinier and Dessein, 1995; Araujo *et al.*, 1996), while very few studies have been reported on *S. haematobium* infection (Grogan *et al.*, 1996; King *et al.*, 1996). The Th1 and Th2 responses in relation to resistance to infection and reinfection have not been defined in humans to the same extent as in experimental animal models. However, the extensive studies in animal models have provided useful pointers toward the types of the immune responses that might be protective in humans. The animal models differ quite markedly from each other in both the extent and the nature of the protective immune responses. None of these animal models can be considered to mimic the situation in

humans in all respects (Colley *et al.*, 1986; Mosmann and Coffman, 1989; Auriault *et al.*, 1990, 1996; Rebeiro de Jesus *et al.*, 1993; Pemberton *et al.*, 1994).

It has become of interest to know if Th1 and Th2 $CD4^+$ T cells could be identified in schistosomiasis patients. Studies of cloned T cells *in vitro* from schistosome-infected humans did not fit into the Th1/Th2 mould leading to proposition that there are Th0 cells which only differentiate into Th1 and Th2 upon prolonged *in vitro* culture (Couissinier and Dessein, 1995). Various groups have attempted to determine if human T cells fit into this Th1/Th2 dichotomy using cytokine profiles from leukocytes (Roberts *et al.*, 1993; Williams *et al.*, 1994; Grogan *et al.*, 1996). To date there is little evidence to support this concept, with many groups reporting T cells producing IL-2, IL-4, IL-5, IL-6 , IL-10 and IFN-γ. The lack of confirmation of the Th1/Th2 concept in human schistosomiasis may suggest that the observed differences in the cytokine production in the mouse are more likely to mark the differences between murine models and humans (Romagnani, 1991, 1992; Cousinier and Dessein, 1995). More work is essential in this area on human schistosomiasis from different transmission areas in endemic zones and on different schistosome species.

The T cell-derived cytokines are produced after an intimate interaction of APC (which can be B cells) and the antigen-specific T cell. For IL-4 production, Poo *et al.*, (1988) showed that the induced cytokine is secreted directionally toward the cell that has presented the antigen. The cytokines produced by the T cells are concentrated in the small spaces between the two interacting cells. Cells in the general environment of the $CD4^+$ T cells that are not participating in the interactions with the T cells are likely to be exposed to little or no cytokine. This accounts for the high degree of specificity of

cytokine-mediated help. IL-4, IL-5, IL-6 and IL-10 are particularly noted for their action on B cells. IL-4 acts as costimulant of B cell growth (Banchereau *et al.*, 1991; Williams *et al.*, 1994), controlling immunoglobulin class switching of the B cells expressing IgM to two isotypes, IgG4 and IgE. In humans it has been shown that both IL-4 and its close family members, e.g. IL-13, can cause immunoglobulin class switching to IgE in *in vitro* systems, IL-5 plays an important role in the development of stimulated B cells into antibody-secreting cells by controlling the induction of high rate secretion of immunoglobulin (Punnonen *et al.*, 1993) and in induction and activation of eosinophils. Both IL-6 and IL-10 have been known to promote immunoglobulin secretion and control allergic inflammation, with IL-10 shown to be particularly prominent in inducing immunoglobulin secretion by human B cells grown in long-term culture on immobilised anti-CD40 together with IL-4 (Rousset *et al.*, 1992). The activated $CD4^+$ T cells interact with macrophages in a similar pattern but do not require a cell surface ligand for the induction of microbicidal activity in response to IFN-γ, it appears that cytokines such as IFN-γ and TNF-β act directly on macrophages to enhance their microbicidal activity; IL-4 and IL-10 block the microbicidal activity induced by IFN-γ (Liew *et al.*, 1989; Pretolani and Goldman, 1997).

Observations in several other host/pathogen interactions have reported that IFN-γ-dominated responses are protective against infections with microbes that are resident within cells (Snapper and Paul, 1987), while IL-4-dominated responses lead to enhanced disease in such cases (as reviewed by Abbas, Murphy and Sher, 1996). Although there have been fewer examples in which IL-4 production by $CD4^+$ cells has been shown to be protective, a few such instances are infection of mice with nematodes *Heligmosomoides polygyrus*, *Trichuris muris* and *T.spiralis*. Mice that have been cured of an *H.polygyrus*

infection fail to display immunity to re-challenge with parasite if IL-4 is neutralised (Urban *et al.*, 1991), while clearance of primary infections with *T.muris* is blocked by neutralising anti-IL-4 antibodies (Finkelman and Urban, 1992). Grencis (1991, 1996), reported two mouse strains which differed in IL-4 production by antigen-stimulated mesenteric lymph nodes cultures and timing of primary expulsion of *T.spiralis*. The mesenteric lymph node cultured secreted increased amounts of Th2 cytokines but low IFN-γ. It was concluded that a Th2 cell response mediates expulsion of *T.spiralis* as well as the associated inflammation pathology.

From the studies cited above it can be argued that IFN-γ-dominated responses are of particular value in intracellular infections through the IFN-γ-mediated enhancement of cellular immunity. By contrast, the products of Th2 cells, IL-4, IL-5, IL-6, IL-10 and IL-13 each potentially enhance some aspect of the B cell response and thus antibody production. Therefore, Th2 responses would be most appropriate in infections with extracellular helminths against which antibody would have greatest efficacy in ADCC. Another function of Th2-type responses may be to ameliorate the tissue damaging effects of immune responses mediated by Th1-like cells. Such damage may occur in the course of the protective function of Th1-like cells responding to an intracellular infectious agent. If the microorganism is already widespread at the time the response develops or if the response does not quickly rid the host of the infectious agent, continued T cell activation inducing both local inflammation and tissue damage may ensue, leading to potential compromise of normal function. A Th2-like response, by opposing the effects of IFN-γ on macrophages through the actions of IL-10 and IL-4 and possibly by suppressing production of IFN-γ and other cytokines by Th1 cells could control such self-inflicted injury (Pretolani and Goldman, 1997).

The recognition of the type of immune response as defined by the pattern of cytokines produced, may directly determine the character of that response, either its tissue-damaging character or capacity to protect or both. This can also assist in determining the factors that control induction of particular cytokine-producing profiles. It is known that naive T cells can develop into effector or memory T cells that have the capacity to produce IFN-γ, IL-4 or both, often described as Th1-like, Th2-like or Th0 effector cells. The question of interest is whether the production of IL-4 and IFN-γ are mutually exclusive, as had been suggested from early studies of long-term mouse T cell clones (Mosmann *et al.*, 1986). Initial studies of clones of human $CD4^+$ T cells failed to show the same degree of striking divergence of cytokine production, clones often produced IL-2, IFN-γ and IL-4, although ratios of IFN-γ to IL-4 often varied markedly. Some antigen-specific human T cell clones have failed to show a greater degree of Th1/Th2-type polarisation in their cytokine-producing phenotype than was true of murine clones that had been examined initially (Romagnani, 1991; Couissinier and Dessein, 1995), while some examples of mouse clones have reported clones that produced IL-2, IFN-γ and IL-4, which have been designated Th0 clones (Street *et al.*, 1990). Effector T helper cells appear to remain in a state in which they produce a large amount of cytokine upon challenge for only a relatively short period. However mice that have been primed will develop effector T helper cells promptly upon rechallenge, indicating that they have memory of the initial immunisation (Bradley *et al.*, 1993).

One of the recent developments in our understanding of T cell effector development is whether an individual precursor T helper cell can give rise both to cells that produce IL-4 as well as to those that produce IFN-γ. More information is required to determine

whether or not cell populations with distinct cytokine-producing phenotypes represent distinct cell lineages that are separated from one another prior to their initial contact with antigen, or are products of a differentiation step that occurs at the time of, or associated with, the antigen-specific activation of the cell. However if an intermediary effector Th0-like state is a requisite step in the acquisition of more restricted patterns of cytokine production, it would seem very likely that some precursor T helper cells can develop into cells that produce both IFN-γ and IL-4, or into cells that produce either IFN-γ or IL-4. The initial event that determines cytokine-producing phenotype may not be the selective outgrowth of already committed cells but rather the differentiation of precursor T helper cells under the influence of factors present at that time of primary stimulation. Some striking correlation of antigen dose and development of immune responses dominated by cellular immunity or by antibody production have been reported (Parish and Liew, 1972), who demonstrated that low doses of antigen favoured cellular immunity; intermediate doses, antibody formation and high doses, cellular immunity. Assuming that cellular immunity represents IFN-γ-dominated responses and the antibody production, IL-4-dominated responses, commitment to IFN-γ, or IL-4 production may be determined in part by the concentration of antigen and the time the antigen persists in the body. This is of major interest in the current work comparing cellular responses and cytokine production of individuals from an endemic region living in two areas of different transmission pattern, in terms of prevalence and intensity, of *S.haematobium* infection.

Besides the $CD4^+$ T cells, $CD8^+$ T cells are also cytokine producers at an equal rate to the $CD4^+$ T cells. Some $CD8^+$ cells have been reported to produce IL-2, IFN-γ, IL-4, IL-6 and TNF-α, which indicates that $CD8^+$ cells produce essentially the same range of cytokines as $CD4^+$ cells (de Vries *et al.*, 1991). T cells have exquisite specificity in terms

of recognition of antigen, but in terms of effector function, T cells have been said not to be specific in their response, liberating whatever cytokines they are capable of producing (Mosmann and Sad, 1996), although the stimulating antigen concentration and the individual's age and duration of infection should be suspected to play an important role in immunological responses of T cell cytokine production and immunoglobulin profiles, which are essential in schistosome infection, susceptibility and protection.

1.11 THE SPECIFIC OBJECTIVES COMPRISE THE FOLLOWING:

1. To examine patterns of infection and reinfection with *S.haematobium* in different communities.
2. To relate levels of infection and reinfection with *S.haematobium* to cellular responses to the parasite antigens -cercarial, worm and egg antigens.
3. To measure cytokine profiles of PBMC collected pre- and post-treatment and relate these to levels of infection or reinfection and resistance to infection.
4. To develop T cell clones, characterise them and study the cytokines produced by them after antigenic stimulation.
5. To examine the effects of repeated chemotherapy on morbidity, development of pathology and resistance to infection.
6. To measure levels of specific antibodies to soluble worm and egg antigens of *S.haematobium* in a population of children receiving repeated chemotherapy.

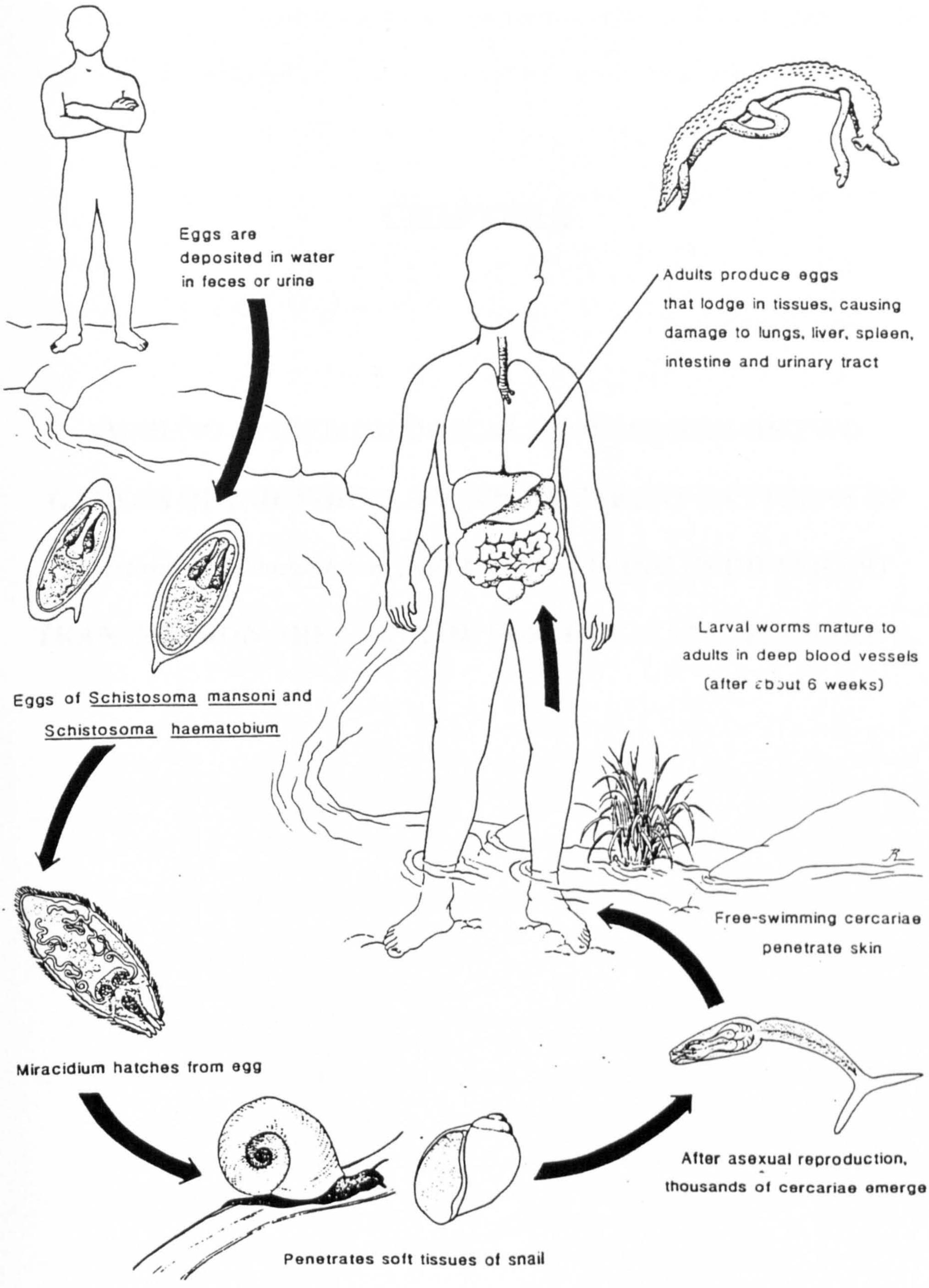
The above objectives will allow to answer the following questions

- do children from different transmission areas have different infection and re-infection intensities?
- do people resident in areas where infection is high develop resistance to infection

earlier due to greater experience of parasites?

- is the response to antigen stimulation influenced by/dependent upon age or history of exposure to infection?
- which type of cytokines are more dominant during infection and re-infection and which antigens stimulate these response?
- are the responses to infection and reinfection of the Th1 or Th2 type?
- does repeated treatment with praziquantel help in the development of protective immunity in young children?

Figure 1.1: The Life Cycle of *Schistosoma*



CHAPTER 2

IMMUNO-EPIDEMIOLOGICAL COMPARISON OF TWO GROUPS OF INDIVIDUALS WITH DIFFERENT PATTERNS OF *Schistosoma haematobium* INFECTION LIVING IN DIFFERENT TRANSMISSION AREAS IN THE BURMA VALLEY, ZIMBABWE.

2.1 INTRODUCTION

The humoral and cellular immune responses of patients with different schistosome infections have been reported from several studies, most of them highlighting the requirement for antibodies such as IgE and effector cells such as eosinophils and mast cells for protective immunity (Hagan *et al.*, 1985a,b, 1991; Butterworth *et al.*, 1985, 1987, 1992; Rihet *et al.*, 1991). The cellular studies have stressed the differences in proliferation between individuals with acute and chronic forms of schistosome infection, due to the long duration of infection which gives rise to chronic infections during which time the host is exposed to large quantities of antigenic materials from worms and eggs of the parasite (Colley *et al.*, 1977a, 1986; Ottesen *et al.*, 1978; Barsoum *et al.*, 1982; Ellner *et al.*, 1985; Gazzinelli *et al.*, 1985). The usual response of the host immune system to the repeated or continuous antigen exposure has been reported as essentially that of downregulating or modulating the immune response to prevent continued over-reactivity which might be harmful to the host (Hagan, 1987; Butterworth *et al.*, 1992).

The immunomodulation of responses during schistosome infection has been investigated usually by using *in vitro* lymphocyte transformation assays, examining the relationship between decreased immune reactivity and clinically defined disease status with levels of infection. The investigations by Colley *et al.*, (1977a, 1986), reported unresponsiveness in chronic infections; while those by Ottesen *et al.*, (1978) and Barsoum *et al.*, (1982) demonstrated vigorous responsiveness in those with acute forms of infection and a return to responsiveness in people treated for *S.mansoni* infection. Some workers have reported the presence of immune factors which are involved in immunomodulation during infections; these include studies by Colley *et al.*, (1977b), Gazzinelli *et al.*, (1980),

Ottesen and Poindexter, (1980) and Ellner *et al.*, (1982), who reported some serum factors inhibiting lymphocyte blastogenesis to schistosome antigens; while Hofstetter *et al.*, (1982, 1983) reported blocking antibodies and some antigen-specific histamine release from basophils sensitised with *S.mansoni*-specific IgE as playing a role in suppression of responses. The investigations cited above concluded that there are multiple suppressor mechanisms involved in immunomodulation which demand closer examination by the use of longitudinal reinfection studies to determine the factors involved in cellular immunity, including cytokines.

Some reports on cellular immunity have included a few selected cytokine types, King *et al.*, (1996) showed that IL-10 inhibits cell-mediated immunity by suppression of IFN-γ, while Grogan *et al.*, (1996) reported an increase in IL-4 and unaltered IL-5 production after treatment in *S.haematobium* infected patients. In contrast, Roberts *et al.*, (1993), Viana *et al.*, (1994) and Araujo *et al.*, (1996), reported contrasting roles of IFN-γ with dominance of IL-5 in subjects from *S.mansoni* endemic areas. Thus, a range of cytokines are now documented to be produced by the effector T helper cells and observations that immune regulatory events develop upon continued exposure to schistosomes and their products, suggests that they are likely to play a role during the establishment and maintenance of the infected condition. A comparison of individuals within an endemic region and areas with different infection patterns, before treatment and followed-up 12 and 18 months after treatment with praziquantel, may help elucidate the cellular modulation of proliferation and cytokine patterns of these individuals. The knowledge thus gained should be helpful in understanding how the immune response is modulated during infection and following treatment with praziquantel.

Since the concept of $CD4^+$ Th1 and Th2 cells was introduced over a decade ago (Mosmann *et al.*, 1986), rapid progress in the knowledge of their physiology has been achieved through some experimental models of infection and vaccination. The importance of these T helper cells in the induction of host responses to *S.mansoni* infection has been reported (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Sher *et al*, 1990, 1991; Pearce *et al.*, 1991; Cheever *et al*, 1992, 1994). In these studies, a number of potential effector killing mechanisms involving both cells and antibodies directed against different stages of the parasite have been suggested. The effector cell functions are now understood to be under the control of cytokines secreted by either Th1 or Th2 cells. However, little work has been conducted to understand cytokine secretions during human schistosomiasis, in particular for *S.haematobium* infection, where only a few studies using mixed lymphocytes have been completed (King, Low and Nutman, 1993; King *et al.*, 1996; Grogan *et al.*, 1996).

Evidence is now available regarding the roles played by cytokines in different schistosome infection stages, including the Th1 and Th2 responses that have been clearly defined in animal models, (mice and rats), from which useful pointers have been obtained for the detailed studies of human schistosomiasis (Del Prete *et al.*, 1988, 1993; Del Prete, Maggi and Romagnani, 1994; Sher *et al.*, 1990, 1991; Pemberton *et al.*, 1993; Cheever *et al.*, 1994; Openshaw *et al.*, 1995). The Th1 and Th2 cytokines identified in murine models of *S.mansoni* are as follows: the Th1 cell products are interferon gamma (IFN-γ), interleukin-2 (IL-2), lymphotoxin, and tumour necrosis factor-β (TNF-β), with the Th2 cells producing IL-4, IL-5, IL-6, IL-10 and IL-13. The major signature

cytokines are IL-4 and IFN-γ which mark each subset and these have a reciprocal cross-inhibitory effects (Mosmann and Coffman, 1989). According to the work cited above, the murine schistosome models have linked Th1 mediators to protection, whereas Th2 responses induced following infection and the onset of egg laying are believed to contribute to the development of pathology (Sher and Coffman, 1992). The relative importance of these functionally distinct T cell subsets and their contribution to protective immunity have yet to be fully established for the different schistosomes affecting humans. However, from recent reports, protection has been said to probably be either Th1- or Th2-dependent (Abbas, Murphy and Sher; 1996; Mosmann and Sad, 1997; Romagnani, 1997).

The evidence from murine models has been utilised mainly in the study of human *S.mansoni*, unlike *S.haematobium* where only a few studies have been reported. In their studies on *S.mansoni*, Roberts and colleagues, (1993), reported high levels of IL-5 and IFN-γ in individuals who were resistant to infection. In another study, Viana and colleagues (1994) observed individuals whom they classified as "endemic normals", having schistosome exposure but being uninfected, who produced high levels of IFN-γ to specific schistosome antigen stimulation. The reports on human cytokine studies during *S.mansoni* infection which are now complete have failed to clarify which particular cytokine(s) play an essential role in aiding protection or susceptibility, with the reports limited to mentioning the dominance of a particular cytokine (Roberts *et al.*, 1993; Viana *et al.*, 1994; Williams *et al.*, 1994; Couissinier and Dessein 1995; Araujo *et al.*, 1996). However, the longitudinal re-infection study described in this chapter will attempt to show how the cytokine profiles appear before treatment and change in the period

following treatment with praziquantel. The cellular immunity experiments were designed to investigate whether cytokine responses could be related to levels of re-infection and whether resistance or susceptibility could be classified as being either Th1- or Th2-like. The comparison of individual residents from areas of different infection intensities and prevalences may help shed some light on the possibility that modulation of immunological responses may alter Th1- and Th2-like cytokine balance and therefore alter the outcome of schistosome infections.

2.2 MATERIALS AND METHODS:

2.2.1 REAGENTS

Incomplete medium: RPMI1640;

Washing medium: RPMI1640, 1% gentamycin, 5% either FCS or human AB (hAB) serum (both from GIBCO-BRL).

Freezing medium: RPMI1640, 25% FCS, 20% DMSO.

Complete medium: RPMI1640 supplemented with 2.4 mM sodium hydrogen carbonate, 1% gentamycin, 2mM L-glutamine, 10% either FCS or hAB serum, 1% non essential amino acids, 1% sodium pyruvate and 25mM β-2-Mercarptoethanol.

S.haematobium antigens: (lyophilised- SEA-soluble egg antigen, SWAP-soluble worm antigen preparation and CAP-soluble cercarial antigen preparation; obtained from Schistosome Biological Supply Programme, Theodore Bilharz Research Institute, Giza Egypt)

Soluble alkaline phosphatase substrate (p-Nitrophenyl phosphate (SIGMA FAST)™.

Monoclonal antibody: anti-human IL-4 clone 82-4; IL-4-biotin clone 12-1 (Mabtech ab).

Mab anti-human IFN gamma Mab1-DIK; 7B6-1 biotinylated (Mabtech ab).

Purified rat-anti-mouse/human IL-5; Biotin rat anti-human IL-5 (Pharmingen).

Purified antihuman/viral IL-10; Biotin antihuman/viral IL-10 (Pharmingen).

Streptavidin alkaline phosphatase conjugate (Amersham Life Sciences).

methyl ^{3}H-thymidine (Amersham, UK)

2.2.2 STUDY AREA AND POPULATION.

The study population was chosen from a farming community of The Burma Valley Area, Eastern Highlands of Manicaland Province in Zimbabwe. The farming activities were based on irrigation and on seasonal rains. In each area there is a permanent river with a dam and several seasonal river tributaries. The two communities are about 10 km apart having low *S.mansoni* prevalence (<1% according to preliminary surveys: Ndhlovu and Mduluza, unpublished results). Kaswa primary school was the centre of the surrounding area in which the children and adults had a low *S.haematobium* infection prevalence and intensity; while Valhalla primary school and the surrounding area had the residents with high *S.haematobium* infection prevalence and intensity.

The children attending each school and the adults living in the surrounding catchment areas of each school were selected. The criteria used for selection of the cohort were provision of a full set of three urine and faecal samples collected over three consecutive days, a blood sample and no record of treatment received within the previous two years. The individuals who were diagnosed as having active malaria were excluded from the cellular studies. Throughout this study, comparisons were made between groups of individuals who were infected or uninfected, taking into account PBMC cytokine levels and proliferation and the variables age, follow-up time point, sex and area.

A total of 205 individuals were selected for detailed study of their cellular immune response and parasitology in relation to schistosomiasis before treatment and 12 months post treatment with the antihelminthic drug, praziquantel. A separate group of 40 school children (aged 7 to 16 years), were examined for parasitological infection and cellular

immune responses at pre-treatment, 12 and 18 months post treatment. The cellular immunity studies of this group included detailed determination of their cytokine profiles measured at each follow-up time.

2.2.3 PARASITOLOGY

Urine samples for parasitological examination for *S.haematobium* infection were collected from all the individuals on three consecutive days and were processed on the day of collection. Samples were collected between 10.00 hours and 14.00 hours to coincide with peak *S.haematobium* egg excretion in urine (Warren, 1978). Urine samples were examined for urinary schistosomiasis by filtration of 10 ml samples after thorough mixing (Mott *et al.*, 1982) using 13 mm polyamide Nytrel™ filters and 5-10% Lugol's iodine stain, to help visualise the eggs. The filters were examined for eggs of *S.haematobium*, and counts were expressed as eggs per 10 ml of urine (ep10ml). Intensity of infection was classified as negative-no eggs detected; very light-<10 ep10ml; light-10 to <50 ep10ml; or heavy-50 or more ep10ml.

Stool samples were examined by the Kato-Katz technique (Katz *et al.*, 1972; as modified by Peters *et al.*, 1980), although the prevalence of *S.mansoni* is known to be very low as earlier described by Taylor and Makura (1985) and according to preliminary surveys. Stool samples were collected and processed to exclude from the study those with *S.mansoni* infection.

2.2.4 COLLECTION OF VENOUS BLOOD

About 20 mls of blood sample were collected from the cubital vein of each child by venepuncture using 21G cannulae into sterile syringes pre-loaded with 5-10 U/ml heparin for later separation and collection of peripheral blood mononuclear cells (PBMC). After blood collection the samples were transported in a cooler box and were processed at the laboratory within two hours of collection.

2.2.5 TREATMENT

All children were treated with praziquantel, given as a single dose of 40 mg/kg body weight, at the end of the first (pre-treatment) examination. All children were finally retreated at the end of the 2 year follow-up. The study design was approved by the Medical Research Council of Zimbabwe and the Zimbabwe Ministry of Health ethical committee.

2.2.6 PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) SEPARATION

Blood was layered gently into sterile LeucoSep tubes, prespun with Lymphoprep™ (Nycomed) and centrifuged at 450xg for 20 min. The method for cell separation employed density gradient centrifugation of anti-coagulated blood on a dense solution of lymphoprep (Nycomed), using a modified method from Boyum (1968); Hunt (1978) and Madsen *et al.*, (1980). The white buffy layer containing the PBMC at the plasma/lymphoprep interface was pipetted into another sterile tube. The remaining lymphoprep with the red cells and granulocytes was discarded. Aspirated PBMC were resuspended in complete media and washed by centrifugation at 350xg for 15 min. The cells were washed again in complete medium and collected by pelleting at 300xg for 10

min. The supernatant was discarded and the cells resuspended into 3 ml of complete media. The resuspended cells were aliquoted and distributed equally into three 1.8 ml sterile Nunc tubes, mixed with 1 ml freezing medium and immediately transferred for freezing by gradient freezing in liquid nitrogen following a given standard curve as described by Hviid *et al.*, (1989).

2.2.7 PBMC PROLIFERATION ASSAYS USING DYE REDUCTION METHODS.

The cryopreserved PBMC were rapidly thawed at 37^{0}C and transferred asceptically into 10 ml RPMI 1640 without serum. The cells were washed at 300xg for 10 minutes to remove the DMSO, resuspended in 1 ml complete medium (RPMI 1640 and 10% AB serum). Cell viability and concentration were determined by counting a portion of the resuspended cells in 0.2% (w/v) trypan blue. A final viable cell concentration of approximately 1 x 10^6 cells/ml was cultured in 100 µl volumes in duplicate or triplicate in 96 well flat bottomed culture plate. The cells from each individual had, as a negative control, unstimulated cells, and as positive control, cells stimulated with 10 µg/ml PHA (Wyler and Oppenheim, 1974), the *S.haematobium* antigens (SEA - soluble egg antigen, SWAP - soluble worm antigen preparation and CAP - soluble cercarial antigen preparation) were used to stimulate the cells at 10 µg/ml. This concentration of antigen/mitogen was determined on the basis of preliminary experimentation as being the optimal level of antigen/mitogen for stimulation of PBMC from *S.haematobium*-infected individuals (data not shown). The plates were incubated in a 5% CO_2 humidified incubator at 37°C for 6 days. On day 6 the cell proliferation index was measured using Non-Radioactive Cell Proliferation Assay (CellTiter 96™ Promega) according to the

manufacturers protocol. After 4 hours incubation with the dye solution, the solubiliser was added to stop any further utilisation of the dye by the cells and the plate reincubated for an hour to allow the formazan formed during the cell proliferation and utilisation of the dye to solubilise. The contents of each well were thoroughly mixed using a multichannel pipette and then absorbance values were determined using an ELISA plate reader at 570 nm, with a reference wavelength at 630 nm using the BIOLINX programme for data handling. For each individual proliferation assay, the results were accepted when the negative control gave a value of 50% or less than the positive control, with a standard deviation of less than 0.02 between the well readings of each duplicate specimen.

Cell proliferation indices (stimulation index) were each expressed as:

$$\text{SI of mean o.d.} = \frac{\text{Mean counts from stimulated cells}}{\text{Mean counts from control (unstimulated) cells.}}$$

The response to each stimulant was classified as low response-when stimulation index (SI) was less than 2 and moderate or high response when SI was ≥2.

2.2.8 PBMC PROLIFERATION ASSAYS USING TRITIATED THYMIDINE.

The cryopreserved PBMC were rapidly thawed at 37°C and transferred asceptically into 10 ml RPMI 1640 without serum, as described in the previous section. The cells were washed by centrifugation at 300xg for 10 minutes to remove the DMSO and resuspended in 1 ml complete medium containing 10% AB serum and 50 μg/ml gentamycin. Cell viability and concentration was determined by counting 20 μl of the resuspended cells in trypan blue (0.2% w/v). A final viable cell concentration of

approximately 1 x 10^6 cells/ml was cultured in 200 µl volumes in duplicate in 96 well flat bottomed culture plates. The cells from each individual had a negative control (unstimulated cells) and as positive control, cells stimulated with 10 µg/ml PHA. The *S.haematobium* antigens SEA and SWAP were used to study specific stimulation of the cells at 10 µg/ml. The plates were incubated in a 5% CO_2 humidified incubator at 37°C for 6 days. The cells were pulsed with 1.0 µCi tritiated thymidine (methyl ^{3}H-thymidine, Amersham, UK) after 5 days incubation, and further incubated for approximately 18 hours. The cells were then harvested onto filters using a semi-automated cell harvester and the filters rinsed with distilled water to remove unincorporated tritiated thymidine.

The amount of tritiated thymidine incorporation induced by antigen or mitogen was determined with a Liquid Scintillation Analyser (TRICAB™, Model 1600 CA Packard USA). Data were expressed as stimulation index of mean counts per minute (SI) calculated as follows:

$$\text{SI of mean cpm} = \frac{\text{Mean counts from stimulated cells}}{\text{Mean counts from control (unstimulated) cells.}}$$

Response to each stimulant was classified as low response-when SI was less than 2 and moderate or high response when SI was ≥2.

2.2.9 PBMC STIMULATION FOR CYTOKINE PRODUCTION.

The frozen PBMC from both the high and low transmission areas were retrieved from liquid nitrogen and rapidly thawed with constant agitation until only a small ice crystal remained and then transferred to incomplete medium with 50 µg/ml gentamycin. After

washing twice by centrifugation, the cells were resuspended in complete medium containing 10% human AB serum and seeded at 2 x 10^6 cells/ml in 24 well microplates in the presence of 10 μg/ml SWAP , SEA or PHA, and without stimulant as control. After 5 days at 37°C in 5% CO_2 in humidified air, culture contents were collected and centrifuged at 350xg for 10 min. Cell free supernatants were collected under sterile conditions and were stored at -70°C before assaying for cytokines.

2.2.10 CYTOKINE DETECTION BY ELISA.

Cytokine detection of IL-4, IL-5, IL-10 and IFN-γ was performed on the supernatants harvested from the PBMC stimulations after 5 days of incubation. Nunc-immunoplates Maxisorp™ (InterMed) were coated with anti-human monoclonal antibodies (mAb) at 0.5 μg/ml for IFN-γ and at 1 μg/ml for IL-4, IL-5 and IL-10 in carbonate/bi-carbonate buffer, pH 9.6 overnight at 4°C on a shaker. The plates were then washed twice with PBS containing 0.05% Tween 20, pH 7.2 and then blocked with complete RPMI or PBS + 3% BSA + 0.05% Tween 20 for 1 hour at room temperature. The blocking solution was removed by washing once and then the PBMC supernatants were added in duplicate, at these dilution: 1/4 for the control, and 1/5, 1/25 and 1/125 for the samples; and incubated overnight at 4°C on a shaker. Standards were added in duplicate for each concentration within the range 15-2000 pg/ml. After overnight incubation, the plates were washed 3x. A second biotinylated monoclonal antibody at 0.5 μg/ml for IFN-γ, 1 μg/ml for IL-4 and IL-5, and 2 μg/ml for IL-10 was added and the plates were incubated for 4 hours at room temperature on a shaker. After washing 3x, streptavidin-alkaline phosphatase conjugate or an alkaline phosphatase-conjugated mAb was added at 1/1000 for IFN-γ and IL-4, and at 1/2500 for IL-5 and IL-10 and then incubated for 2 hours at

room temperature. Enzymatic activity was revealed by incubating the plates with p-nitrophenyl phosphate for 10-15 minutes. Absorbance was read at 405 nm with 630 nm as reference wavelength on a DIAS Multiscan reader (Dynatech Laboratories). The absorbances were interpolated from a standard curve to obtain cytokine concentrations (pg/ml) taking into account the dilution factor of the culture supernatants. The standard curves were calculated for each plate according to the sigmoid curve equation:

$$Y=D + (A-D)/(1 + (X/C)^{B})$$

Where X is the cytokine concentration and Y is the absorbance at 405 nm

A, B, C and D are constants.

Examples of the standard curves for each cytokine are shown in Figure 2.1.

2.2.11 STATISTICAL ANALYSES

The data sets were mostly not normally distributed, in view of which non parametric tests were applied. Spearman rank correlation was used for the correlations of infection intensity and proliferation and cytokine production. Kruskal-Wallis one-way ANOVA was used to compare the cytokine levels within infection intensity groups and within age groups. The Mann-Whitney (Rank sum) test was used to compare the infected and uninfected categories at each follow-up time point, between the two areas and the response groups. The Rank sum test was also used to compare the infected or uninfected groups at any two of the follow-up time points.

To reduce the risk of type 1 errors arising from making multiple pairwise comparisons, Bonferroni's method was applied which uses the function $p=\alpha/m$, where α is a p(probability) constant from statistical comparison and m is the number of comparisons.

When comparisons were made of levels of proliferation or production of a particular cytokine between infected and uninfected individuals, the revised significance level was; p=0.05/3 = 0.017; because three comparisons (PHA, SWAP and SEA) were made at each time point. When comparisons were made of a particular category of respondent at different time points (e.g. PHA stimulated responses of uninfected individuals at 12 and 18 months post-treatment), the revised significance level was p = 0.05/6 = 0.008.

Each individual immune response was classified as a T helper-like cytokine response according to the ratios of the signature cytokines produced as being of Th0/1 or Th0/2 phenotypes. The cytokines studied were IFN-γ, IL-4, IL-5 and IL-10 and the ratios derived as, IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ. If the ratio obtained, x, satisfied the following expression then the individual cytokine environment response was classified accordingly:

$x \leq 0.1$	**strong Th0/1-like response**
$0.1 < x \leq 1$	**Th0/1-like response**
$1 < x \leq 10$	**Th0/2-like response**
$x > 10$	**strong Th0/2-like response.**

A similar classification has been proposed for classification of human T cell clones during Leishmaniasis and schistosomiasis mansoni (Couissinier-Paris *et al.*, personal communication), although it is used here to classify the general cytokine responses of PBMC.

2.3 RESULTS:

2.3.1 STUDY POPULATION AND PARASITOLOGY OF THE COHORT FOR THE DYE REDUCTION PROLIFERATION ASSAYS.

Two hundred and five participants were recruited from the two areas for detailed study of their PBMC proliferation using the dye reduction method. 95 participants were from the Low Transmission area and 110 from the High Transmission area. Of the participants, 81.5% (167) were school children from both areas and 13.7% (28) were adults recruited from the Low Transmission area while 4.9% (10) were adults recruited from the High Transmission area. The distribution of the participants by age, sex and transmission area is shown in Figure 2.2. At the start of the study, 21.5% (44) and 42.4% (87) were infected with *S.haematobium* from the Low and High Transmission areas, respectively. By 12 months post-treatment only 5.4% (11) and 14.1% (29), were re-infected with *S.haematobium* from the Low and High Transmission areas, respectively (Figure 2.3).

Parasitologically, the populations from the two areas showed a distinct difference in infection intensity, before treatment (LT: n=95, U=2763.5 and HT: n=110, U=7686.5; p=0.0001) and 12 months post-treatment (LT: n=88, U=2765.5 and HT: n=83, U=4538.5; p=0.0062). For the prevalence of infection there was also a significant difference between the two areas at both examinations (before treatment, LT: n=95, U=3512.5 and HT: n=110, U=6937.5; p=0.0001 and after treatment, LT: n=88, U=2791.0 and HT: n=83, U=4513.0; p=0.0114). The distributions of infection prevalence and intensity with age groups are shown in the Figures 2.4 and 2.5.

In the Low Transmission area, the distribution of infection prevalence showed no statistically significant difference between the age groups before treatment using Kruskal-Wallis one way ANOVA. However, at 12 months post-treatment the peak prevalence was observed in the 12-16 age group (Figure 2.4). In the High Transmission area the infection prevalence showed no statistically significant differences between the age groups (Figure 2.4 at both pre- and post-treatment examinations. However, the peak prevalence of infection after treatment was reached in the 9-11 age group. The prevalence of infection remained high in the 12-16 year olds while those 17 and above years old were not reinfected (Figure 2.4).

The infection intensity in both areas showed interesting features. In the Low Transmission area before treatment, the age groups below 17 years old showed no difference from each other, but they were different from adults 17 and over years old who had low infection levels. At 12 months post treatment, peak infection intensity was found in the 12-16 age group while those 17 and over years old were only lightly re-infected (Figure 2.5). In the High Transmission area before treatment, the 8 years old and below age group had lower infection intensity compared to those over eight years old (Figure 2.5), and there was no difference between the other three age groups. By 12 months post-treatment, the 8 years old and below age group had high re-infection intensity of 20 ep10ml, which was different from the other age groups. There was no detectable reinfection taking place in those above 17 years of age (Figure 2.5).

2.3.2 PBMC PROLIFERATION RESULTS USING THE DYE REDUCTION ASSAYS

Detection of proliferation after antigenic and mitogenic stimulation of PBMC over six days using the chromogenic dye-based MTT Promega™ kit, gave no statistical differences between infected and uninfected individuals from the Low Transmission area before treatment (Figure 2.6). Before treatment PBMC from uninfected 5-8 years old age group showed high levels of proliferation to both egg and cercarial antigen stimulation as did the PBMC from infected 9-11 and 17+ years old age groups. At 12 months post-treatment PBMC from uninfected 9-11 years old age group showed high levels of proliferation to both worm and egg antigens stimulation as did PBMC from individuals uninfected aged 17 and above (Figure 2.7), while the PBMC from the uninfected 5-8 age group children gave high levels of proliferation to cercarial antigen. However, the PBMC from 9-16 years old infected children post treatment, gave higher levels of proliferation to CAP than the PBMC from younger children, 5-8 and adults, 17+ (Figure 2.7b). None of the above trends approached statistical significance.

When comparing the overall pattern of responses in the Low Transmission area before and after treatment there was no statistical difference within each age group for all stimulants. However, when looking at the trends in responses, the PBMC from the 9-11 age group gave raised levels of proliferation with worm, egg and cercarial antigens after treatment compared with the other age groups where proliferation indices were usually lower after treatment and this could have some biological importance (Figure 2.8).

Before treatment, there was no statistically significant difference in infection intensity between low and moderate/high responder categories, where responders are defined according to their proliferative stimulation index when PBMC samples were stimulated with antigen or mitogen. However, on mitogenic stimulation low responders tended to have higher infection intensities than moderate/high responders (Figure 2.9a). After treatment, there was no statistically significant difference between the egg output of the two response categories for the mitogen. However, PBMC from those excreting eggs gave low responses to schistosome antigen whereas the PBMC from those not excreting eggs had moderate or high responses (Figure 2.9b).

The PBMC from individuals living in the High Transmission area gave no statistical differences in responses to the stimulating agents when comparing infected and uninfected individuals within each age group (Figure 2.10). However, some biological differences were observed between age groups. Infected 9-11 year olds gave a higher response to worm antigen stimulation relative to those of other age groups (Figure 2.10a). Responses to the egg antigen were high in the PBMC from infected individuals in the 9-11 and 17+ age groups. The PBMC from uninfected children below 8 years old gave a higher response to cercarial antigen than those from the other age groups (Figure 2.10b). At 12 months post-treatment the PBMC from the 9-11 age groups provided some differences between those from infected and uninfected for the egg and cercarial antigens (Figure 2.11b). Interestingly, most of the individuals in the 17 and above age group were not re-infected.

A comparison was made of proliferative response categories of individuals from the High Transmission area before and after treatment (Figure 2.12). The PBMC from the individuals who were in the 9-11 and 17+ age groups gave a higher response to the egg and cercarial antigen stimulation than the other age groups before and after treatment (Figure 2.12).

Before treatment, there was no statistically significant difference in infection intensity between low and moderate/high responders categories. However, the trends on cercarial antigen stimulation showed that low responders had higher infection intensities than moderate/high responders (Figure 2.13a). In contrast, at 12 months post-treatment the individuals with moderate and heavy infection gave high levels of proliferation to all the stimulants except cercarial antigen (Figure 2.13b).

In summary, the data from the dye reduction assays were difficult to interpret due to the lack of sensitivity of the method. All the stimulants showed similar effects on the PBMC from infected and uninfected individuals before and after treatment within age groups and in both areas. Isolated raised levels of proliferation were observed on stimulation of PBMC with egg and cercarial antigens from uninfected individuals in the 9-11 age group in the High Transmission area before treatment, but none of these was statistically significantly different from their infected counterparts.

Despite the lack of sensitivity of the method, an interesting comparison was apparent between the infection intensities of infected individuals who were low or moderate/high responders. The changes in these patterns resulting from chemotherapy were very

different in the two areas, when using both worm and egg antigens to generate proliferative responses (Figure 2.9 and 2.13). In both areas before treatment, the worm burden (as detected by egg output) was fairly evenly distributed between individuals with PBMC of different proliferative abilities. After treatment however, most infections were in Low responders in the Low Transmission area but in the moderate/high responders in the High Transmission area.

2.3.3 STUDY POPULATION AND PARASITOLOGY OF THE COHORT FOR PBMC CYTOKINE PRODUCTION.

Forty children were selected for the detailed study of proliferation and cytokine production of PBMC from the two transmission areas. 21 were from the High Transmission area and 19 were from the Low Transmission area. Prior to treatment, 66.7% (14) children from the High Transmission area were infected with *S.haematobium*, with a mean egg count of 85 eggs per 10 ml of urine. 47.4% (9) from the Low Transmission area cohort were infected with mean infection intensity of 36 eggs per 10 ml of urine (Figure 2.14). Drug treatment markedly reduced prevalence and intensity of infection at 12 months, with a partial recovery to pre-treatment levels at 18 months, in both areas. In the High Transmission area the prevalence of infection increased to pre-treatment levels by the 18 months post-treatment, while infection intensity increased to about a quarter of the pre-treatment level by the 18 months post treatment (18 ep10ml of urine). For those from the Low Transmission area, the prevalence and intensity of infection both increased slightly between 12 and 18 months post treatment examination (Figure 2.14). The prevalence and intensity of infection in the children from the High Transmission area was significantly higher than in those from the

Low Transmission area before treatment (LT: n=19, U=1134.0, HT: n=21, U=2106.0; p=0.0059 and LT: n=19, U=1104.0, HT: n=21, U=2136; p=0.0034, respectively) and at 18 months post-treatment (LT: n=18, U=94.0, HT: n=20, U=266.0; p=0.0124 and p=0.0124, respectively).

2.3.4 PBMC PROLIFERATION USING TRITIATED THYMIDINE

The PBMC from infected children from the Low Transmission area gave higher levels of response than those from uninfected children to both the schistosome antigens at all three times examined (Figure 2.15). However, response to the mitogen showed no differences between the infected and uninfected children at any time point (Table 2.1). Comparing infected or uninfected children at different time points (e.g. PHA stimulated PBMC from infected children), no significant differences were observed.

In the High Transmission area, proliferative response patterns were very different to those observed in the Low Transmission area. The PBMC from uninfected children had highly significantly increased levels of proliferation when compared to those from children who were infected before treatment, for both worm and egg antigens (Figure 2.16 and Table 2.2). There were no differences between the responses of PBMC from uninfected and infected children at both the post-treatment stimulations. There was an overall increase in cellular response to all the stimulating agents amongst infected children after treatment compared with those for the equivalent children before treatment (Figure 2.16), although these increases were not statistically significant, except response to egg stimulation (Table 2.2).

The differences in proliferative responses of children living in the two areas were reinforced by egg intensity in low versus moderate/high proliferative responders in the Low Transmission (Figure 2.17) and High Transmission (Figure 2.18) areas. In the Low Transmission area, the biological trends showed that the groups giving moderate/high responses to the stimulants were those excreting eggs at both pre- and post-treatment examination, while the group with low responses were not infected (Figure 2.17). This result was observed with all three stimulants. In contrast, in the High Transmission area before treatment, some biological differences were observed where the PBMC which gave moderate/high responses to the worm and egg stimulants were from children with very light intensities of infection. After treatment this pattern was dramatically reversed with PBMC from more heavily infected children giving moderate/high responses to all the stimulants (Figure 2.18). It is noteworthy that the PBMC from infected children living in the Low Transmission area gave considerably higher levels of proliferation on stimulation with both schistosome worm and egg antigens than the PBMC from infected children living in the High Transmission area, compare Figure 2.15 and 2.16.

In summary, the cohort comprised of 40 school children aged 7-16 years from both Low and High Transmission areas. The patterns of PBMC responses as determined by incorporation of tritiated thymidine were very different in the two areas. In the Low Transmission area, the PBMC from infected children gave higher levels of proliferation than the PBMC from uninfected children on stimulation with both schistosome antigens, this pattern was not changed following chemotherapy and most eggs were produced by children with moderate/high proliferative responses. In the High Transmission area, before treatment, PBMC from uninfected children had higher levels of proliferation to all

stimulants than those of infected children and most eggs were produced by children with low proliferative responses. These proliferative responses were reversed after treatment, higher levels of proliferation were observed in the infected than in the uninfected children and most eggs were produced by children with higher proliferative responses.

2.3.5 PBMC CYTOKINE PRODUCTION PROFILES.

PBMC samples were stimulated in *ex vivo* cultures using the schistosome egg and worm antigens and the mitogen, PHA, and production of four cytokines was measured in the culture supernatants: IFN-γ, IL-4, IL-5 and IL-10.

The PBMC from infected children in the Low Transmission area showed trends of producing higher levels of IFN-γ than uninfected children to all stimulants (Figure 2.19) but, after application of a Bonferonni correction ($p<0.017$), none of these differences were statistically significant except for the comparison at 18 months when PBMC were stimulated with PHA (Table 2.3).

The PBMC from the infected and the uninfected children in the Low Transmission area produced similar levels of IL-4 and IL-5 at all time points for all the stimulants (Figures 2.20 and 2.21; Tables 2.4 and 2.5), but there was a non statistically significant trend towards higher levels of IL-4 production in PBMC from uninfected children. The levels of production of IL-10 were higher from the PBMC of uninfected children than those of infected children, using all three stimulants before treatment and 12 months after treatment (Figure 2.22). These differences approached statistical significance when PBMC were stimulated with PHA and egg antigen before treatment and for the worm

and egg antigens at 12 months post treatment (Table 2.6). A comparison was made of the levels of IL-10 produced by PBMC from either infected or uninfected children between any two of the time points. Only one difference, PHA stimulation of PBMC from infected children at pre- and 18 months post-treatment, approached statistical significance, with the PBMC from the re-infected children after treatment producing higher levels of IL-10 (Table 2.6).

The PBMC from the infected children in the High Transmission area produced higher levels of IFN-γ compared with those from uninfected children at all three time points examined (Figure 2.23). The PHA stimulations showed significant differences at 12 months post treatment (Table 2.7), but there was no statistically significant difference before treatment and at 18 months post-treatment (Table 2.7). The schistosome antigen-specific stimulation of the PBMC from infected children resulted in high production of IFN-γ (Figure 2.23) for both antigens before treatment and 12 months after treatment. The comparison between infected or uninfected groups at any two of the time points showed some differences which were not statistically significant, in which higher levels of IFN-γ were produced by the PBMC obtained before treatment, and at 12 months post-treatment, when compared with 18 months post-treatment (Table 2.7).

Higher levels of IL-4 were produced by the PBMC from infected children compared to the uninfected children before treatment but this pattern was reversed at 12 months and 18 months post-treatment with the uninfected children producing higher levels of IL-4 than the re-infected children (Figure 2.24). The differences between levels of IL-4 production in infected and uninfected children approached statistically significance (Table

2.8). In terms of IL-4 production the PBMC from uninfected children when compared between time points showed differences between pre-treatment and post-treatment, with the post treatment groups producing higher IL-4 levels at both 12 and 18 months (Figure 2.24 and Table 2.8). No difference in IL-4 levels could be observed when PBMC obtained from the uninfected children at 12 months post-treatment were compared with those from the equivalent children 18 months post-treatment (Figure 2.24 and Table 2.8).

The pattern of results for IL-5 and IL-10 were, in general terms, similar to those observed for IL-4. The PBMC from infected children obtained before treatment produced higher levels of IL-5 compared with the uninfected children, statistically significant for schistosome antigens but not PHA (Figure 2.25 and Table 2.9). A comparison of IL-5 production by PBMC between infected and uninfected children with PBMC obtained at other time points indicated no statistically significant differences in responses to any stimulant at other time point, but the trends showed that the PBMC from uninfected children produced higher levels of IL-5 cytokine. The PBMC from the uninfected children before treatment produced lower levels of IL-5 when compared with those from uninfected children at both 12 and 18 months post-treatment (Figure 2.25) but these differences were not statistically significant (Table 2.9).

The IL-10 levels produced by PBMC from infected children were higher than those produced by non-infected children before treatment but not at either 12 or 18 months post-treatment, but there was a trend towards lower production by the PBMC from infected children after treatment, such that there was little difference in levels between

infected and uninfected children after treatment (Figure 2.26). The only statistically significant differences in this data were comparisons of IL-10 levels produced by the PBMC from the infected children before treatment and 18 months post-treatment which showed higher levels were produced before treatment on stimulation with both PHA and egg antigen (Table 2.10).

These cytokine data can be summarised as follows: Cytokine levels produced by PBMC from children in High Transmission area were several fold higher than the levels produced by PBMC from children in Low Transmission area at all time points. The PBMC from infected children from both areas produced higher levels of IFN-γ than uninfected children to all stimulants at all time points. The trends of IL-4 and IL-5 production by PBMC from children in Low Transmission area showed similar levels between infected and uninfected children before treatment, but higher levels of IL-4 were produced by PBMC from uninfected than infected children after treatment. The trends of IL-10 production showed uninfected children in Low Transmission area producing higher levels than infected children at all time points. In contrast, the PBMC from infected children in High Transmission area produced higher levels of IL-4, IL-5 and IL-10 than uninfected children to all stimulants before treatment. 12 and 18 months after treatment, these patterns were reversed and the cytokine levels were higher from uninfected children than the infected children to all stimulants.

2.3.6 CYTOKINE RATIOS

In addition to investigating the changes in absolute levels of cytokines, described above, the changes in ratios of 'signature' cytokines were also analysed. This analysis was based on the premise that Th1 and Th2 cytokines cross-regulate the other T cell subset and changes in the relative abundance of Th1 and Th2 cytokines may be more sensitive indices of the *in vivo* T cell environment and how it is changed in response to infection, treatment and re-infection.

In both areas, the PBMC obtained from infected children before treatment produced significantly higher levels of IFN-γ, IL-4 and IL-5 than PBMC from uninfected counterparts. The mean ratios of the cytokines produced by the PBMC from the Low Transmission area, however, gave no statistical difference between infected and uninfected groups for IL-4:IFN-γ and IL-5:IFN-γ (Tables 2.11 and 2.12). However, there was a clear trend toward a higher ratio among uninfected than infected children 12 months post-treatment (Figure 2.27 and 2.28). The trends in the IL-10:IFN-γ ratio data indicated higher ratios among uninfected than infected children before treatment and 12 months post-treatment for all the stimulants but these also were not statistically significant (Figure 2.29 and Table 2.13).

In the High Transmission area the ratio of IL-4:IFN-γ showed no difference between infected and uninfected children for all the stimulants before treatment (Figure 2.30). 12 months post-treatment there was higher ratio of IL-4:IFN-γ in uninfected than infected children and on stimulation with egg antigen these differences approached statistical significance (Table 2.14). The IL-5:IFN-γ and IL-10:IFN-γ ratios showed no statistically

significant differences between infected and uninfected children before treatment and in addition the IL-5:IFN-γ ratio showed no statistically significant differences after treatment (Figures 2.31 and 2.32). There were however, some marked differences for IL-10:IFN-γ between the groups at 12 and 18 months post treatment for all the stimulants; uninfected children produced statistically significantly higher ratios of IL-10:IFN-γ. The trends of the IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ ratios all indicated that the PBMC from the uninfected children produced high ratios of IL-4, IL-5 and IL-10 to IFN-γ for all the stimulants (Figures 2.30, 2.31, 2.32 and Tables 2.14, 2.15, 2.16).

In summary, the mean ratios of the cytokines IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ produced by PBMC from children in the Low Transmission area gave no significant differences between infected and uninfected groups. But the trends showed higher ratios in uninfected than infected children, particularly 12 months after treatment. The mean ratios of IL-4:IFN-γ and IL-5:IFN-γ between infected and uninfected children in the High Transmission area showed similar patterns. Before treatment, there were no differences but 12 and 18 months after treatment the ratios were higher in uninfected than infected children to all stimulants. IL-10:IFN-γ ratios were higher in uninfected than infected children at all time points.

2.3.7 PROLIFERATION AND CYTOKINE LEVELS

Children from the Low Transmission area were grouped according to their proliferative responses and their cytokine levels compared. No differences between low and moderate/high proliferative responders were observed in their cytokine levels on PHA stimulation both before and after treatment (Figure 2.33). Equivalent analyses comparing

proliferative responses and cytokine production in response to worm and egg antigens stimulation produced a slightly different pattern (Figures 2.34 and 2.35). Before treatment, the PBMC giving moderate/high proliferative responses to both worm and egg antigens produced higher levels of IFN-γ and IL-5, whilst the PBMC giving low proliferative responses produced higher levels of IL-10 (Figure 2.34a and 2.35a). After treatment, the PBMC giving low proliferative responses to worm, but not egg antigen produced higher levels of IL-5 than the PBMC giving moderate/high responses (Figure 2.34b). The levels of IL-10 were higher in the PBMC that had low responses to the egg antigen stimulation after treatment (Figure 2.35b), whilst the levels of IFN-γ, IL-4 and IL-5 were similar for the response groups.

A comparison of proliferative responses and cytokine production in the High Transmission area was also conducted. PBMC samples giving low proliferative responses on stimulation with PHA produced higher levels of IFN-γ and IL-5 than those giving moderate/high responses before treatment (Figure 2.36a). After treatment, the levels of IL-4 were higher among the PBMC giving low proliferative responses. The response to the worm antigen stimulation gave high production of IFN-γ, IL-5 and IL-10 in the PBMC giving low proliferative responses before treatment, but after treatment no differences could be found between the two response groups for each cytokine (Figure 2.37). The PBMC which had low proliferative responses to egg antigen stimulation produced higher levels of all four cytokines when compared to the PBMC which had moderate/high responses, before treatment (Figure 2.38a). After treatment, high levels of IFN-γ were produced by the PBMC giving moderate/high proliferative response to egg

antigen, whilst no differences could be found between the two groups of responders for the other three cytokines (Figure 2.38b).

In summary, proliferation and cytokine levels of PBMC from children in Low Transmission area showed that similar levels of cytokines were produced from PBMC showing low and moderate/high proliferative responses to PHA stimulation, before and after treatment. The responses to both schistosome antigens resulted in similar patterns of cytokine levels at both time points with the moderate/high responders producing higher levels of IL-5 and IFN-γ than low responders before treatment and lower levels of IL-10 both before and after treatment. Low responding PBMC from children in High Transmission area gave higher levels of IFN-γ, IL-5 and IL-10 to all stimulants before treatment, while only the egg antigen stimulation of low responding PBMC gave high IL-4 levels. After treatment, the trend was for higher levels of IL-4 from low responders to PHA stimulation and higher levels of IFN-γ produced by moderate /high responding PBMC to egg antigen.

2.3.8 T HELPER PHENOTYPE RESPONSES

An attempt was made to classify the T helper response phenotypes of individuals in this study according to the ratios of IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ in response to stimulation with mitogen, egg or worm antigens. The data for children from the Low Transmission area are given in Table 2.17. These data suggest that uninfected children show a greater tendency to a Th0/2-like response than infected children with this difference most clearly seen in the IL-5:IFN-γ and IL-10:IFN-γ ratio in response to antigens. A similar pattern was seen in the equivalent data for children from the High

Transmission area (Table 2.18). Uninfected children showed a greater tendency to a Th0/2-like responses than infected children with the difference most clearly seen in IL-4:IFN-γ and IL-5:IFN-γ ratio in response to schistosome antigen stimulation.

2.4 DISCUSSION

The Low Transmission area was characterised by a low prevalence and intensity of infection in the resident population. There is only restricted access to the perennial river and the dam and as a result only seasonal water contact is likely. Most of those infected were the young and the adult males who access the restricted water points in the perennial river and the dam. As might be expected for a low transmission area, there was little difference between the age groups in levels of infection before giving treatment. After treatment, however, the pattern of infection was observed to reach the peak prevalence and intensity of infection in the 12-16 years old age group, and some adults 17 and above years old were reinfected, perhaps indicating an absence of protective immune responses in those individuals. These data suggest a possible slow development of immunity in areas where transmission and exposure is low.

The High Transmission area, where uncontrolled water contact took place and several open water bodies were widely available for domestic and recreational use was characterised by very high infection intensity and prevalence. However, at 12 months post treatment the peak of infection prevalence occurred in the 9-11 years old age group which is earlier than observed in the Low Transmission area. The lower age group, 8 years old and below, had the highest infection intensity at the examination after

treatment. The adults, 17 years old and above who had been infected prior to treatment, generally remained uninfected post-treatment, suggesting that intense transmission might provide a greater level of protective immunity.

A comparison of the reinfection data for the two areas is consistent with an interpretation that immunity plays a role in preventing the acquisition of infection, which has been reported by others to be age-related (Khalife *et al.*, 1986; Wilkins *et al.*, 1987; Butterworth *et al.*, 1987, 1992; Haggi *et al.*, 1990; Hagan *et al.*, 1987, 1991; Rihet *et al.*, 1991; Woolhouse *et al.*, 1991; Dunne *et al.*, 1992; Ndhlovu *et al.*, 1996). The parasitology data from the two cohorts, one for which proliferation was determined by the dye reduction method and the other for which proliferation and cytokine production was determined, showed similar patterns of infection prevalence and intensity. Infection prevalences and intensities were very low after treatment in both areas for both cohorts. However, the individuals from the two different areas showed different infection prevalences and intensities even when re-infection was low at 12 months post-treatment.

The data on proliferative responses assessed using tritiated Thymidine uptake and on cytokine production are limited by the small size of the cohort studied. It is encouraging, therefore, that the comparison of parasitological data between the smaller and larger cohorts suggests that the smaller cohort which was subjected to detailed analysis was likely to have been representative of the larger group. There were several instances where analysis of the data for the smaller cohort indicated clear biological trends (for instance in the similarity of patterns for Th2 'signature' cytokines) but these differences were not statistically significant, especially after necessary application of a Bonferonni

correction. It seems reasonable to assume that the main source of these statistical limitations is the small n values. While remaining aware of this important statistical limitation the data were generally interpreted emphasising the biological trends observed, rather than the statistical significances and the limitations this imposed.

The results from the two methods used in determining the proliferative responses of PBMC from children in Low and High Transmission areas showed the sensitivity of the two methods to be markedly different with the tritiated thymidine being the most appropriate, reliable and sensitive assay. This difference may be due to the fact that one assay measures the amount of tritiated thymidine that is incorporated into the DNA during blastogenesis and proliferation, whilst the other dye reduction assay measures only viable cells and these may not be proliferating. Therefore, the results discussed here are restricted to the tritiated thymidine incorporation assays.

Proliferative responses to the specific schistosome antigens of PBMC samples from the Low Transmission area before treatment, showed that only those from infected individuals had a marked response to stimulation. Those from uninfected individuals did not react to the stimulus, perhaps indicating that they had not previously encountered schistosome infection. After treatment, the proliferative responses in uninfected individuals remained lower, perhaps indicating a lack of exposure to schistosome infection in uninfected individuals, than those in reinfected children. Nevertheless, after treatment uninfected individuals showed several fold increases in proliferative responses to stimulation when compared with pre-treatment proliferative responses for the uninfected individuals. At 18 months after treatment, some infected individuals

responded giving low proliferative responses to stimulation with worm antigen even though they were infected, and this may indicate that immuno-modulation had taken place, as reported in other studies (Domingo and Warren, 1968; Barsoum *et al.*, 1982; Hofstetter *et al.*, 1983; Feldmeier, Gastl and Poggensee, 1985a,b; Gazzinelli *et al.*, 1985, Bahia-Oliveira *et al.*, 1992; Auriault *et al.*, 1996).

The individuals in the High Transmission area had high infection intensities, including those re-infected after treatment. On testing PBMC samples for proliferative responses to schistosome-specific stimulation, uninfected individuals before treatment showed higher responses than infected individuals, suggesting the possibility of an acquired protective immunity. The infected children revealed a frequently reported immuno-modulation of their responses resulting in cellular unresponsiveness. This seems to occur when infections are intense and as the parasite and the host defences establish an apparent equilibrium enabling continued coexistence. Similar results were reported in *S.mansoni*-infected patients by Colley and colleagues (1977a, 1986), Ottesen and colleagues, (1978), Ellner and colleagues (1985), Gazzinelli and colleagues (1985), Roberts and colleagues (1993) and Ribeiro de Jesus and colleagues (1993). Grogan and colleagues (1996) also reported similar findings to this study of low proliferative responses to stimulation with schistosome antigens before treatment in *S.haematobium*-infected patients.

Infected children from the High Transmission area (in the age range 7 - 16 years old), often reported showing highest infection intensity and prevalence, were found to have lower proliferative responses to worm and egg antigen stimulation before treatment.

After treatment, the levels of proliferative responses were restored, as previously observed by Grogan and colleagues (1996). A possible interpretation of these data is that antigens became available for immune recognition after treatment, and thus treatment may have promoted the development of a protective immune response. 12 and 18 months after treatment, there was a general increase in proliferative responses in both infected and uninfected groups of individuals.

Cytokine production in response to the stimulating antigens and mitogen showed contrasting features between the two sites. The PBMC from infected children in the Low Transmission area, showed raised levels of proliferation but produced only small quantities of cytokines when compared with the infected children in the High Transmission area who, in turn had very low levels of proliferation but gave high levels of cytokine production. These data may indicate that infected children in the two areas are at different stages of an immune response in which there is a marked proliferative responses during low infections, whilst during heavy infections the deleterious effect of cellular proliferation is modulated (switched-off) early but maintains a sufficient cytokine flow in the environment. Such an interpretation is consistent with the age/prevalence and age/intensity data for the two areas.

The involvement of raised IFN-γ levels in driving the cellular immune response was demonstrated in individuals who were infected. The same individuals showed raised proliferative responses and taken together this may be an indication of a newly initiated (naive) immune response to the infection which is less prevalent in this Low Transmission area. Such patterns of response may mark the existence of acute infection

in individuals living in the Low Transmission area. Confirmation of the low infection intensities and prevalences of infection was observed by the cytokines that drive the humoral responses, IL-4 and IL-5 levels which were similar between infection groups, although IL-10 was raised in the uninfected children before treatment and 12 months after treatment.

In the High Transmission area, a similar cytokine pattern to that shown in the Low Transmission area was shown by the raised IFN-γ in those who were infected, during all the examination times. In the same individuals, raised levels of IL-4 and IL-5 were also observed, which is consistent with the dominance of IL-4, IL-5 and IL-10 later in chronic infections. The data is indicative of the counter-balancing of IL-4, IL-10 and IFN-γ, opposing or downregulating each other's effects, especially before treatment was administered. IL-4 and IL-5 were markedly raised in uninfected children after treatment.

Similar results were reported by Grogan *et al.*, (1996) of restored and increased IL-4 levels post treatment from *S.haematobium* patients, also from the studies by Roberts *et al.*, (1993) on *S.mansoni* patients who had elevated levels of IL-5 in those who were said to be resistant to infection. The same effect was shown for IL-10 after treatment. It is reasonable to suggest therefore that these raised levels of IL-4, IL-5 and IL-10 indicate that drug treatment has had an immunising effect.

The patterns of the production of the cytokines IFN-γ, IL-4 and IL-5 in individuals from the two different areas in relation to infection intensity and prevalence are likely to be indicative of the way the immune response is evoked and matures in different transmission situations. In the High Transmission area the children were heavily infected

and possibly had cellular responses that matured earlier and are being driven by IFN-γ. At the same time, the cytokines IL-4 and IL-5 were raised to drive the humoral immunity which may potentially be involved in the attrition of the parasite through ADCC mechanisms. In addition IL-4 and IL-10 could serve to downregulate the harmful effects of any otherwise uncontrolled and rapid cellular mobilisation which is being driven by the higher levels of IFN-γ (Del Prete *et al.*, 1988, 1993; Fiorentino *et al.*, 1991; Kamogawa *et al.*, 1993; Del Prete, Maggi and Romagnani, 1994).

Before treatment with praziquantel, most of the ratios of IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ were lower than 2.5 for most groups of children in both areas and irrespective of stimulant. This may indicate the involvement of all the cytokines in trying to limit infection and indicate their co-operative role which functions in part through cross-regulation. However, an exception to this finding is IL-5. Higher IL-5:IFN-γ ratios were common, perhaps reflecting the role IL-5 may play in populations living in areas where they are exposed to a variety of other helminths (Lopez *et al.*, 1988; Mahanty et al., 1997), in this study area exposure was to *S.haematobium.*

This observation supports the reports of eosinophilia as one of the hallmarks of schistosome infections (Butterworth *et al.*, 1982; Hagan *et al.*, 1985a,b). The individuals in the current study showed higher ratios of IL-10:IFN-γ, perhaps indicating the involvement of this cytokine in modulating the effects of IFN-γ, as suggested in the context of parasite-specific anergy in human urinary schistosomiasis by King and colleagues, (1996). At 12 and 18 months post-treatment, the effects of the mechanisms that drive humoral responses could be dominating, which is a possible interpretation of

the high ratios of IL-4:IFN-γ and IL-5:IFN-γ which were above 2.5 for the specific schistosome antigens stimulation in uninfected children living in the High Transmission area.

The cytokine phenotypes, as determined using the ratios of the 'signature' cytokines known to mark each T helper subset, showed that individuals living in different areas of transmission in an endemic region, have similar cytokine profiles marking their immune responses. The PBMC produced a mixture of cytokines suggesting both Th1 and Th2 cell phenotypes were involved during infection. All the uninfected and non re-infected children produced a pattern of Th0/2-like or a strong Th0/2-like cytokine responses. The infected children had mixed classes of cytokines in their peripheral blood environment, showing some individuals with a Th0/1-like and a few with Th0/2-like responses. The infected children showed the balance tipping towards the Th0/1-like response which could have contributed to the individuals with the Th0/1-like cytokine phenotype being susceptible to infection.

The cytokine balances determined from PBMC stimulation with the schistosome antigens and phytohaematoglutinin, can help in determining the parameters that control humoral and cellular immune responses during *S.haematobium* infection. The PBMC stimulation by schistosome -specific antigen can produce cytokines whose quantitative levels may show the dominating T helper type of response. Human T helper subsets being classified as Th0/1 and Th0/2, the balance and imbalance points to either susceptibility or protection, with a Th0/2-like cytokine response dominating in uninfected individuals. However, any small quantitative change in a particular parameter of the cytokine profiles

of the immune response can switch the host from susceptibility to protection, or vice versa, depending on the balances of the contrasting 'signature' cytokine profiles in the environment which are believed to be cross-inhibitory to each other. The Th0/2-like subset cytokines are believed to enhance secretion of antibodies either by causing selective proliferation or maturation of B cells already committed to the isotype production or increasing the frequency with which B cells switch to the expression of those isotypes that maybe involved in ADCC, the cytokine phenotype thus denoting a protecting immune status (Finkelman et al., 1986; Coffman *et al.*, 1988; King, Low and Nutman, 1993; Del Prete, Maggi and Romagnani, 1994).

The Th0/1-like cytokine phenotype observed in this study can not be considered as inducing susceptibility to schistosome infection but may possibly indicate a driving role of Th0/1-like cytokines in the early responses to infection. IFN-γ has been suggested to be involved in conferring protection when found in high levels in resistant individuals infected with *S.mansoni* in a Kenyan study by Roberts *et al.*, (1993). But in this present study, it was observed that when a Th0/1-like phenotype dominates, the individuals were liable to infection. Perhaps due to lack of the effectors which mediate protection and which are marked by a Th0/2-like phenotype. IL-4 and IL-5 which mark the Th0/2-like response are known to stimulate B cells to selectively mature and proliferate for production of particular antibody isotypes, which could ultimately be involved in ADCC reactions resulting in multicellular parasite killing and preventing any further invasion. The Th0/1-like cytokine shown by IFN-γ, was found to exist during infection but the cytokine persisted at lower levels in uninfected individuals before and after treatment, indicative of a dynamic system which possibly begins with raised levels of IFN-γ, a Th0/1

cytokine; and Th0/2-like responses dominating later in the response to infection (Vella and Pearce, 1992), with this shift in response phenotype being enhanced by praziquantel treatment. This study has shown the existence of a cytokine "see-saw" balance in humans infected or uninfected with *S.haematobium*.

Although dominance of IFN-γ may be associated with susceptibility to schistosome infection, the findings of this study also suggest that it may be involved in some early stages of the immune response to schistosome infection. IFN-γ is generally involved in DTH reactions dominating in individuals having acute infections. During this period the immune response is involved in antigen processing and mobilisation of the cellular wing in which IFN-γ plays a key role. Also IFN-γ has been reported to help APCs express MHC class II required for antigen processing (Marrack and Kappler, 1994; Sprent, 1994).

As reported in several other studies and reviews cited in Section 2.1 for both *S.mansoni* and *S.haematobium* infections, Th2-like responses are considered protective and the data presented in this study support this conclusion. The potential role played by Th1 and Th2 cytokines in individuals from areas of low and high transmission have been further clarified by the data presented in this chapter. Individuals in high transmission areas of *S.haematobium* are likely to be sensitised more than individuals in Low Transmission areas, as shown by high cytokine production to schistosome-specific antigen stimulation. However, infection with *S.haematobium in* individuals in the Low Transmission area may exist in an acute phase such that levels of proliferation to schistosome-specific antigen stimulation remain raised. The reports of T cell anergy, shown by T cell

unresponsiveness to stimulation, was observed in heavily infected individuals from the High Transmission area. These data may be helpful in future vaccine distribution to individuals living in different transmission areas. Further studies are required however on larger populations which should consider the cytokine profiles in adults as well as children with follow-up at closer time intervals.

Figure 2.1: Examples of standard curves used in the determination of the cytokine concentration, (i) IFN-y, (ii) IL-4, (iii) IL-5 and (iv) IL-10.

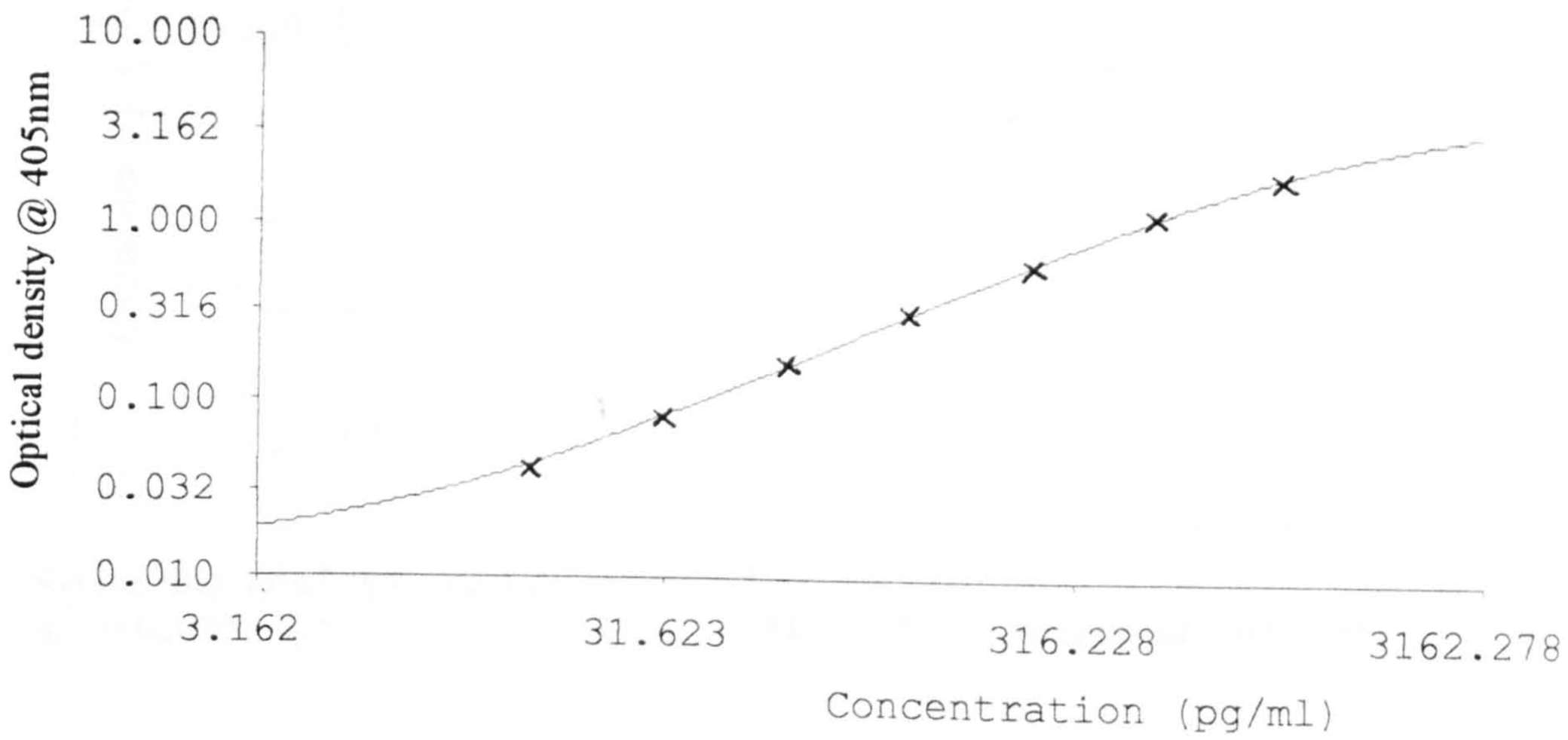


Figure (i): Log/log sigmoid curve for IFN-γ

A = 0.01318 B = 1.07 C = 1534 D = 4.533 R-Squared = 0.99945

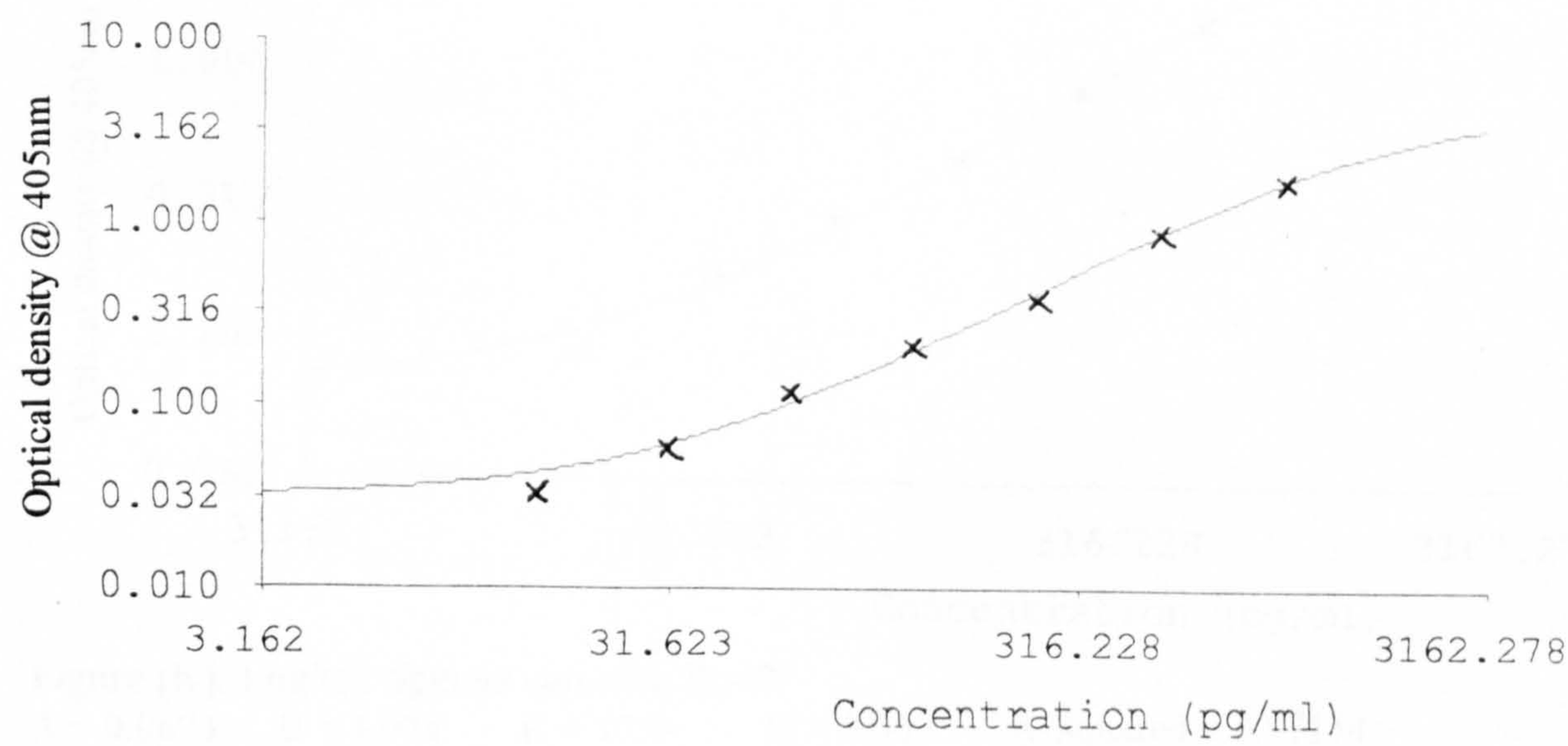


Figure (ii): Log/log sigmoid curve for IL-4:

A = 0.03152 B = 1.279 C = 1671 D = 5.013 R-Squared = 0.94561

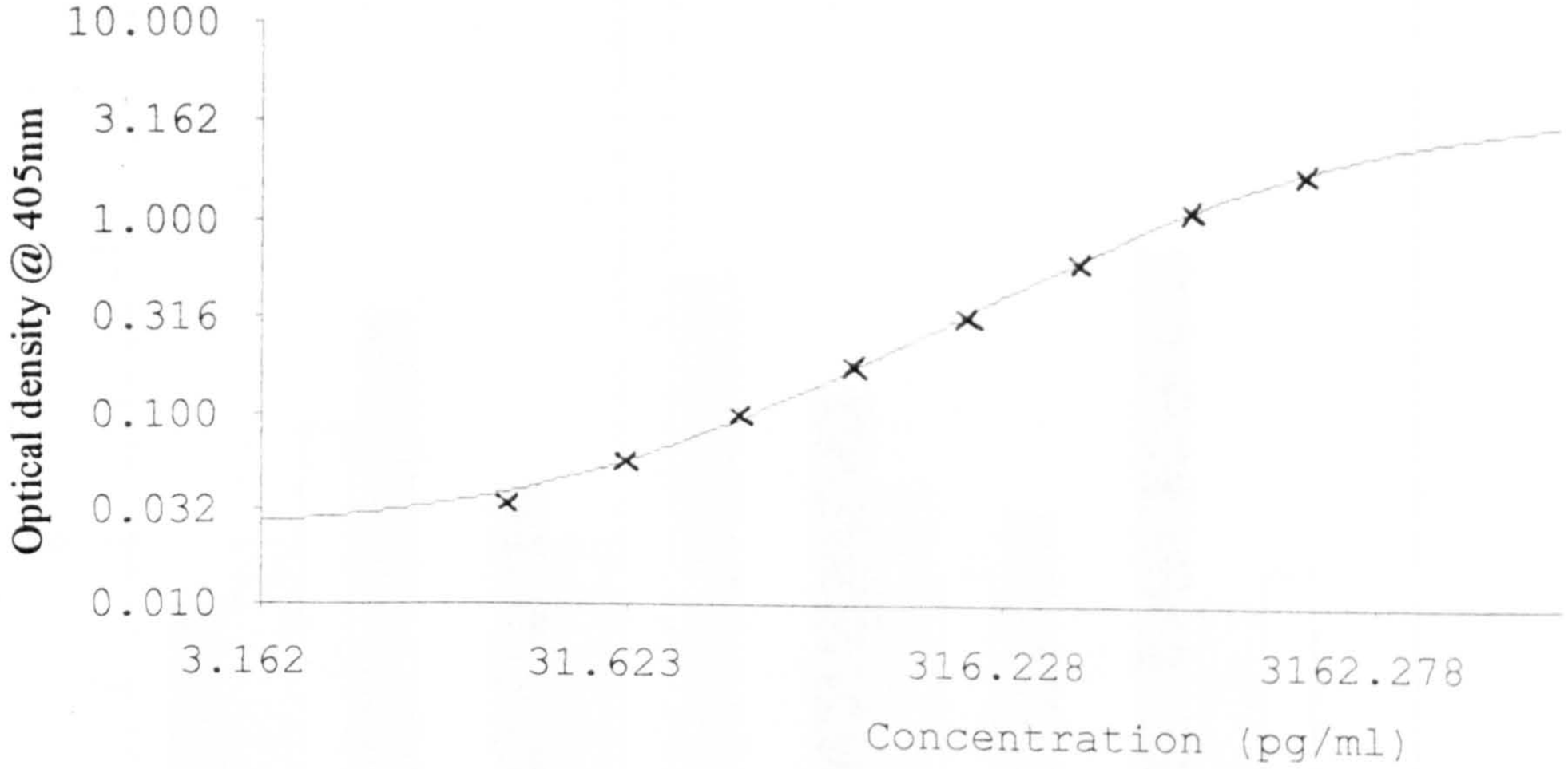


Figure (iii): Log/log sigmoid curve for IL-5:
A = 0.02438 B = 1.1 C = 2154 D = 3.643 R-Squared = 0.99369

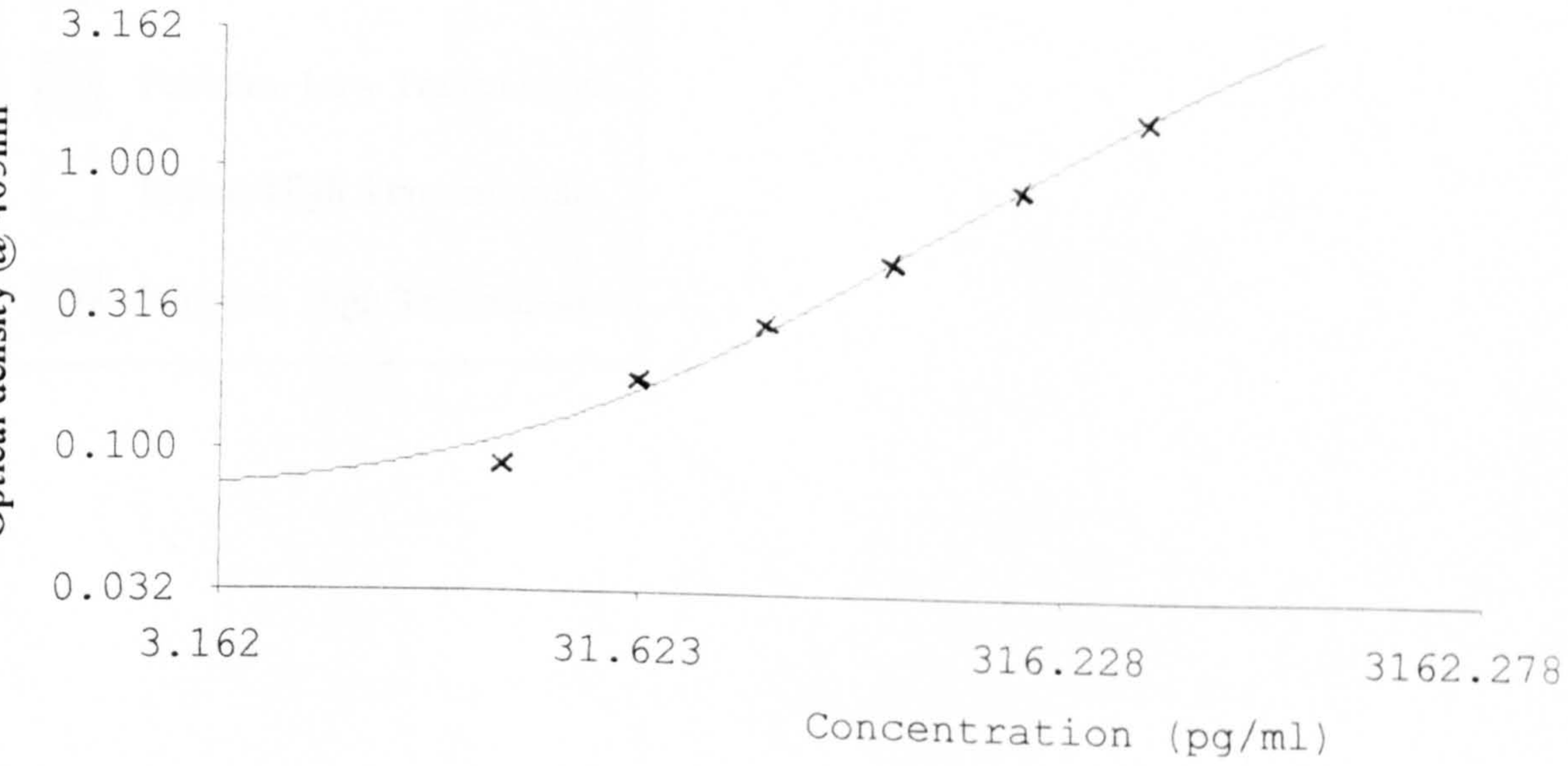


Figure (iv): Log/log sigmoid curve for IL-10:
A = 0.0673 B = 1.074 C = 1753 D = 7.603 R-Squared = 0.97154

Figure 2.2: The distribution of the study population before treatment examination according to age, sex and transmission area.

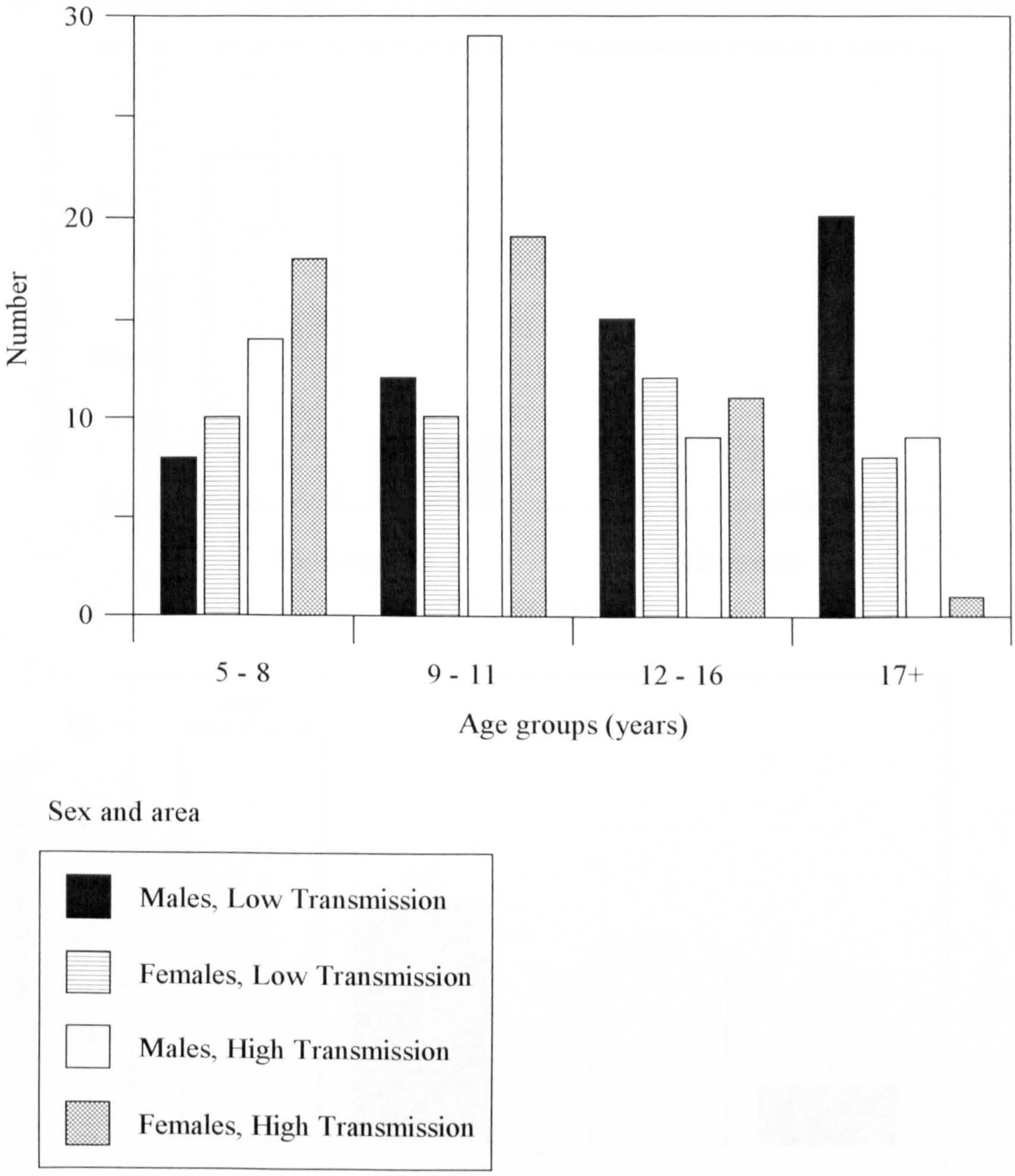


Figure 2.3: Intensity (mean ±S.E)and prevalence of infection in the cohort for which proliferation of PBMC was determined by the dye reduction method from the two study areas, before and 12 months after treatment with praziquantel. Intensity and prevalence were determined by filtration of 10 ml urine samples on each of three consecutive days.

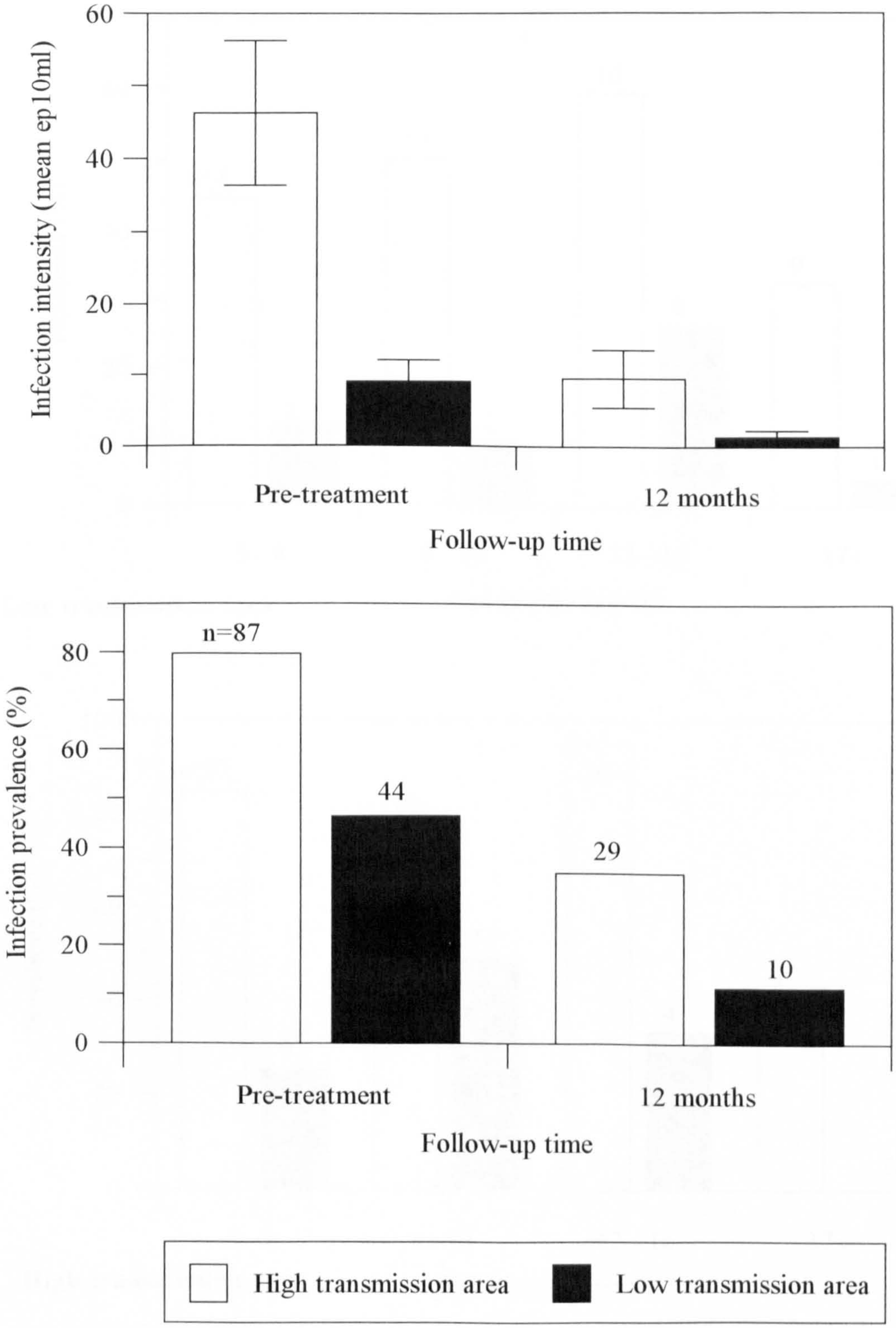


Figure 2.4: Prevalence of infection according to age groups in the cohort from the two study areas for which proliferation of PBMC was determined by the dye reduction method, before and 12 months after treatment with praziquantel. Prevalence was determined by filtration of 10 ml urine samples on each of three consecutive days.

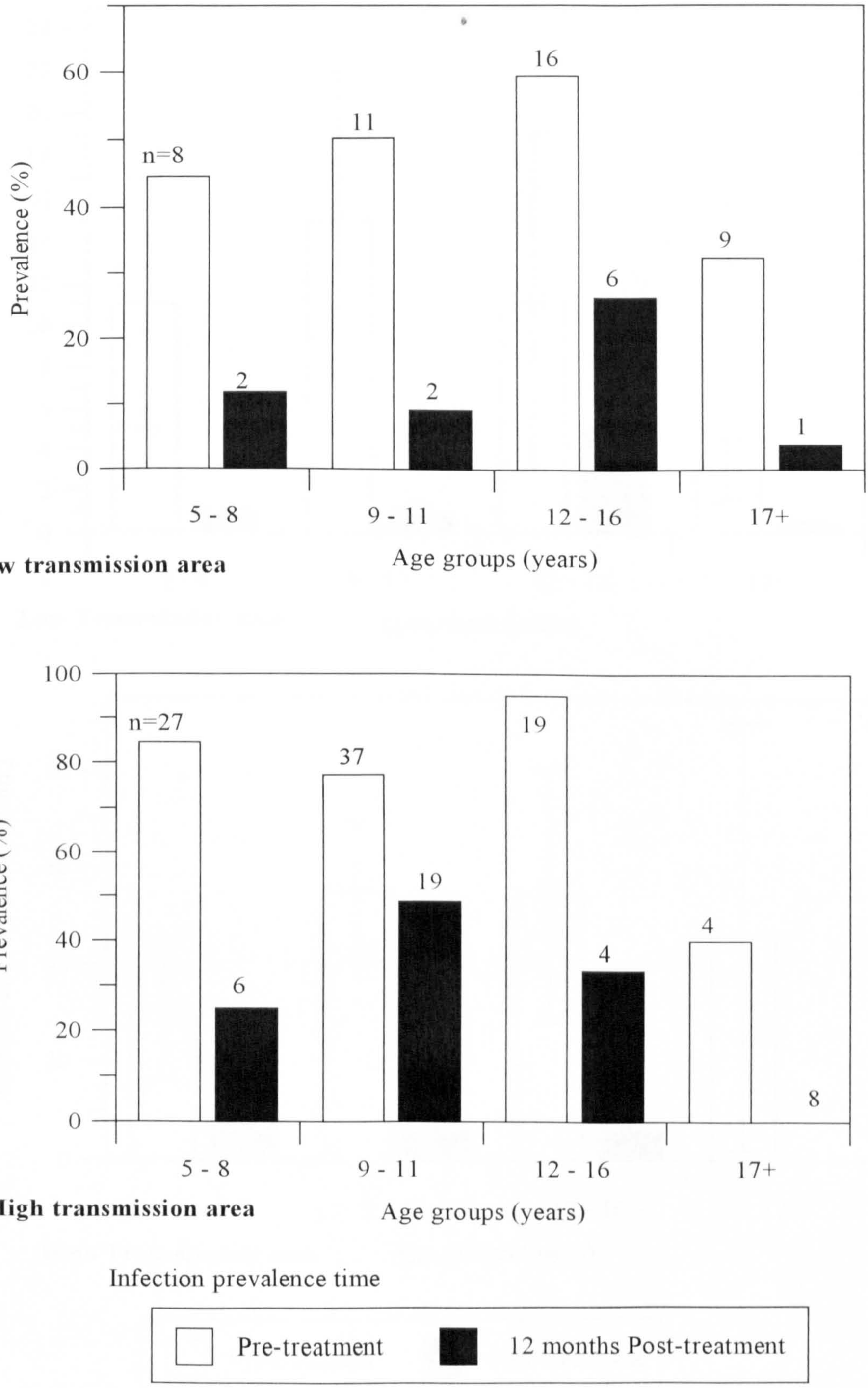


Figure 2.5: Intensity of infection according to age groups in the cohort from the two study areas for which proliferation of PBMC was determined by the dye reduction method, before and 12 months after treatment with praziquantel. Intensity was determined by filtration of 10 ml urine samples on each of three consecutive days.

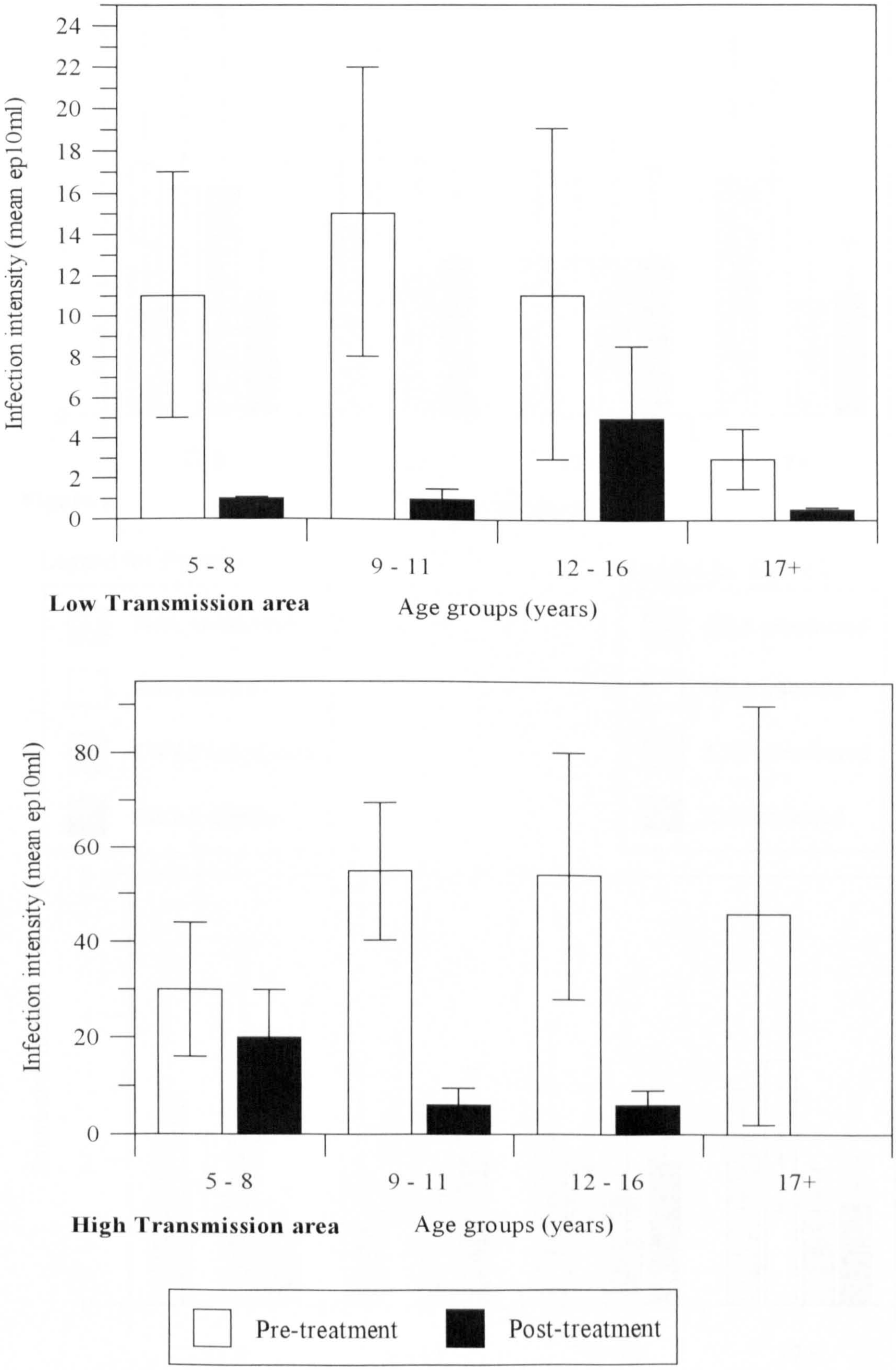


Figure 2.6: Proliferation (mean SI ± SD) of the PBMC from individuals living in the Low Transmission area as detected by the dye reduction assay, showing the response according to infection status, stimulant and age groups before treatment.

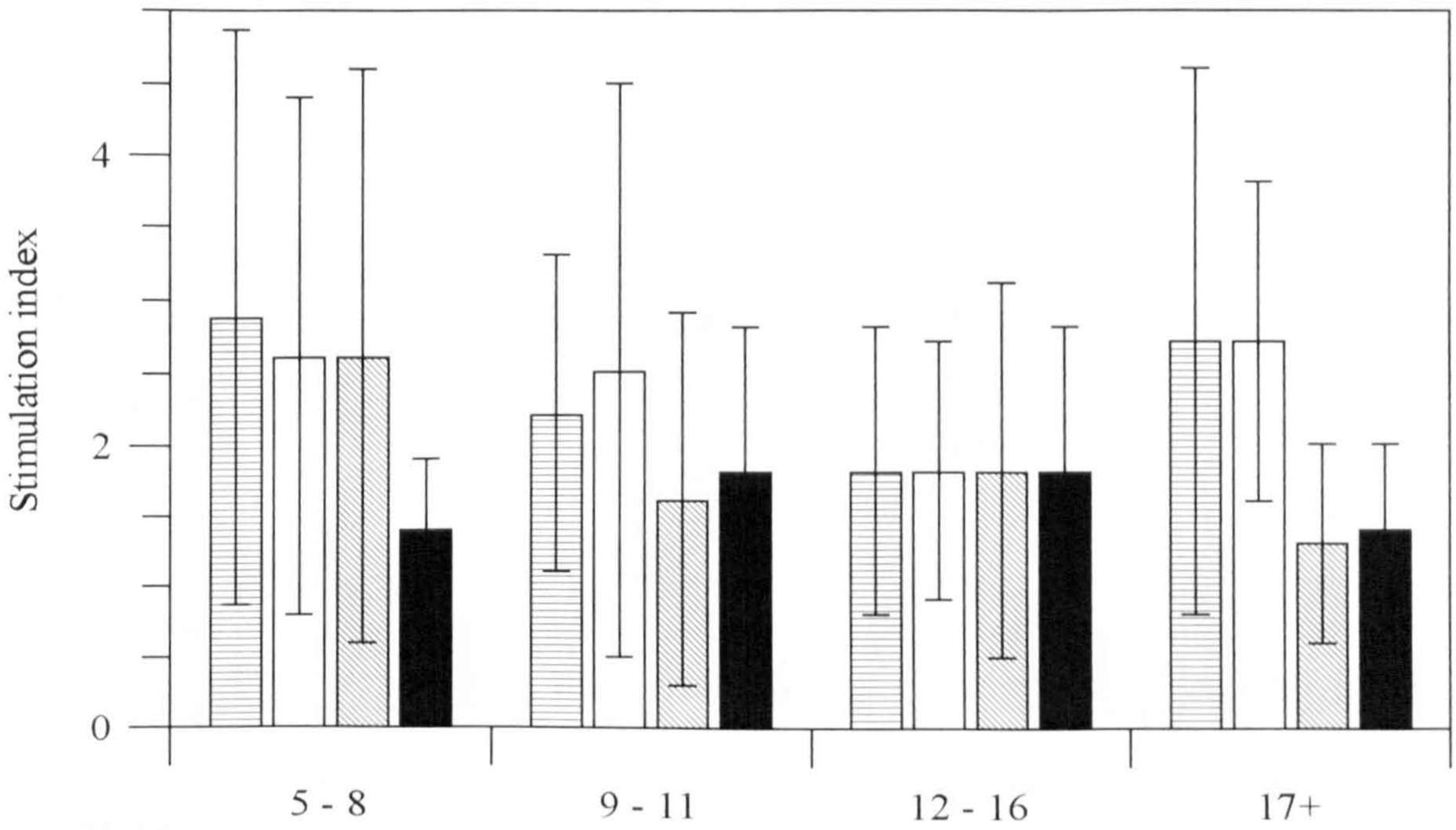
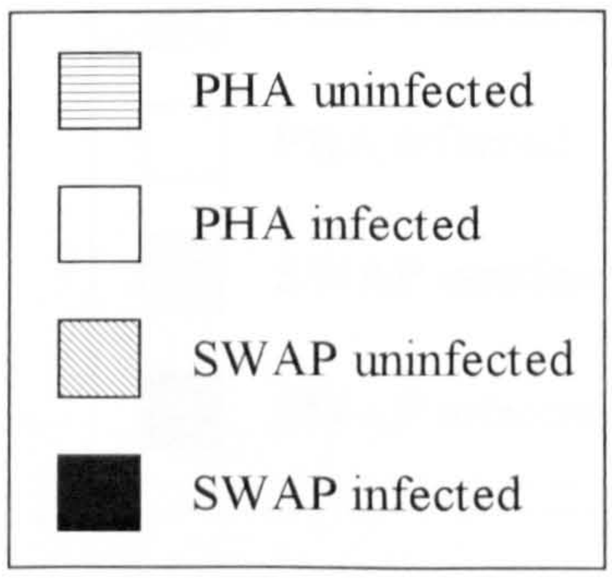


Figure a

Legend for Figure a



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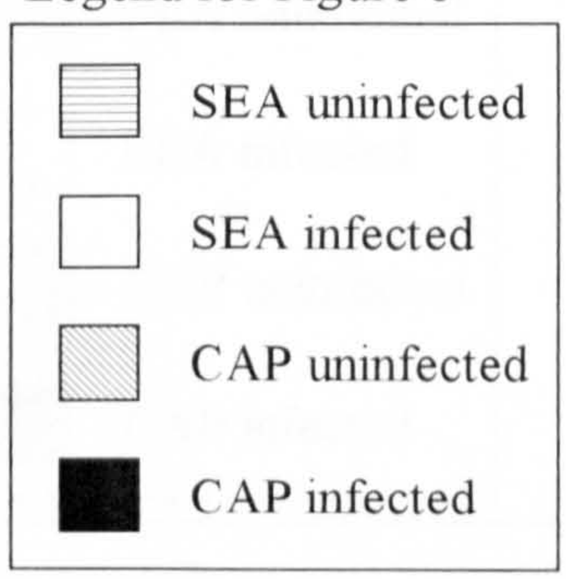


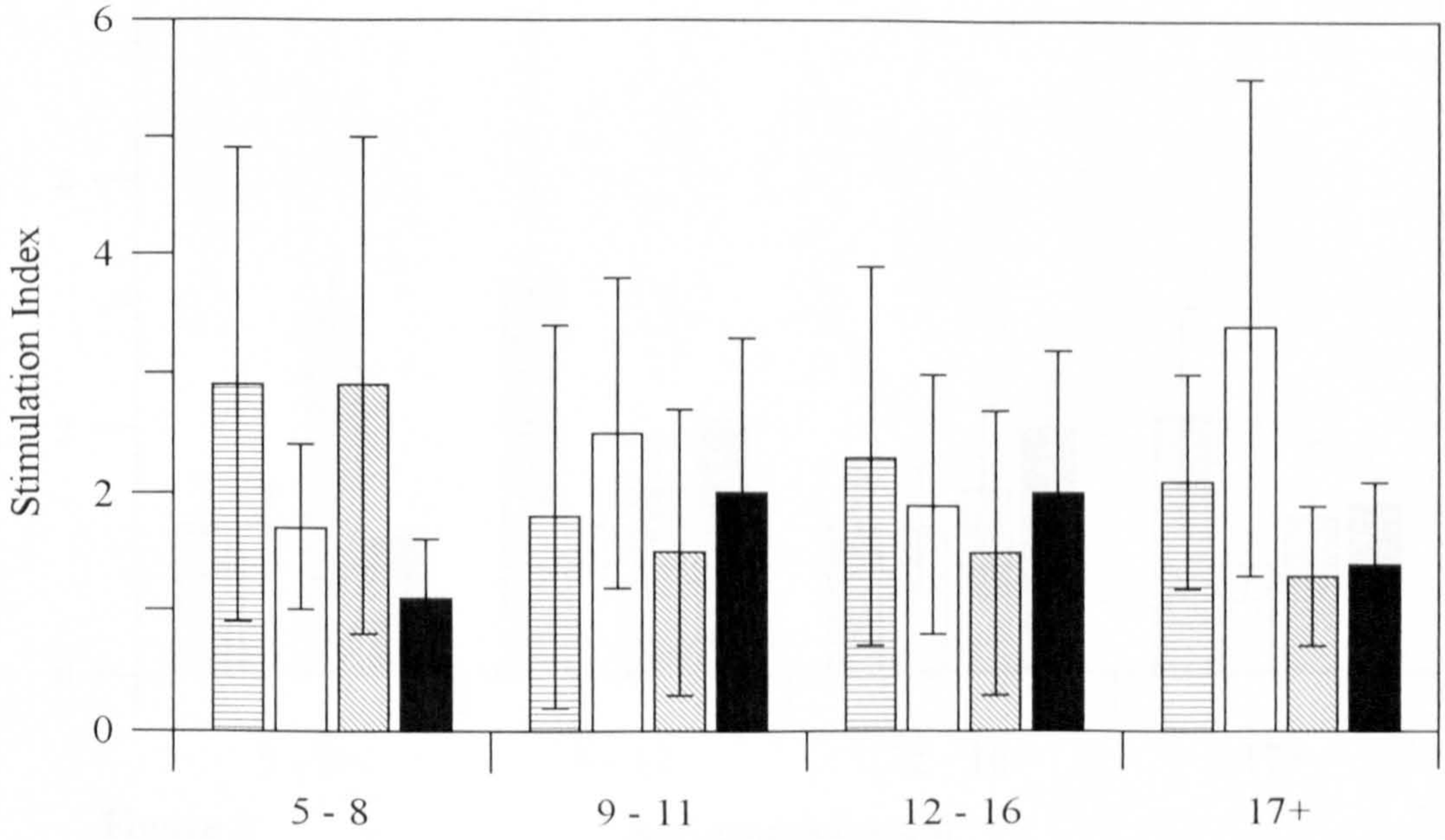


Figure b

Figure 2.7: Proliferation (mean SI ± SD) of the PBMC from individuals living in Low Transmission area as detected by the dye reduction assay, showing the response according to infection status, stimulant and age groups after treatment with praziquantel.

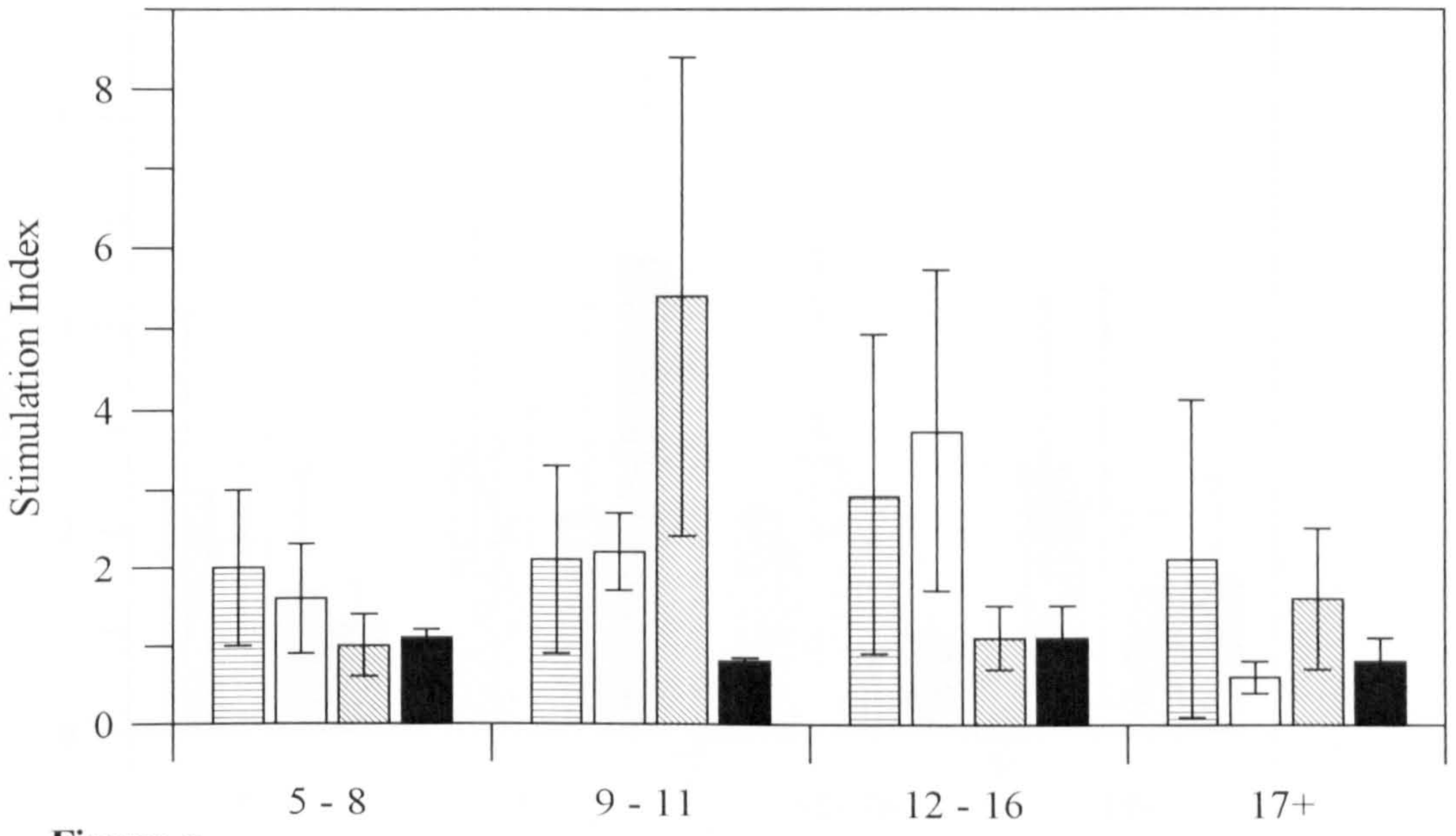
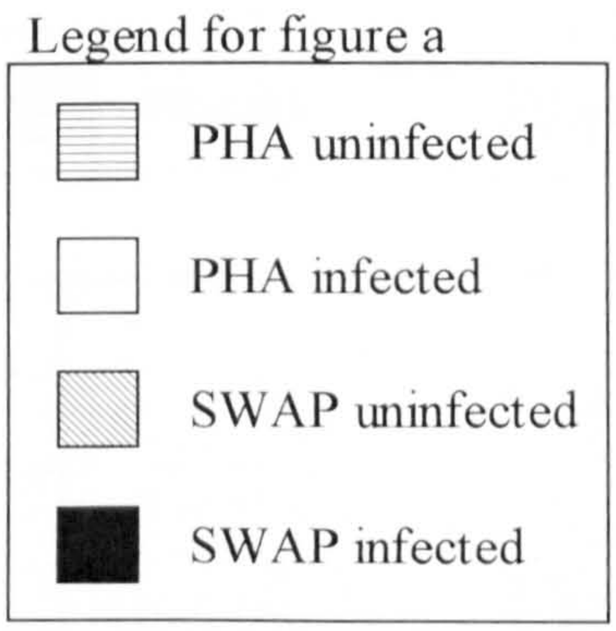
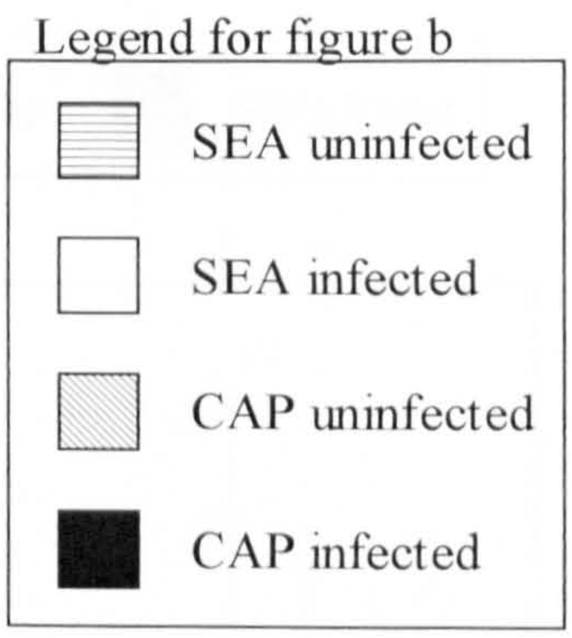


Figure a





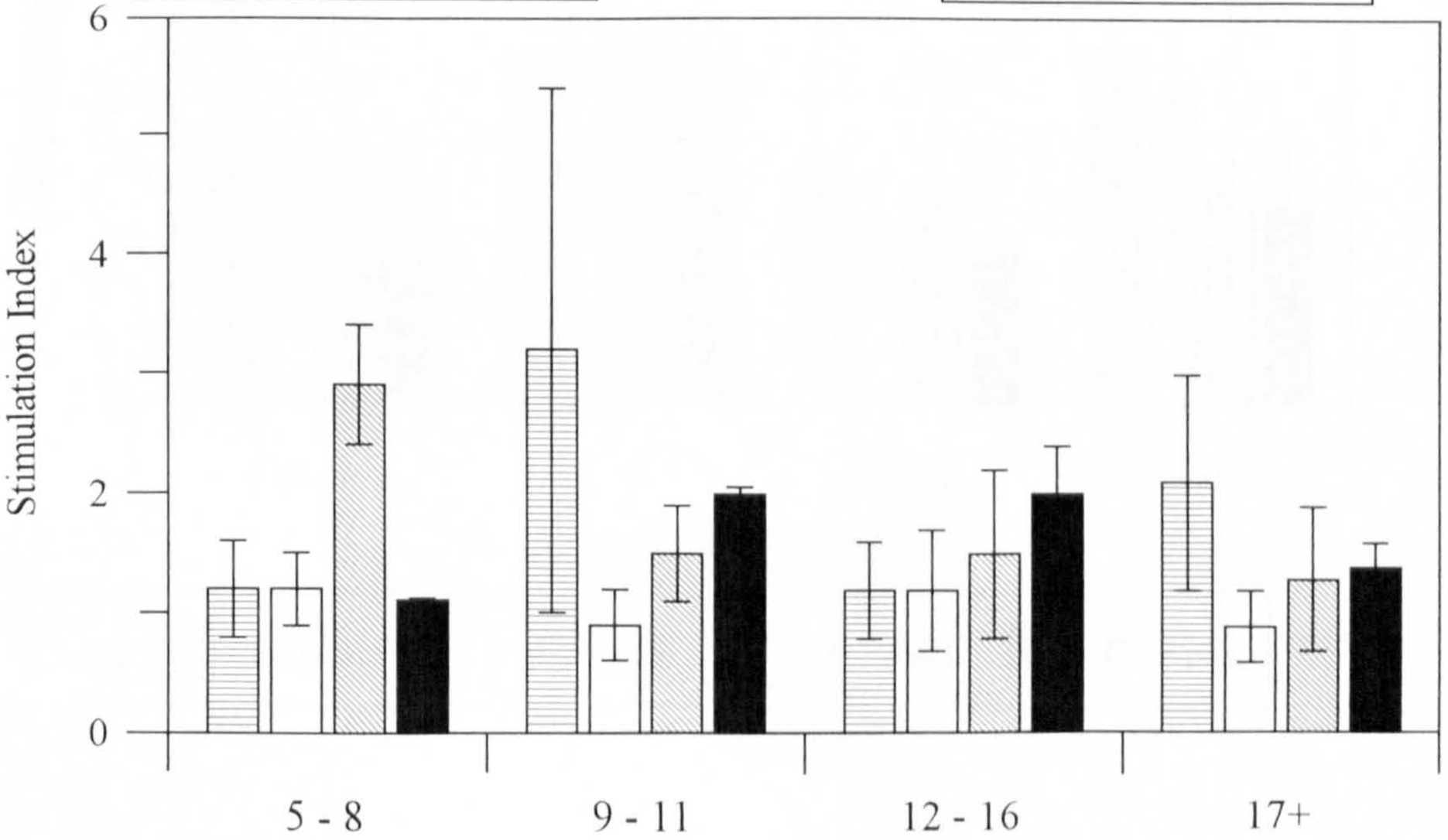
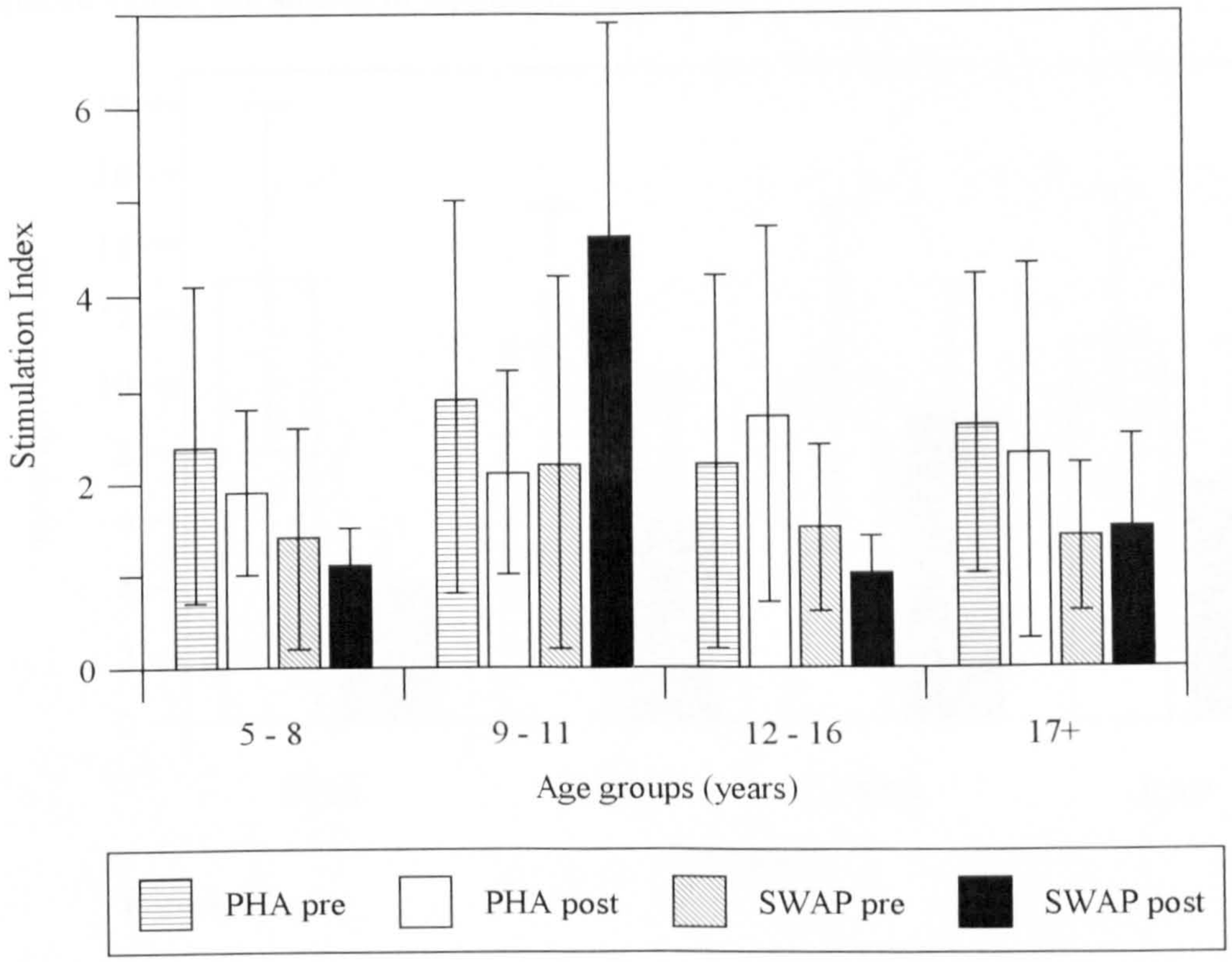


Figure b

Figure 2.8: Proliferation (mean SI ± SD) of the PBMC from infected and uninfected individuals living in Low Transmission area as detected by the dye reduction assay comparing responses before and after treatment with praziquantel.



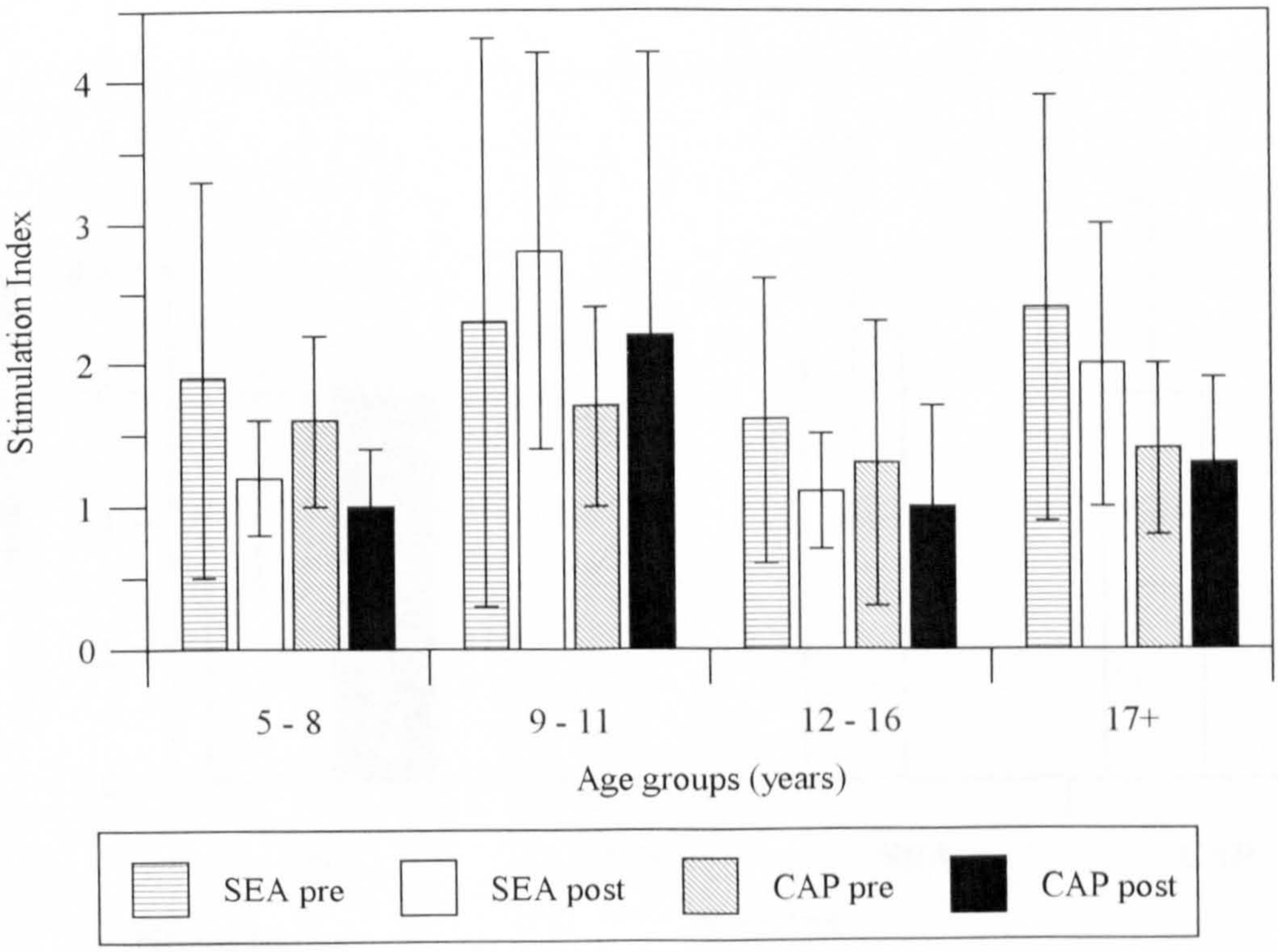


Figure 2.9: Infection intensity (ep10ml urine, mean ±S.E.) and proliferation classes (low and moderate/high) of the PBMC from individuals from the Low Transmission area,as detected by the dye reduction assay, before (a) and after (b) treatment with praziquantel. (more values are shown in Appendix Table 6.1).

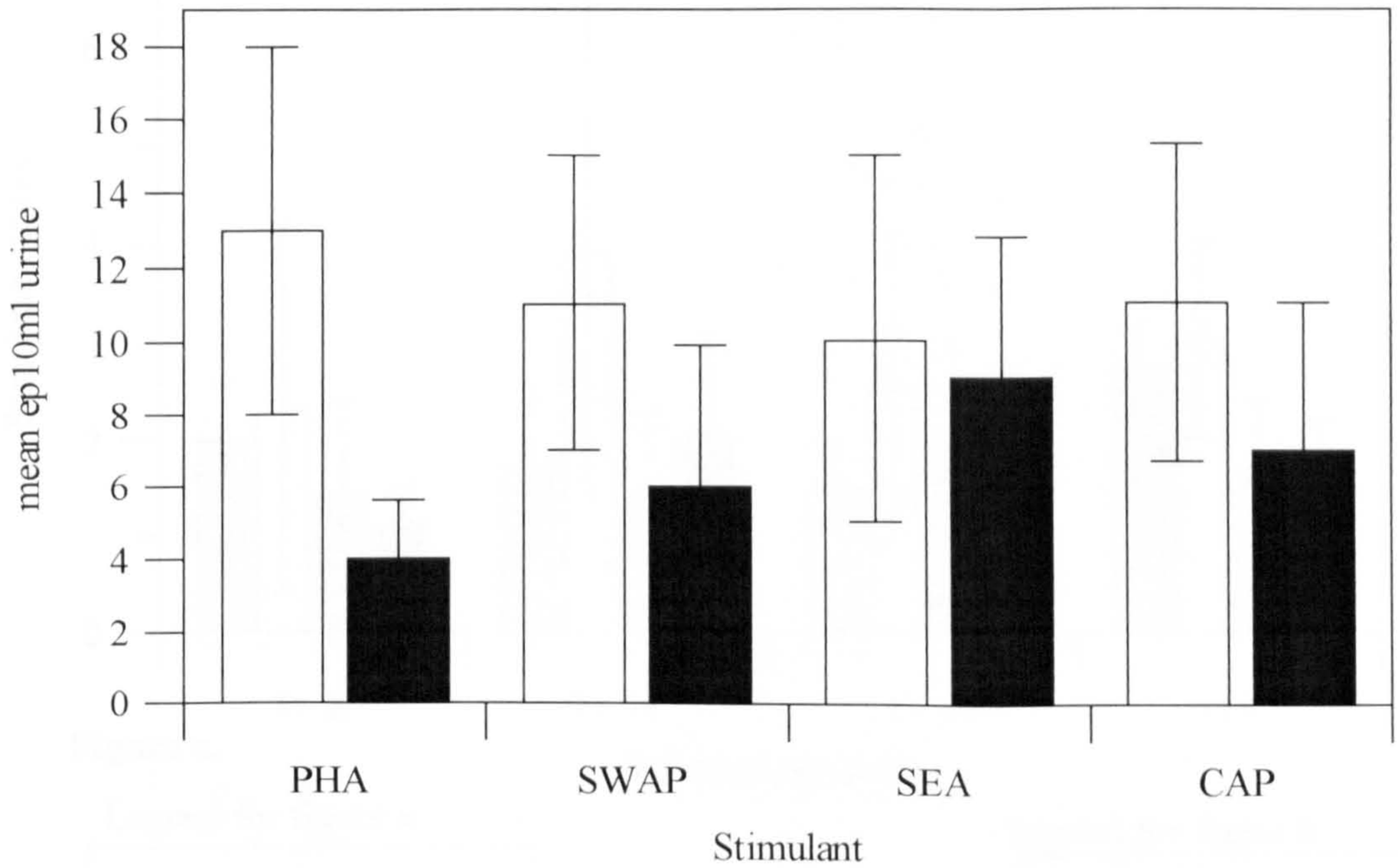


Figure a

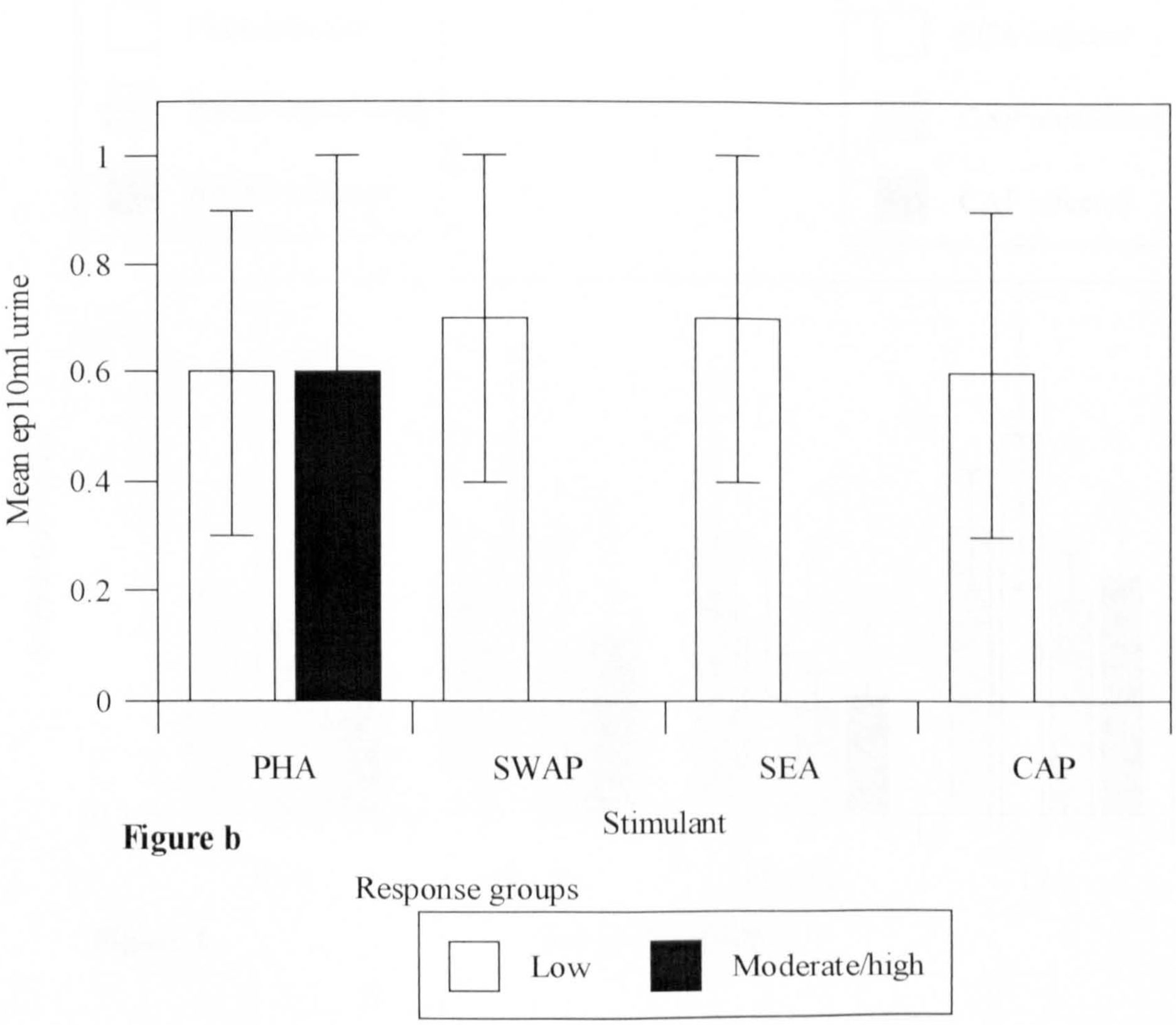


Figure b

Figure 2.10: Proliferation (mean SI ± SD) of the PBMC from individuals living in the High Transmission area as detected by the dye reduction assay, showing the response according to infection status, stimulant and age groups before treatment.

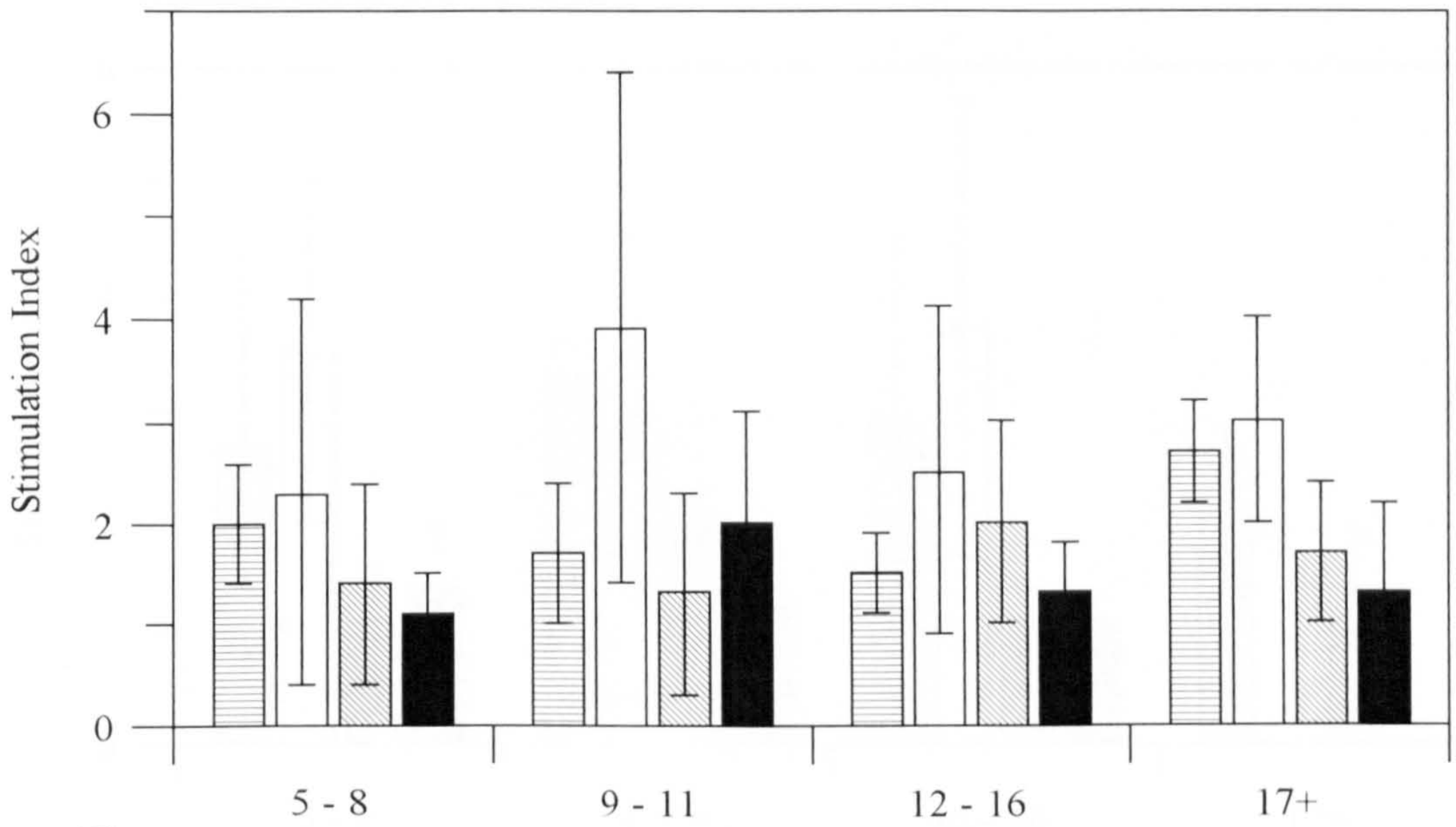


Figure a.

Legend for figure a.

PHA uninfected
PHA infected
SWAP uninfected
SWAP infected

Legend for figure b.

SEA uninfected
SEA infected
CAP uninfected
CAP infected

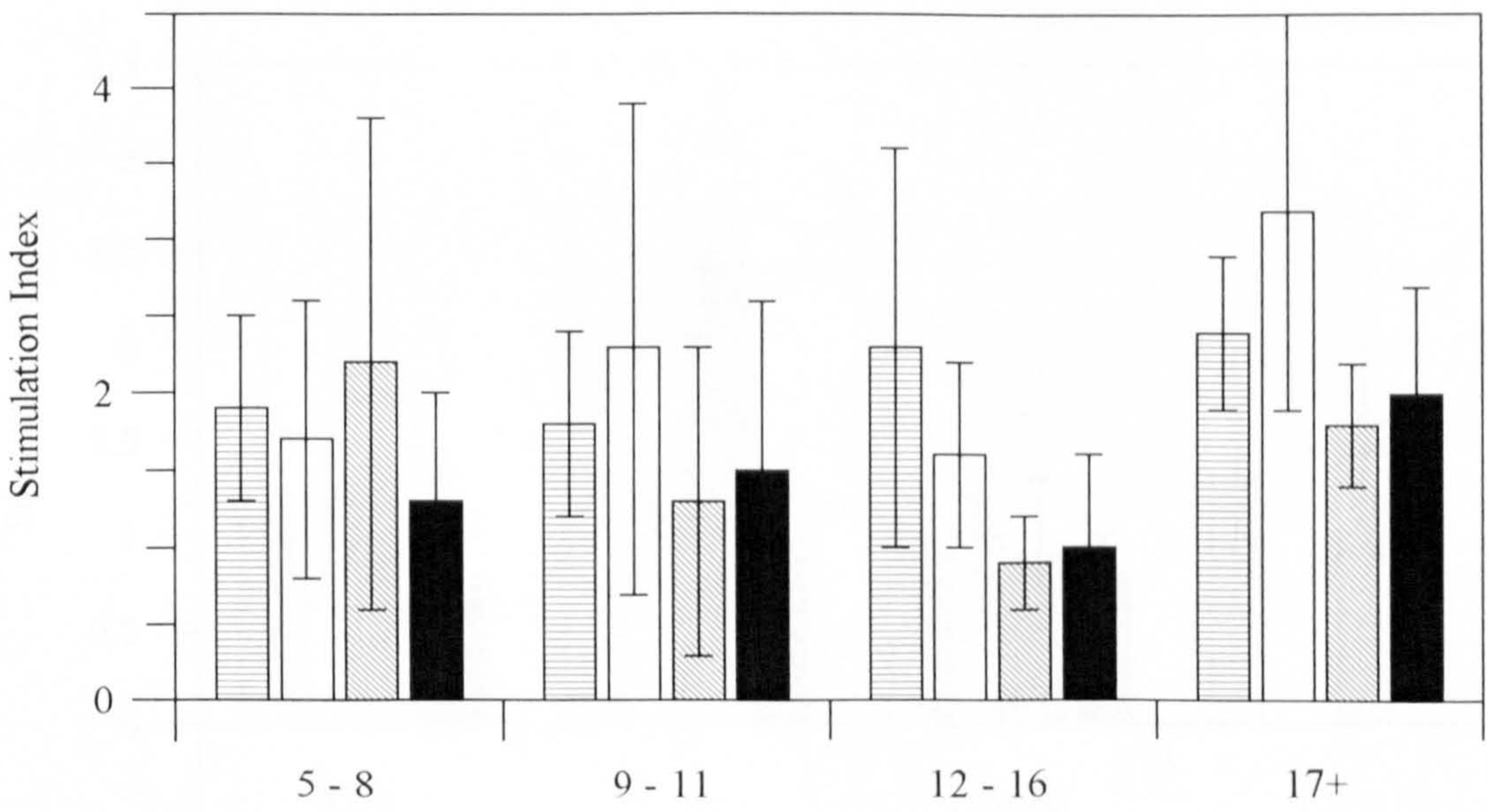


Figure b.

Figure 2.11: Proliferation (mean SI ± SD) of the PBMC from individuals living in the High Transmission area as detected by the dye reduction assay, showing the response according to infection status, stimulant and age groups after treatment with praziquantel.

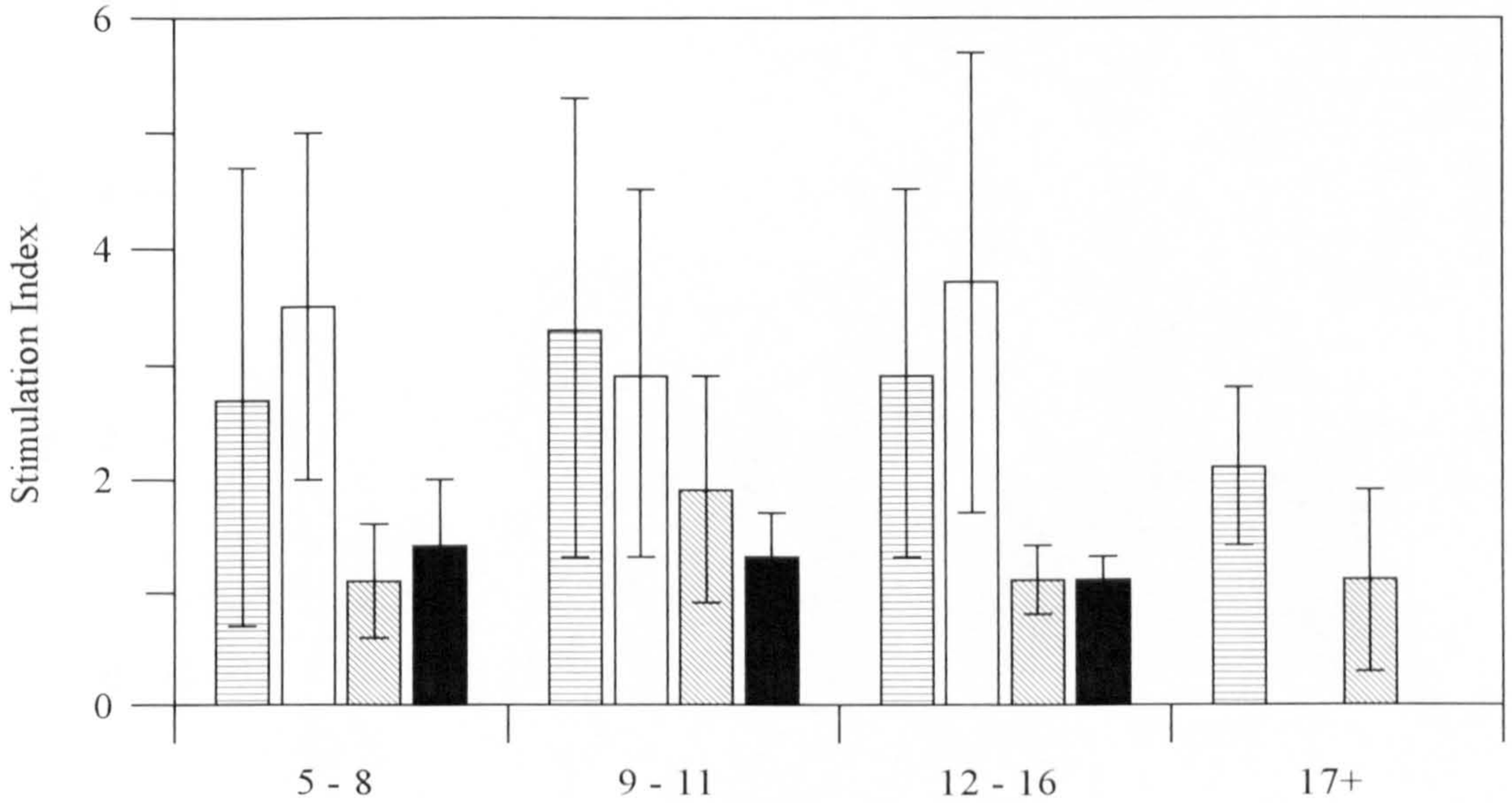


Figure a

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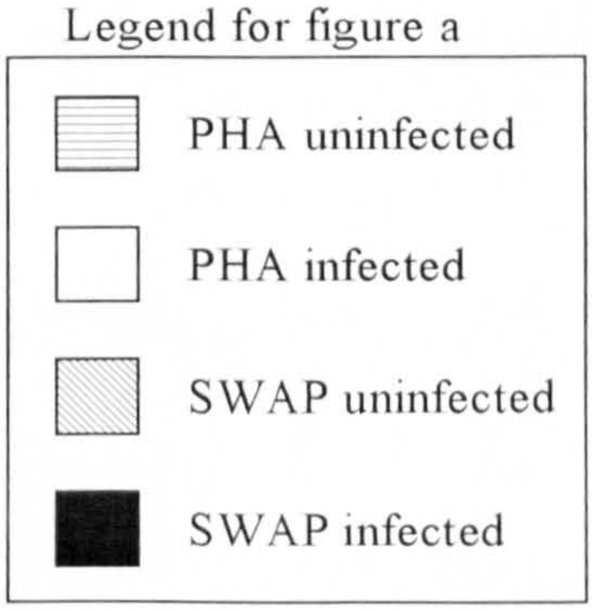

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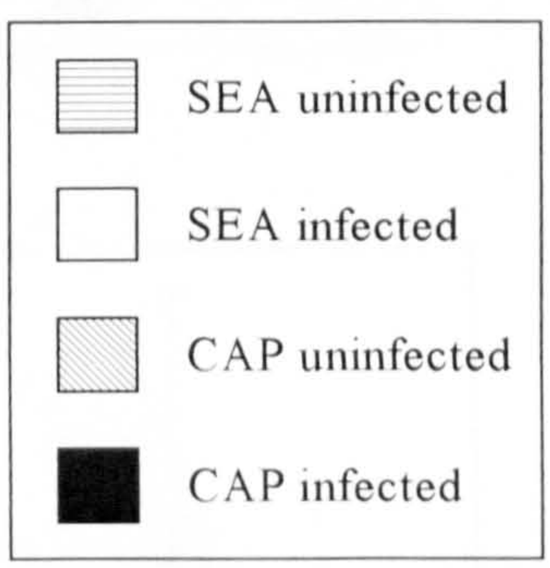


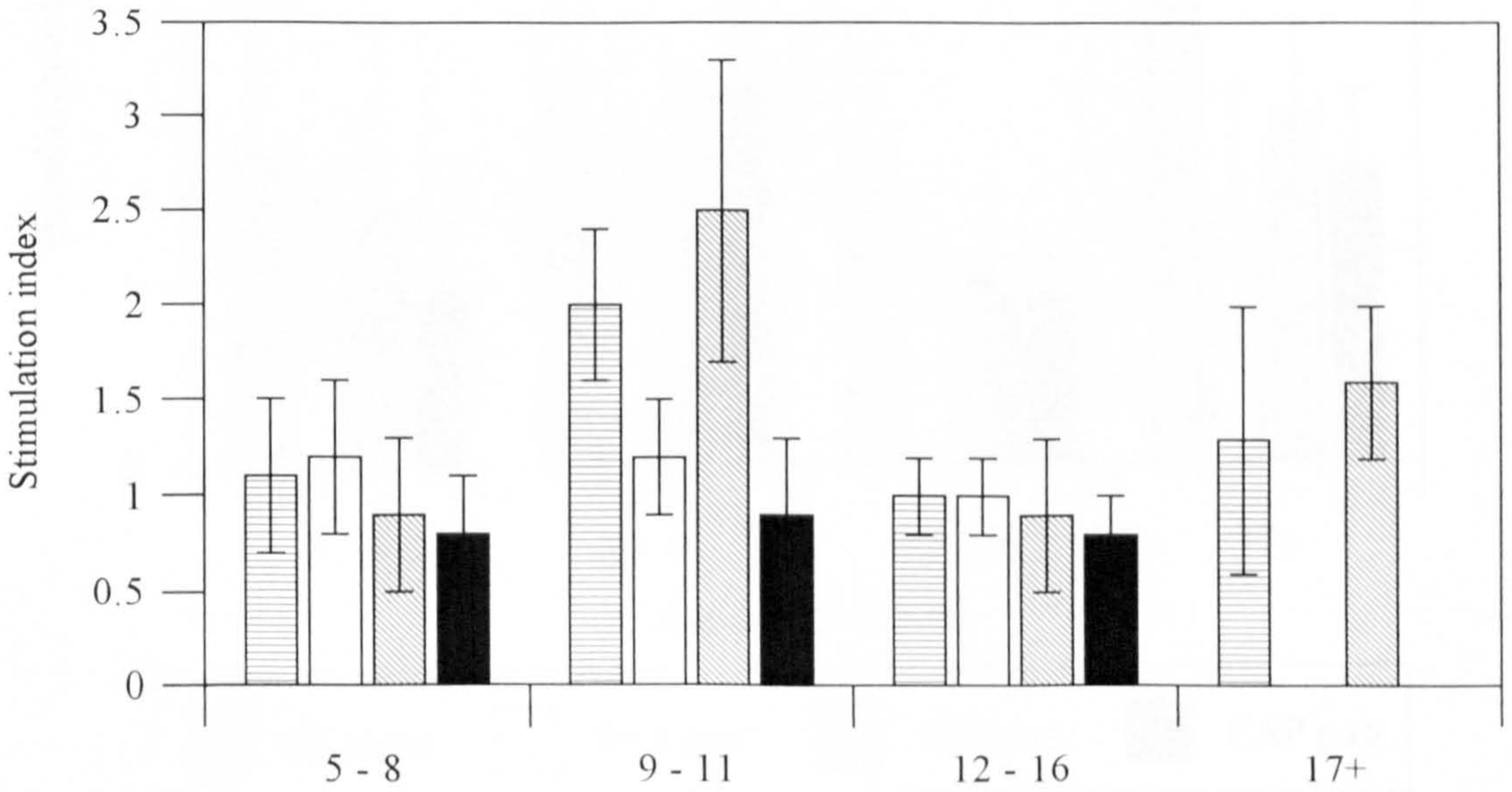
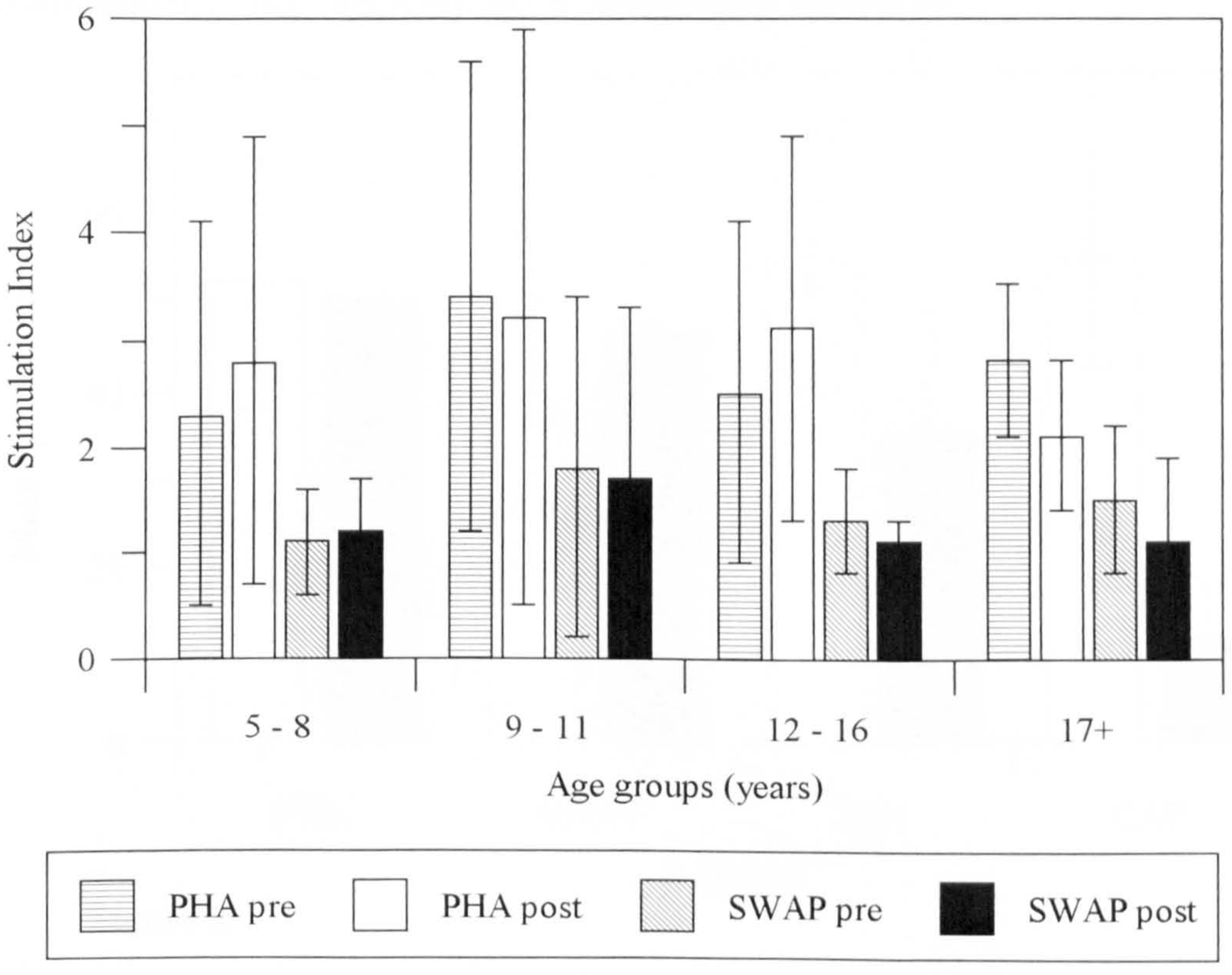


Figure b.

Figure 2.12: Proliferation (mean SI ±SD) of the PBMC from infected and uninfected individuals living in High Transmission area as detected by the dye reduction assay comparing responses before and after treatment with praziquantel.



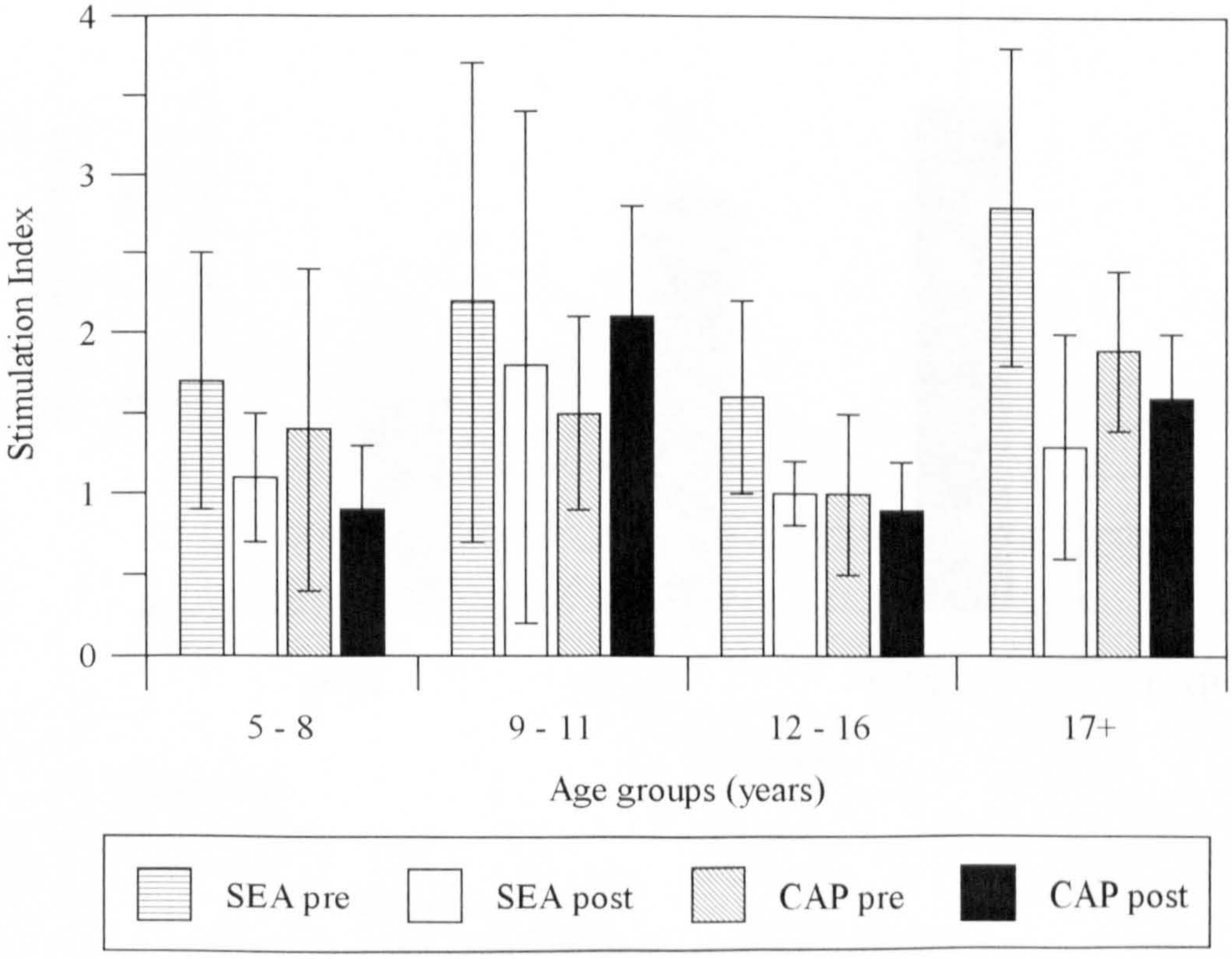


Figure 2.13: Infection intensity (ep10ml urine, mean ±S.E) and proliferation classes (low and moderate/high) of the PBMC from individuals from the High Transmission area, as detected by the dye reduction assay, before (Fig. a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.2)

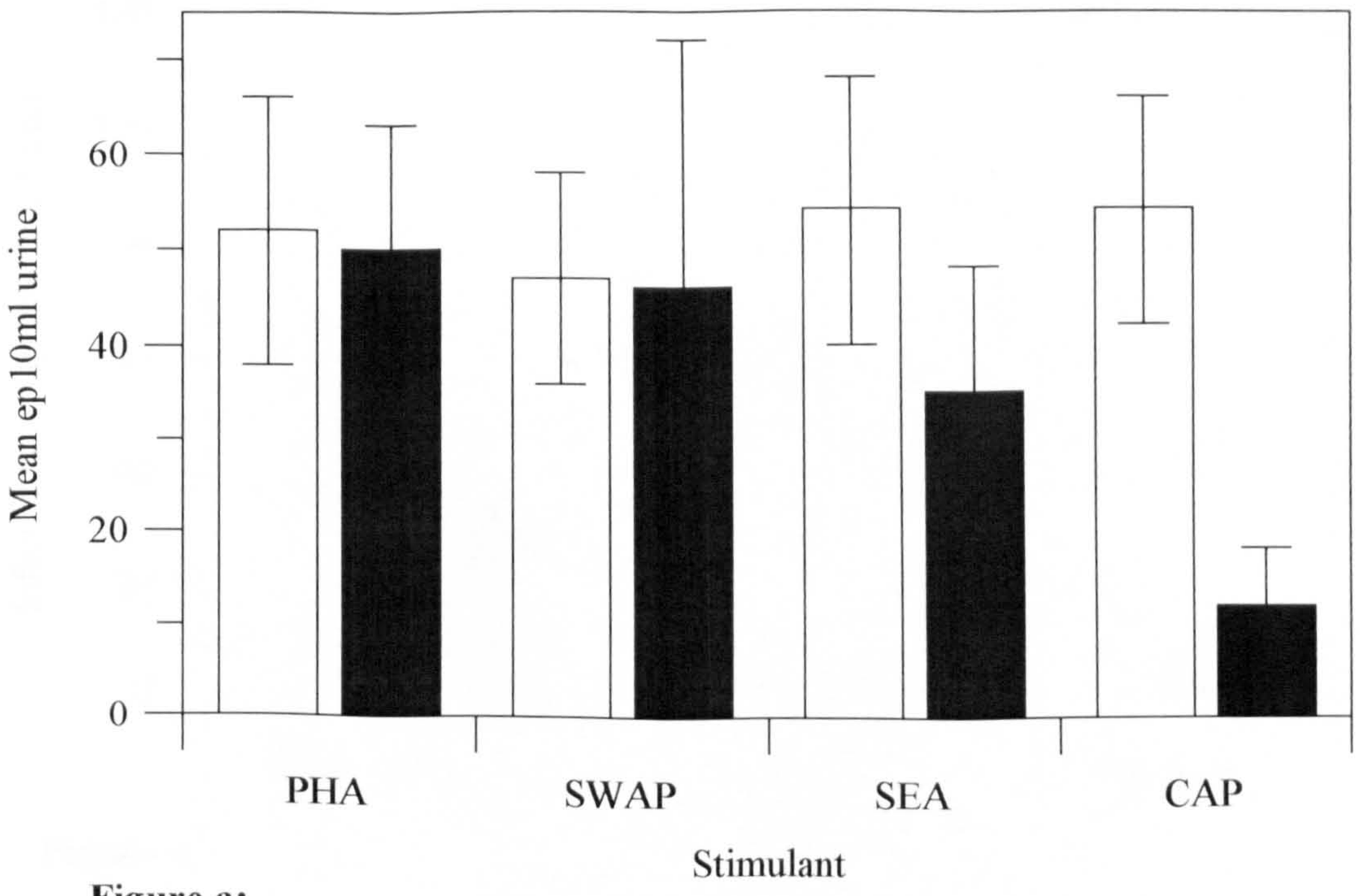


Figure a:

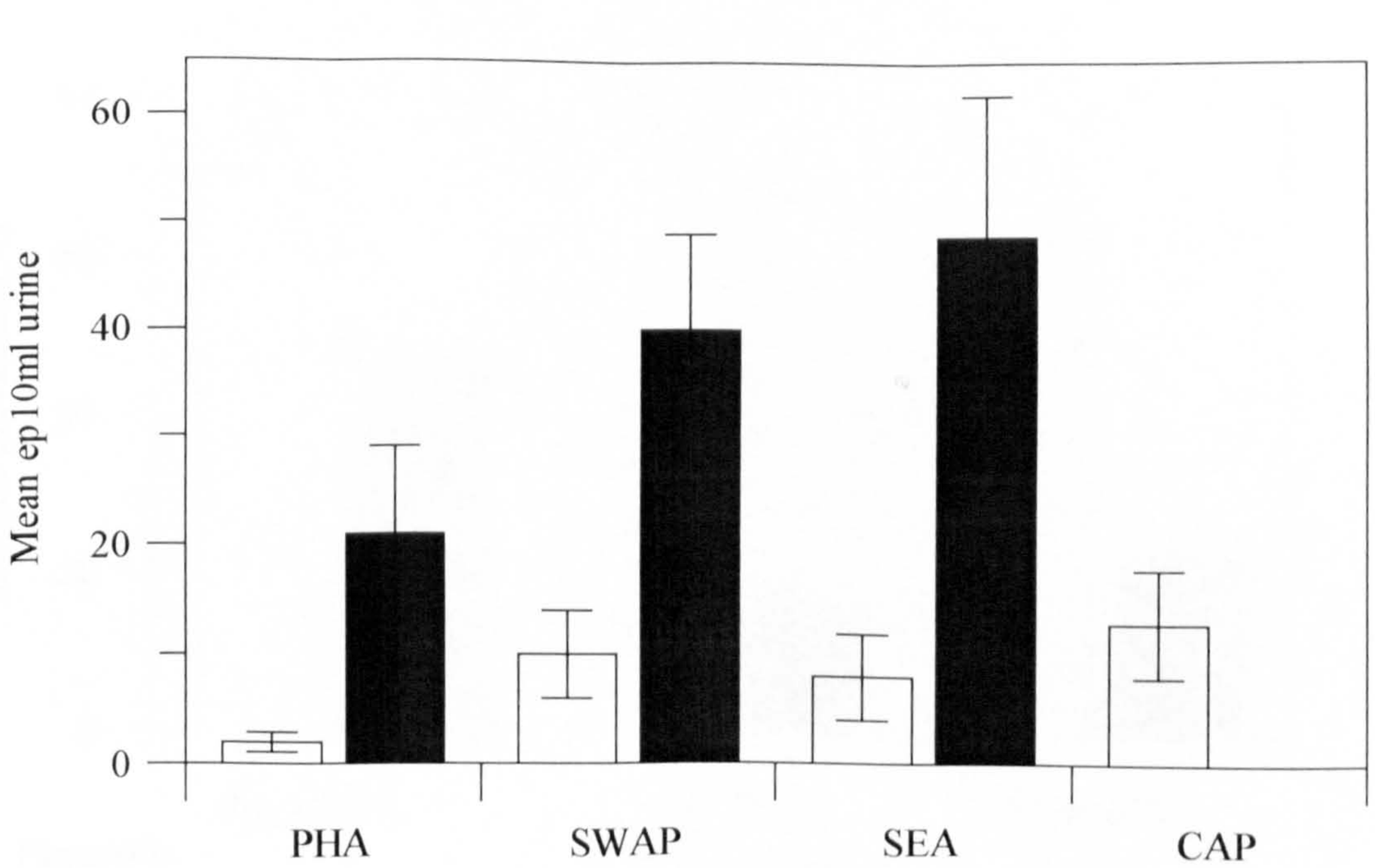


Figure b:

Response groups

Low Moderate/high

Figure 2.14: Intensity and prevalence of infection in the cohort for which proliferation and cytokine production of PBMC were determined from the two study areas, before, 12 and 18 months after treatment with praziquantel. Intensity and prevalence were determined by filtration of 10 ml urine samples on each of three consecutive days.

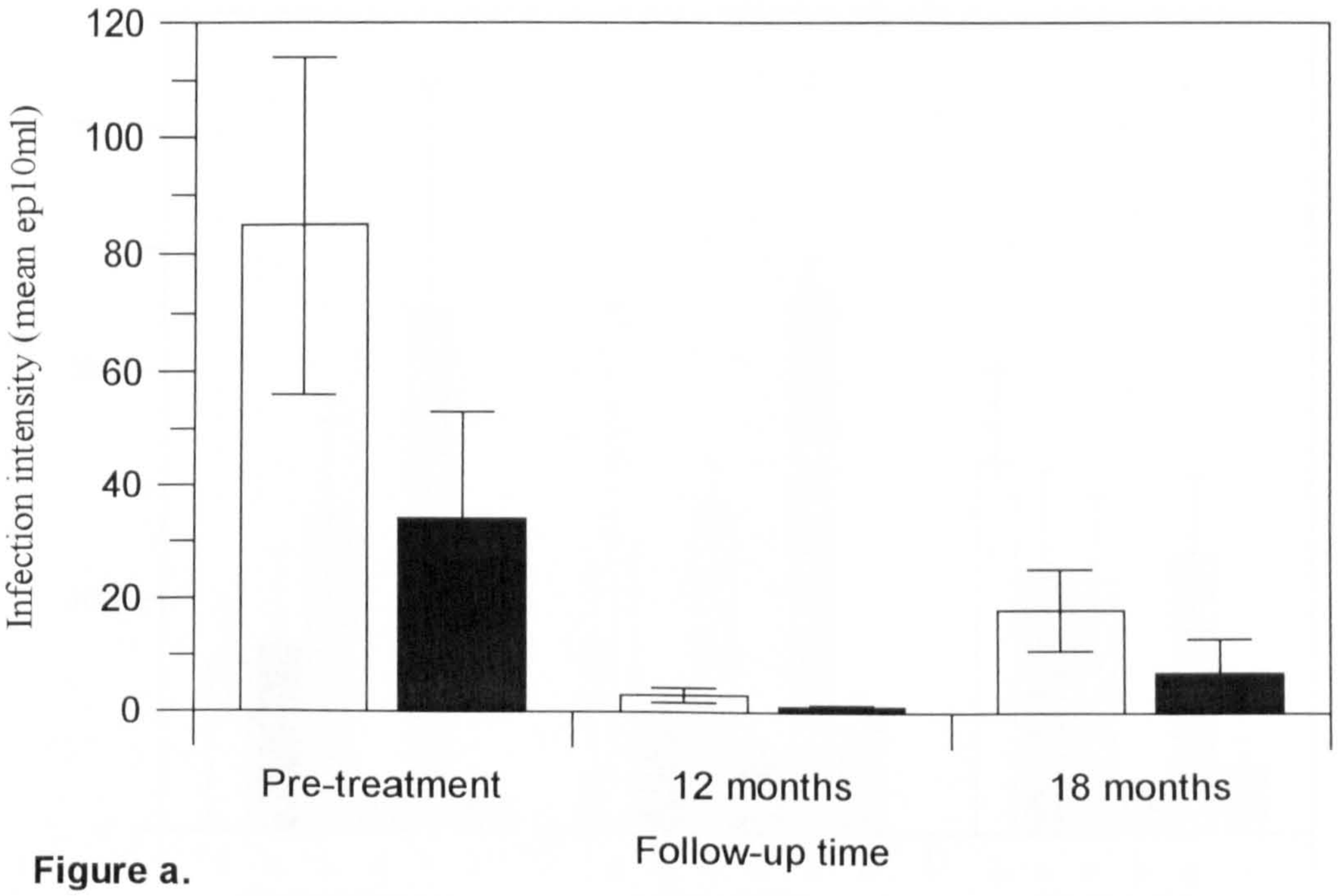


Figure a.

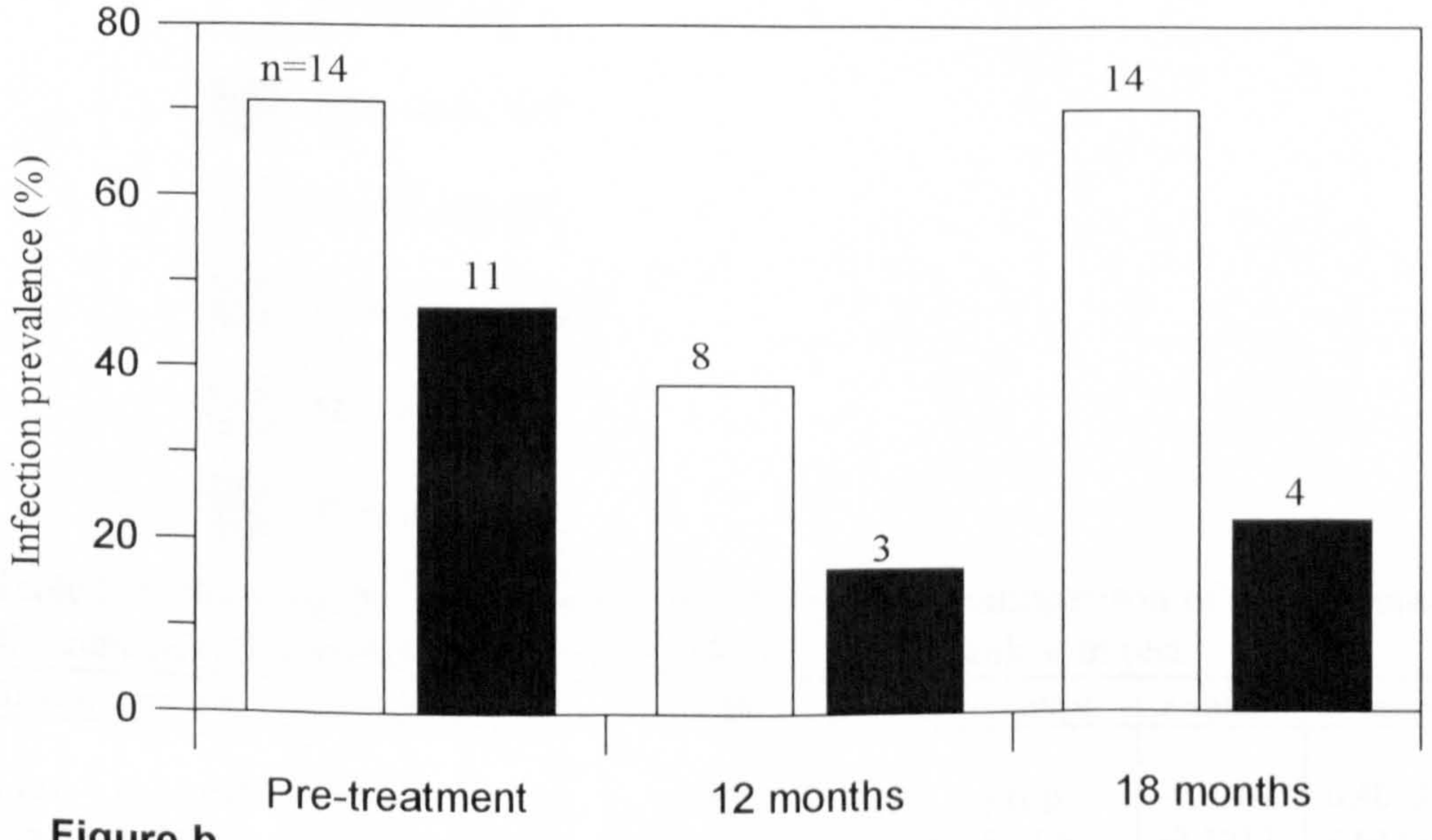


Figure b.

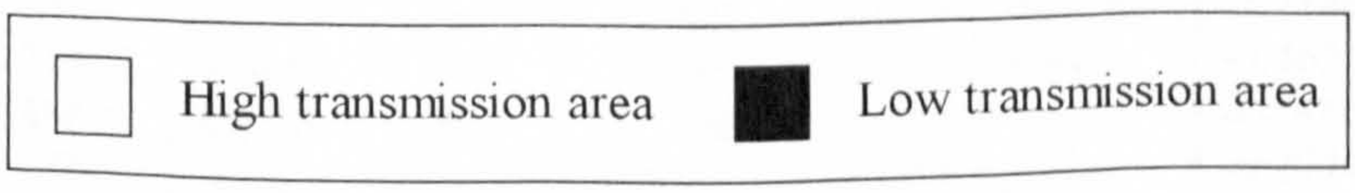


Figure 2.15: Proliferation (mean SI, ± S.E of mean) of the PBMC from the cohort living in Low Transmission area for which proliferation and cytokine production was etermined according to infection status with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.3).

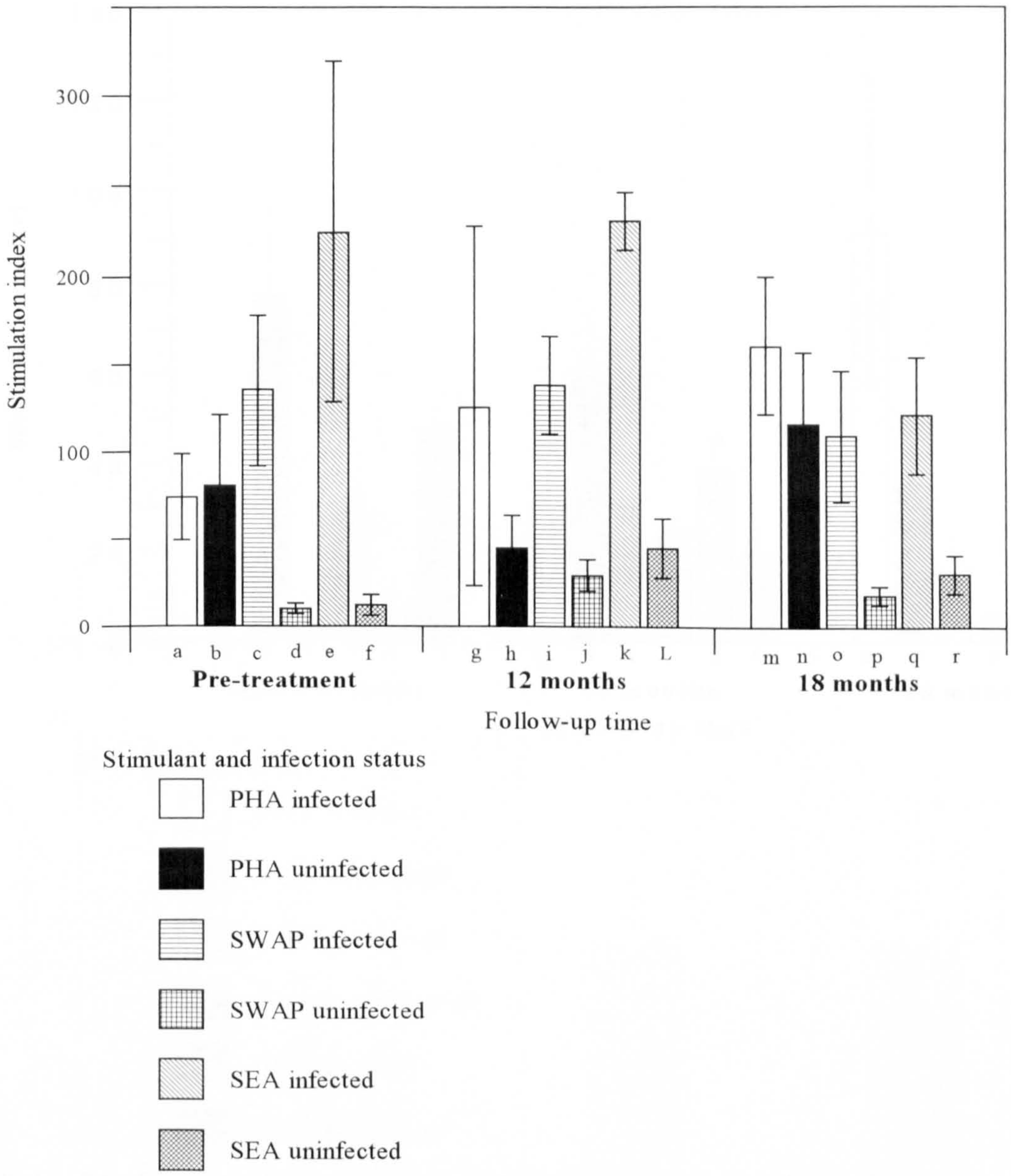


Table 2.1: Showing the significance levels between the comparison of two groups of different infection status or follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-0.1491	0.8815	a vs g	-0.7454	0.4561	j vs p	-0.8389	0.4015
c vs d	-2.2361	**0.0253**	b vs h	-1.0170	0.3092	k vs q	-2.1213	0.0339
e vs f	-2.2361	**0.0253**	c vs i	-0.7454	0.4561	L vs r	-0.7506	0.4529
g vs h	-1.3868	0.1655	d vs j	-1.2019	0.2294	m vs a	-1.7146	0.0861
i vs j	-2.3113	**0.0208**	e vs k	-0.4472	0.6547	n vs b	-0.4623	0.6439
k vs L	-2.4962	**0.0126**	f vs L	-1.3868	0.1655	o vs c	0.0000	1.0000
m vs n	-1.0801	0.2801	g vs m	-0.3536	0.7237	p vs d	-0.2774	0.7815
o vs p	-2.1602	**0.0308**	h vs n	-1.4570	0.1451	q vs e	-0.2449	0.8065
q vs r	-2.1602	**0.0308**	i vs o	-0.7071	0.4795	r vs f	-0.6472	0.5175

Figure 2.16: Proliferation (mean SI, ± S.E of mean) of the PBMC from the cohort living in High Transmission area for which proliferation and cytokine production was determined according to infection status with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.4).

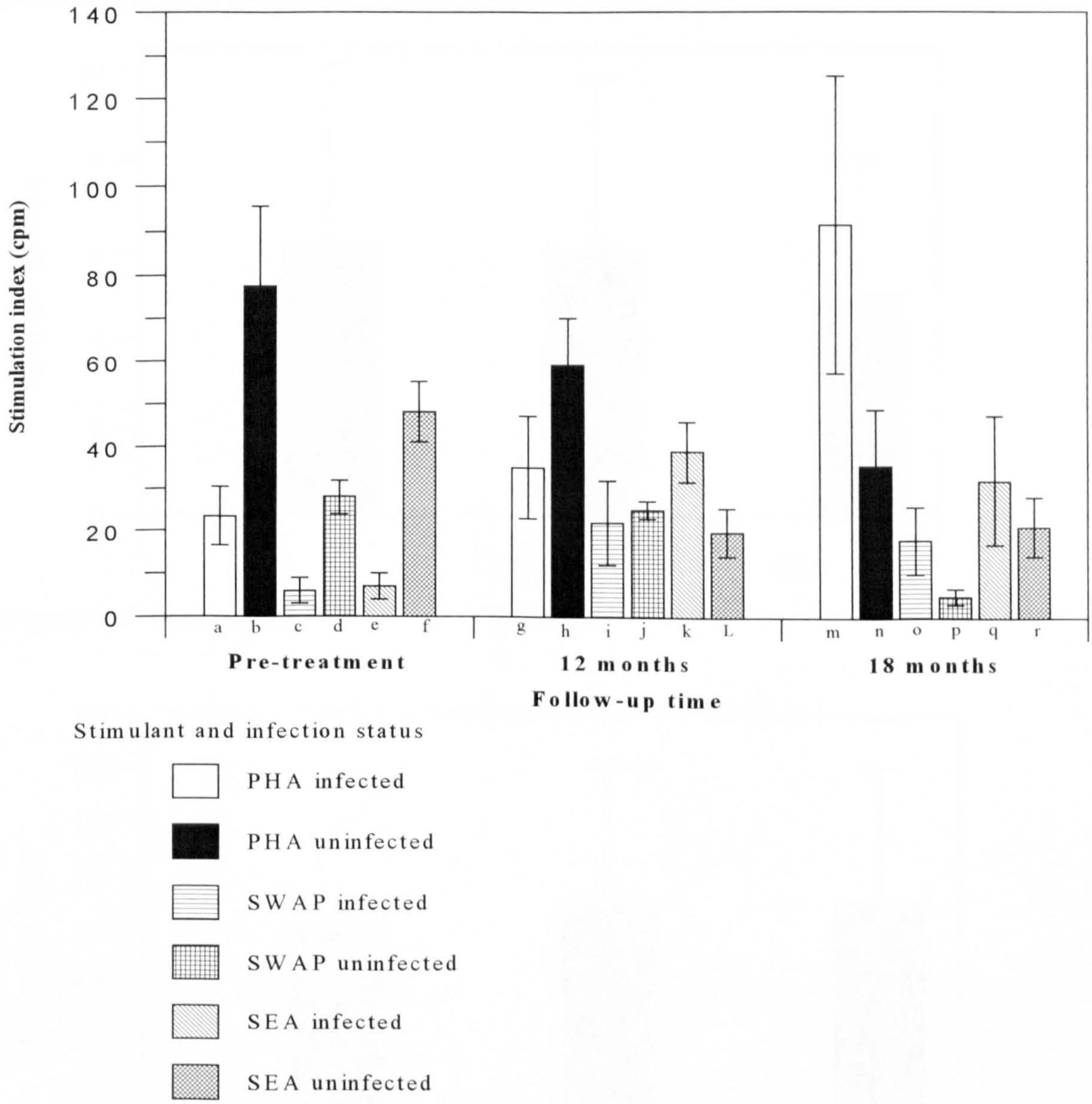


Table 2.2: Showing the significance levels between the comparison of two groups of different infection status or follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-2.6112	**0.0090**	a vs g	-0.9139	0.3608	j vs p	-2.6112	**0.0090**
c vs d	-2.7417	**0.0061**	b vs h	-0.2447	0.8065	k vs q	-1.5286	0.1264
e vs f	-2.8723	**0.0041**	c vs i	-1.9584	0.0502	L vs r	-0.9400	0.3472
g vs h	-1.7146	0.0864	d vs j	-0.9798	0.3272	m vs a	-1.3212	0.1864
i vs j	0.0000	1.0000	e vs k	-2.7417	**0.0061**	n vs b	-1.4697	0.1416
k vs L	-1.7146	0.0864	f vs L	-2.2045	0.0275	o vs c	-1.0734	0.2831
m vs n	-0.5855	0.5582	g vs m	-0.5095	0.6104	p vs d	-2.4495	0.0143
o vs p	-0.2928	0.7697	h vs n	-1.3578	0.1745	q vs e	-2.2295	0.0258
q vs r	-0.5855	0.5582	i vs o	-0.8492	0.3958	r vs f	-1.7146	0.0864

Figure 2.17: Infection intensity (ep10ml urine, ± S.E. of mean) and proliferation classes (low and moderate/high) of the PBMC from individuals from the Low Transmission area, for which proliferation (determined using tritiated thymidine) and cytokine production was determined, before (a) and 12 months after (b) treatment with praziquantel, (more values are shown in Appendix Table 6.5).

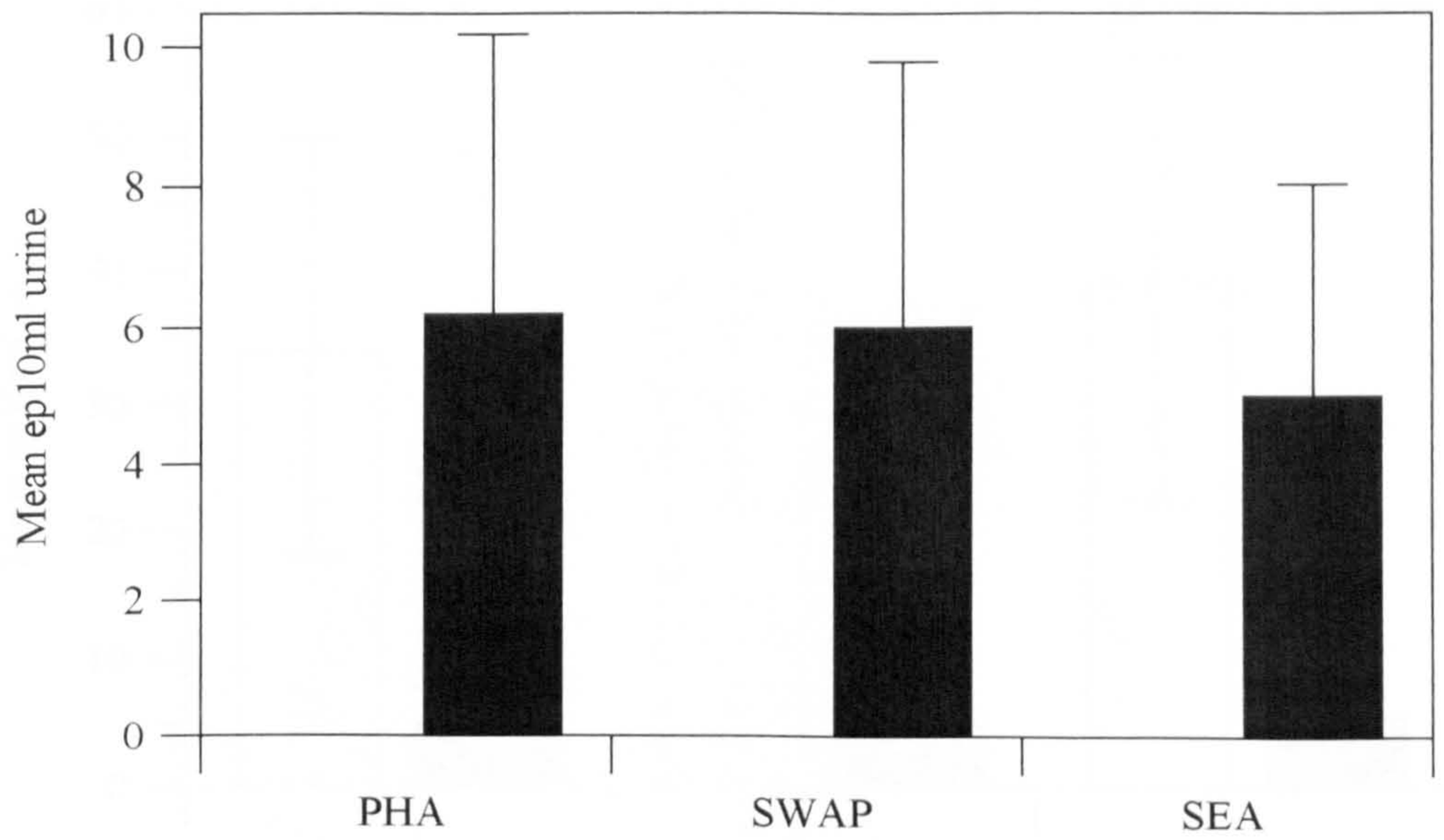


Figure a: Pre-treatment

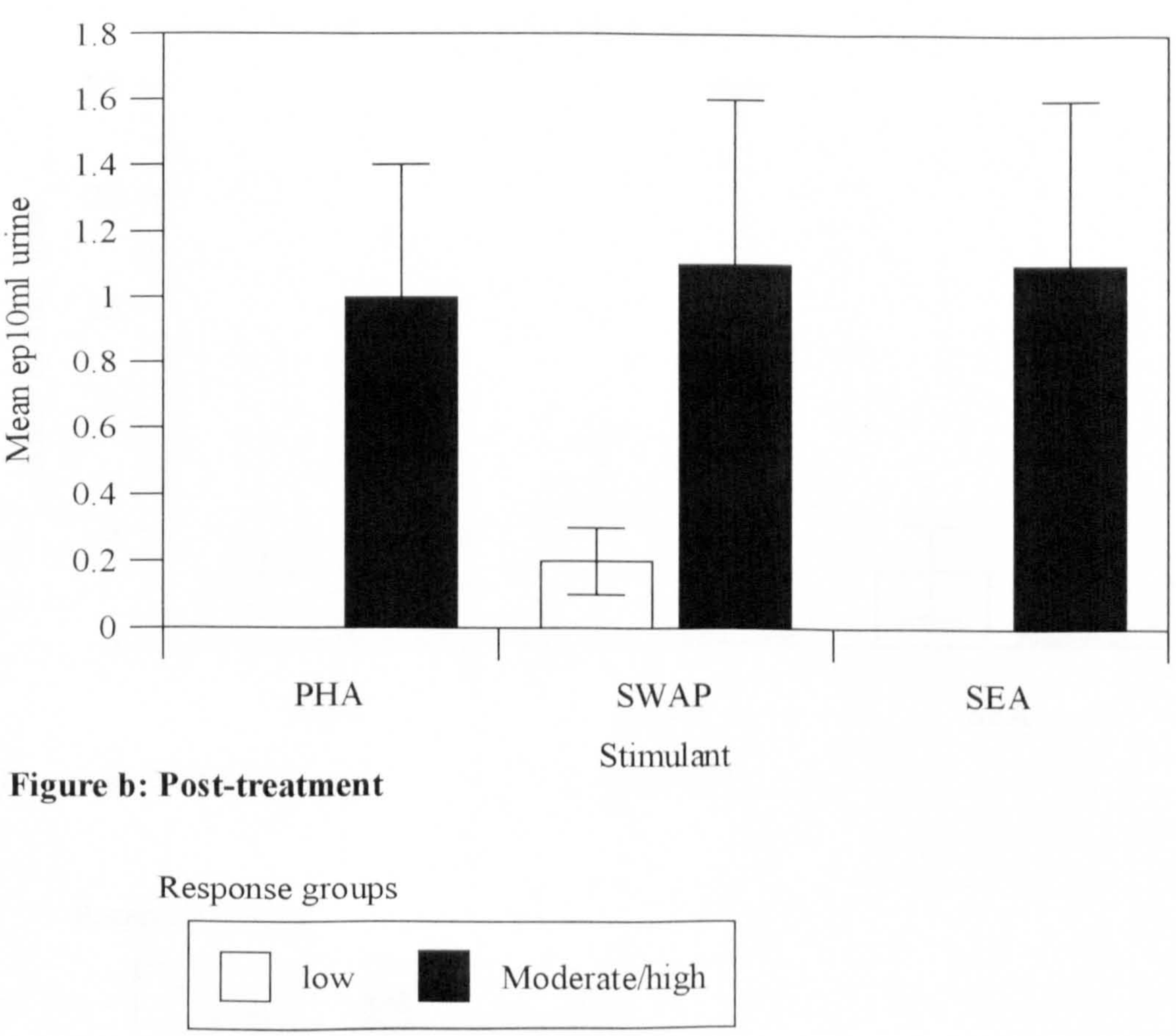


Figure b: Post-treatment

Figure 2.18: Infection intensity (ep10ml urine, ± S.E. of mean) and proliferation classes (low and moderate/high) of the PBMC from individuals from the High Transmission area, for which proliferation (detected using tritiated thymidine) and cytokine production was determined, before (a) and 12 months after (b) treatment with praziquantel, (more values are shown in Appendix Table 6.6).

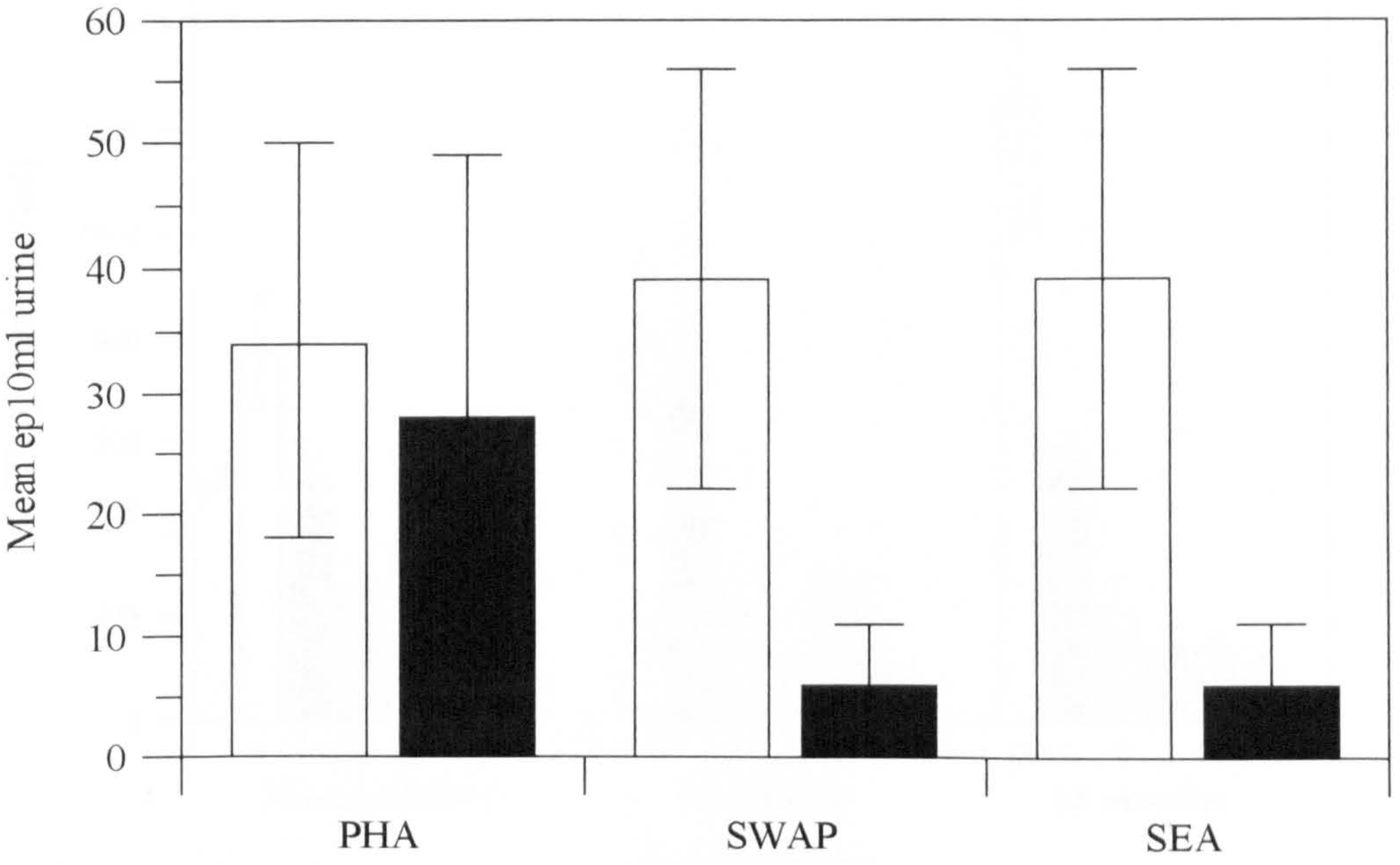


Figure a: Pre-treatment

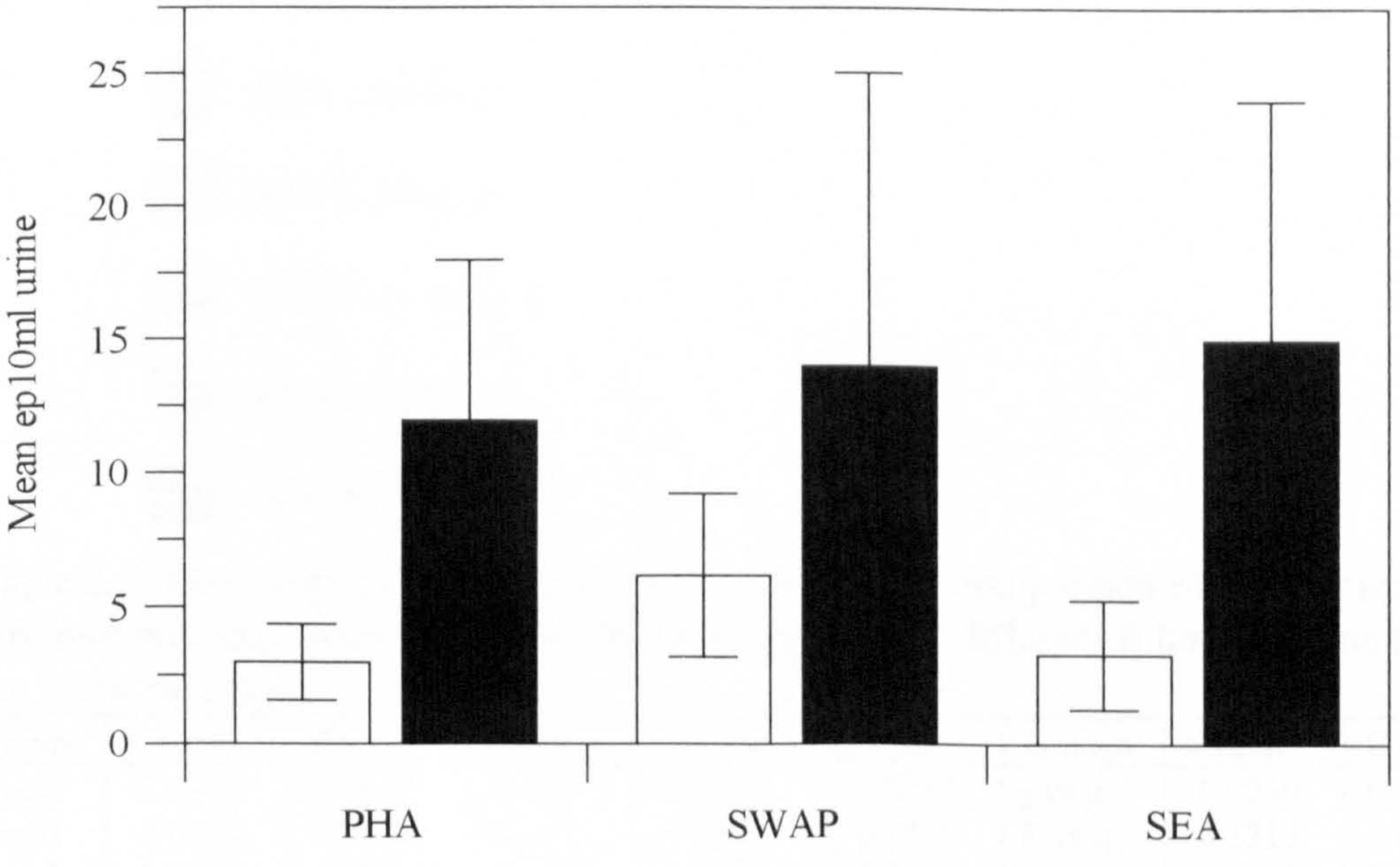


Figure b: Post-treatment

Response groups

low Moderate/high

Figure 2.19: IFN-gamma levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the Low Transmission area with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.7).

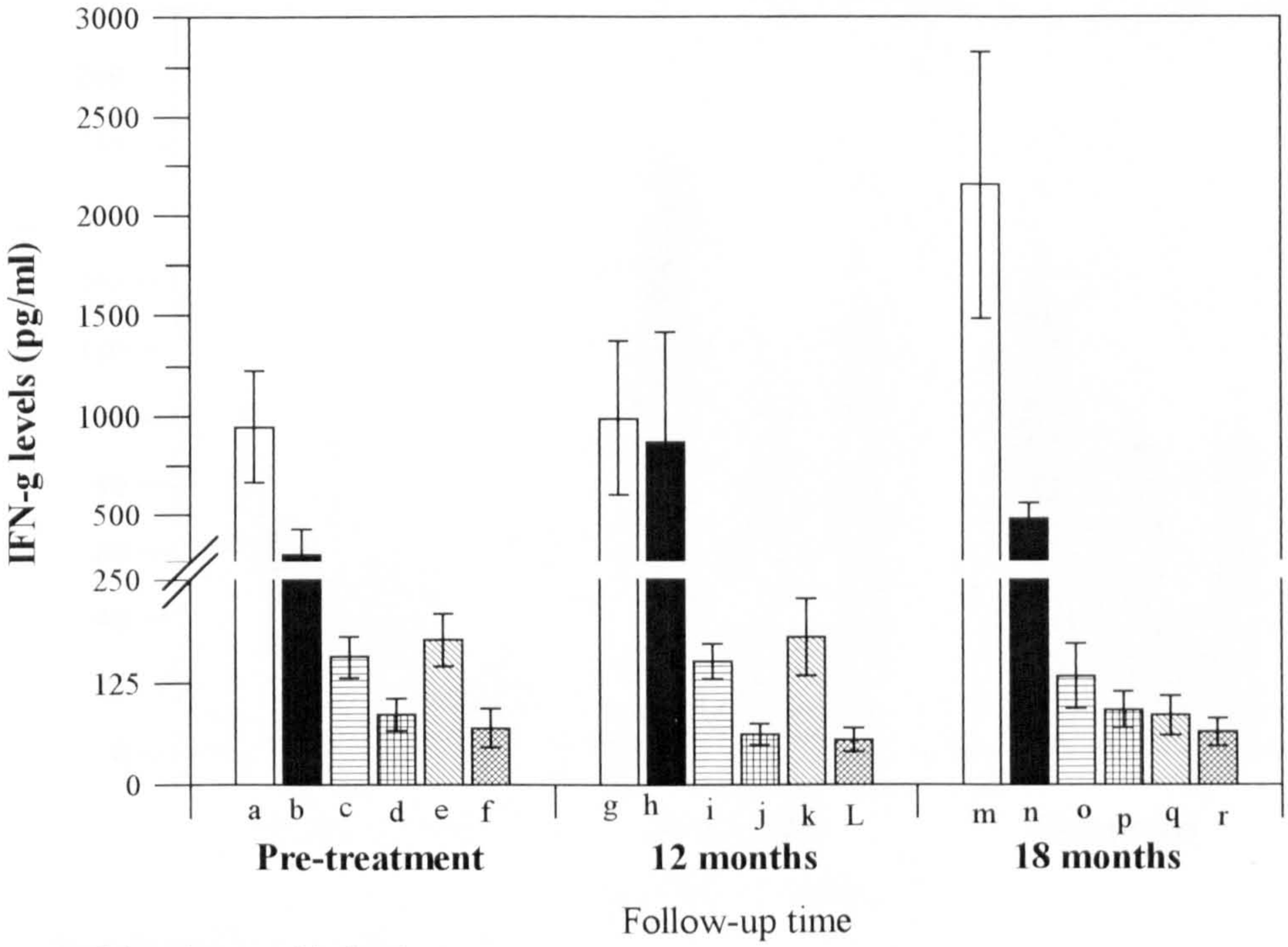


Table 2.3: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time (using the Rank sum test).

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-1.9432	0.0520	a vs g	-0.3429	0.7317	j vs p	-0.9239	0.3556
c vs d	-1.6003	0.1095	b vs h	-0.8660	0.3865	k vs q	-2.1213	0.0339
e vs f	-2.1652	0.0304	c vs i	-0.4572	0.6475	L vs r	-0.4267	0.6696
g vs h	-1.0835	0.2786	d vs j	-1.4434	0.1489	m vs a	-1.3259	0.1849
i vs j	-2.3094	0.0209	e vs k	-0.2286	0.8196	n vs b	-1.3868	0.1655
k vs L	-2.3115	0.0208	f vs L	-1.0113	0.3119	o vs c	-1.1890	0.8501
m vs n	-2.6232	**0.0087**	g vs m	-0.7071	0.4795	p vs d	-0.4623	0.6439
o vs p	-0.6172	0.5371	h vs n	-1.2081	0.2270	q vs e	-2.4568	0.0140
q vs r	-0.7726	0.4398	i vs o	-0.1784	0.8584	r vs f	-0.6483	0.5168

Figure 2.20: IL-4 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the Low Transmission area with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.8).

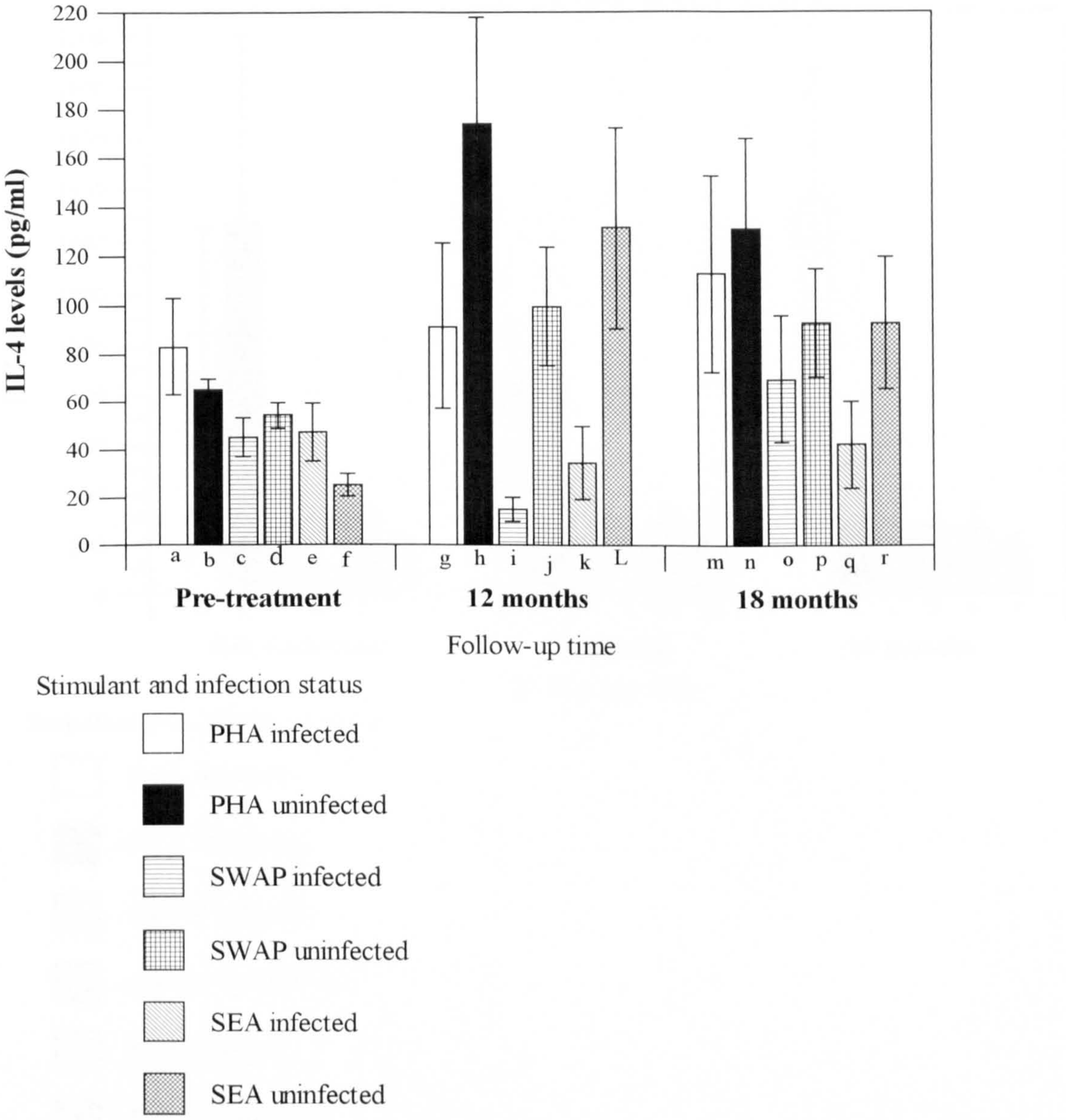


Table 2.4: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-1.0435	0.2967	a vs g	0.0000	1.0000	j vs p	0.0000	1.0000
c vs d	-0.7498	0.4534	b vs h	-1.3416	0.1797	k vs q	0.0000	1.0000
e vs f	-1.3416	0.1797	c vs i	-1.7748	0.0759	L vs r	-0.9798	0.3272
g vs h	-0.7746	0.4386	d vs j	-1.3416	0.1797	m vs a	-0.4899	0.6242
i vs j	-1.5492	0.1213	e vs k	-0.3873	0.6985	n vs b	-1.0607	0.2888
k vs L	-1.1619	0.2453	f vs L	-1.3416	0.1797	o vs c	-0.4920	0.6228
m vs n	0.0000	1.0000	g vs m	0.0000	1.0000	p vs d	-1.0607	0.2888
o vs p	-0.5774	0.5637	h vs n	-0.7348	0.4624	q vs e	-0.1230	0.9021
q vs r	-1.1547	0.2482	i vs o	-1.8516	0.0641	r vs f	-1.0607	0.2888

Figure 2.21: IL-5 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the Low Transmission area with follow-up time following treatment with praziquantel. (more values are shown in Appendix Table 6.9)

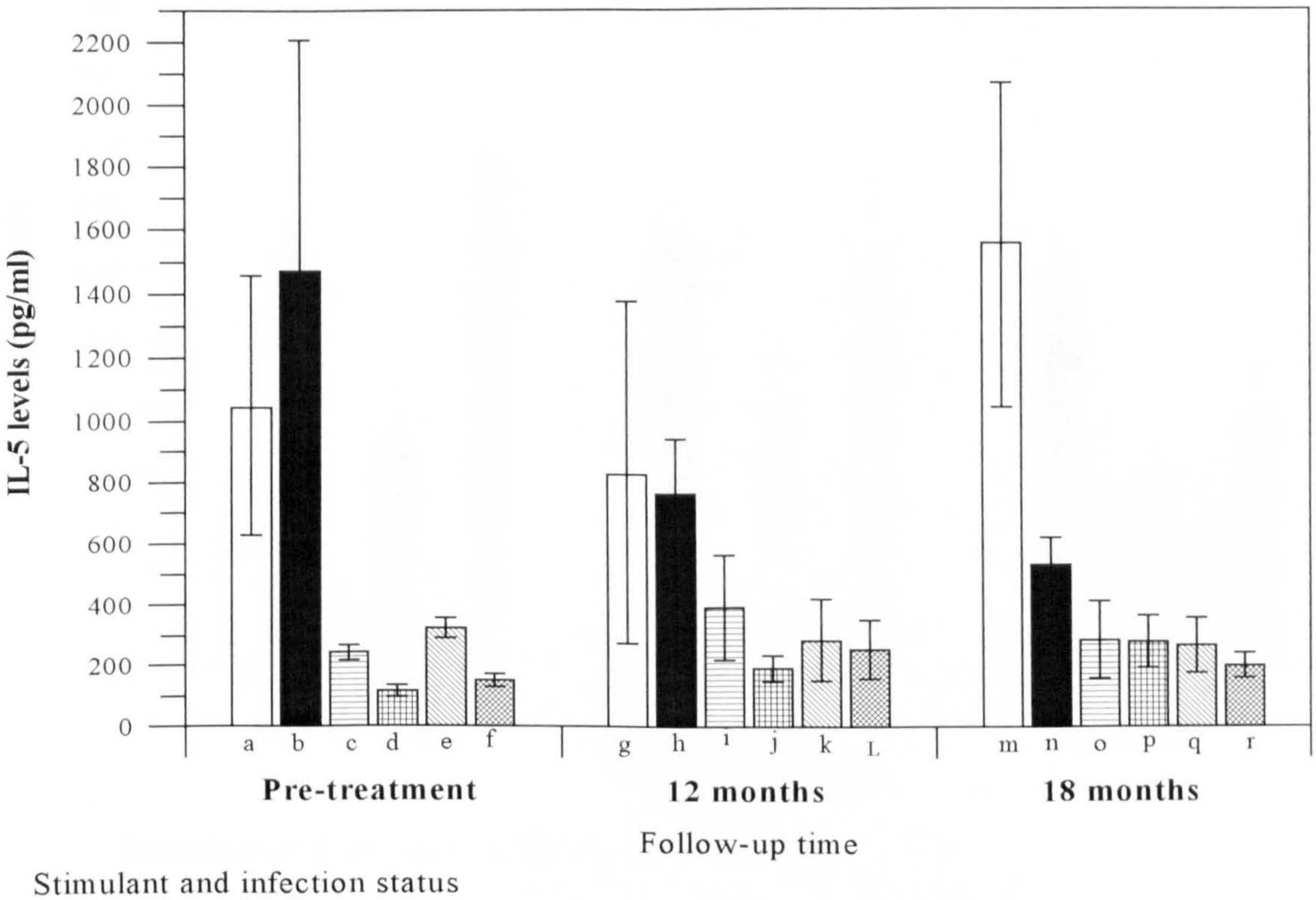


Table 2.5: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-0.2582	0.7963	a vs g	-0.5164	0.6056	j vs p	-0.9891	0.3226
c vs d	-1.5492	0.1213	b vs h	-0.7217	0.4705	k vs q	0.0000	1.0000
e vs f	-0.7746	0.4386	c vs i	-0.7746	0.4386	L vs r	-0.0330	0.9737
g vs h	-0.4330	0.6650	d vs j	-1.0104	0.3123	m vs a	-0.6396	0.5224
i vs j	-1.1547	0.2482	e vs k	-0.2582	0.7963	n vs b	-1.0142	0.3105
k vs L	-0.4330	0.6650	f vs L	-0.4330	0.6650	o vs c	-0.2132	0.8312
m vs n	-1.5556	0.1198	g vs m	-1.0607	0.2888	p vs d	-1.5213	0.1282
o vs p	-0.1414	0.8875	h vs n	-0.7913	0.4288	q vs e	-0.2132	0.8312
q vs r	-1.4142	0.1573	i vs o	-0.3536	0.7237	r vs f	-0.5924	0.5536

Figure 2.22: IL-10 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the Low Transmission area with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.10).

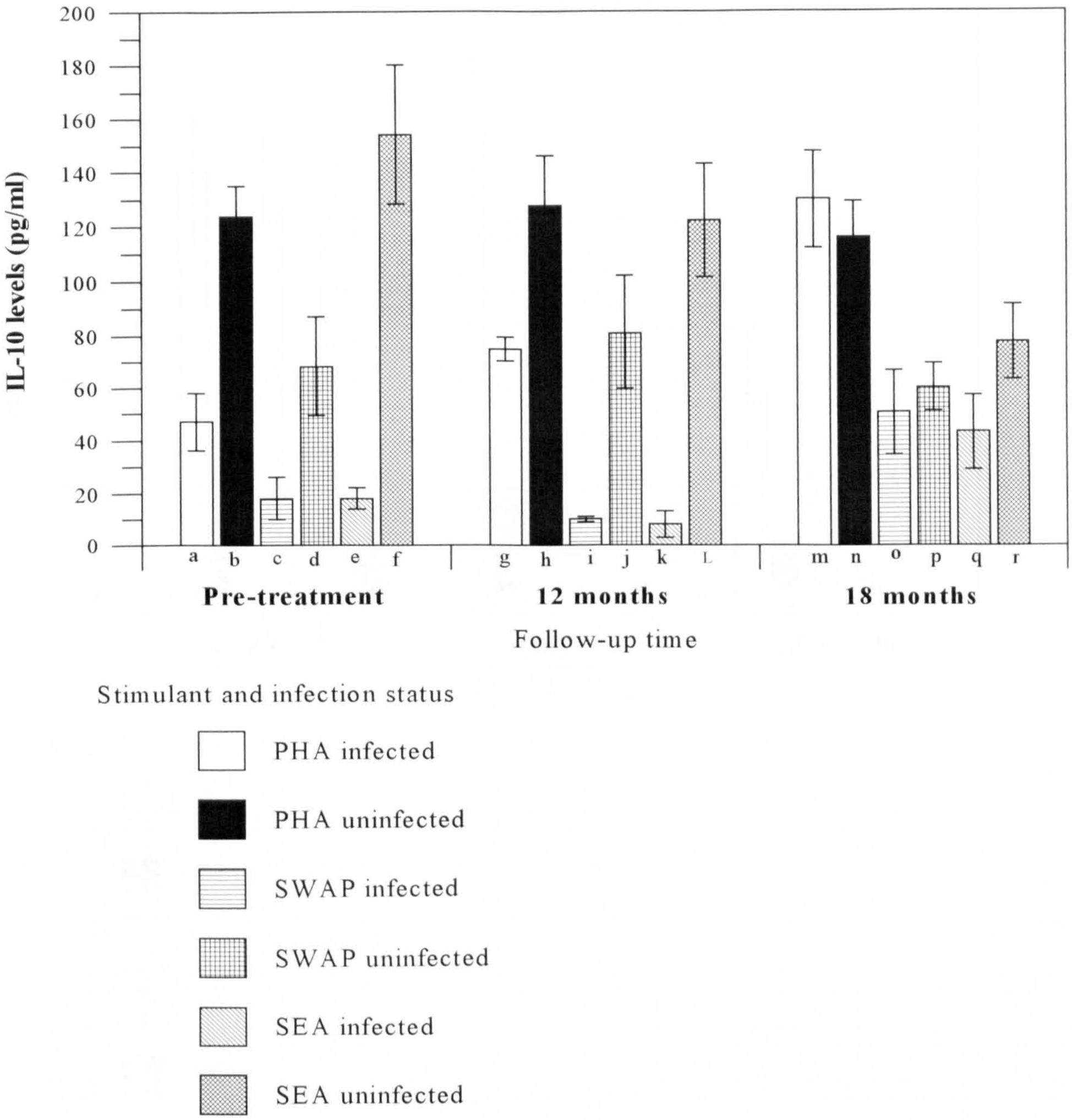


Table 2.6: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-2.2361	0.0253	a vs g	-1.1619	0.2453	j vs p	-0.6630	0.5074
c vs d	-1.9496	0.0512	b vs h	-0.4623	0.6439	k vs q	-1.8516	0.0641
e vs f	-2.2361	0.0253	c vs i	-1.2058	0.2279	L vs r	-1.6336	0.1023
g vs h	-1.1785	0.2386	d vs j	-0.4631	0.6433	m vs a	-2.4495	0.0143
i vs j	-2.1262	0.0335	e vs k	-1.9365	0.0528	n vs b	-0.2774	0.7815
k vs L	-2.1213	0.0339	f vs L	-0.8321	0.4054	o vs c	-1.9678	0.0491
m vs n	-0.3086	0.7576	g vs m	-1.8516	0.0641	p vs d	-0.4631	0.6433
o vs p	-0.8510	0.3948	h vs n	-0.6630	0.5074	q vs e	-1.2247	0.2207
q vs r	-1.2344	0.2170	i vs o	-1.8516	0.0641	r vs f	-2.1264	0.0335

Figure 2.23: IFN-gamma levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the High Transmission area with follow-up time following treatment with praziquantel, (more values are shown in Appendix Table 6.11).

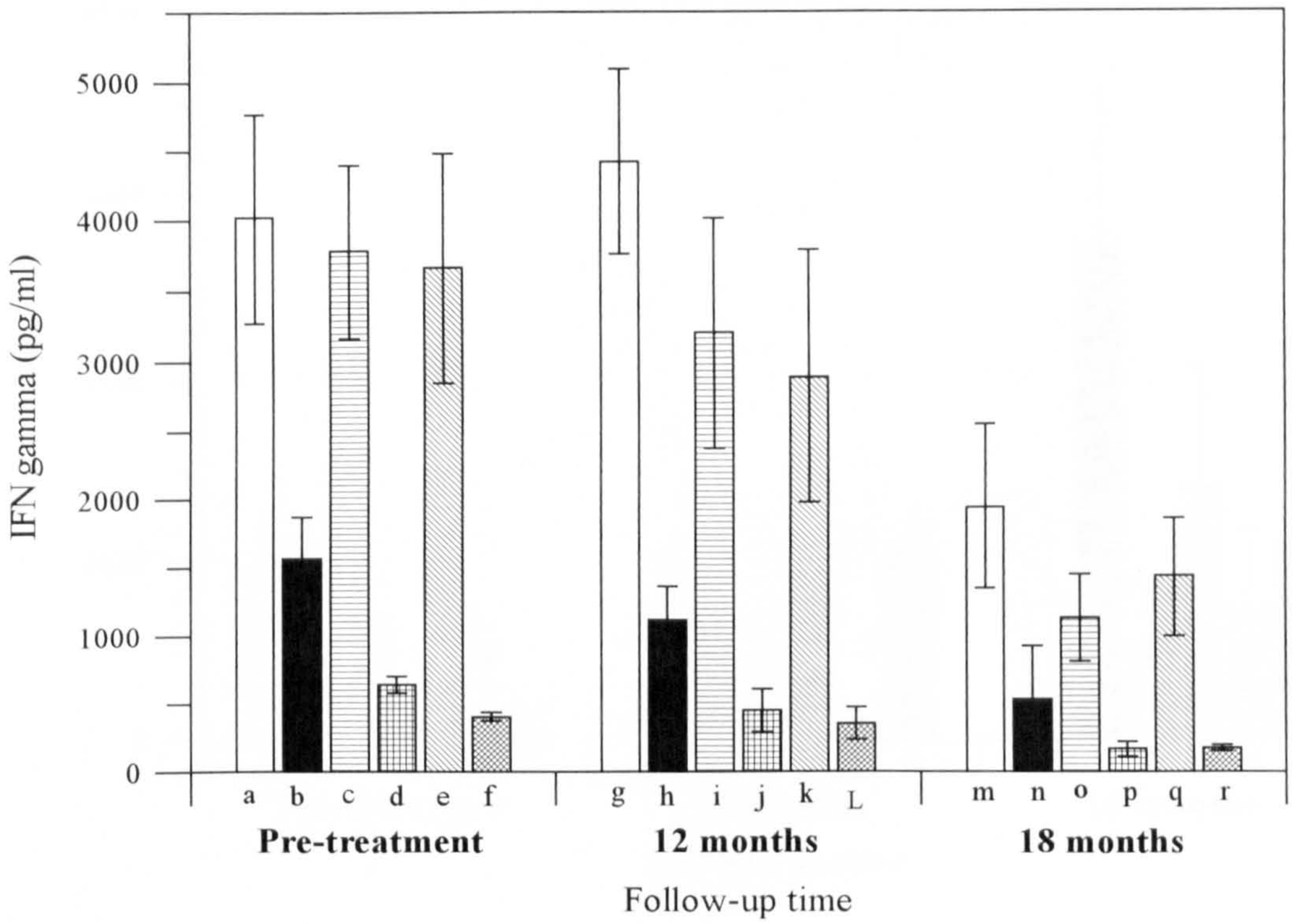
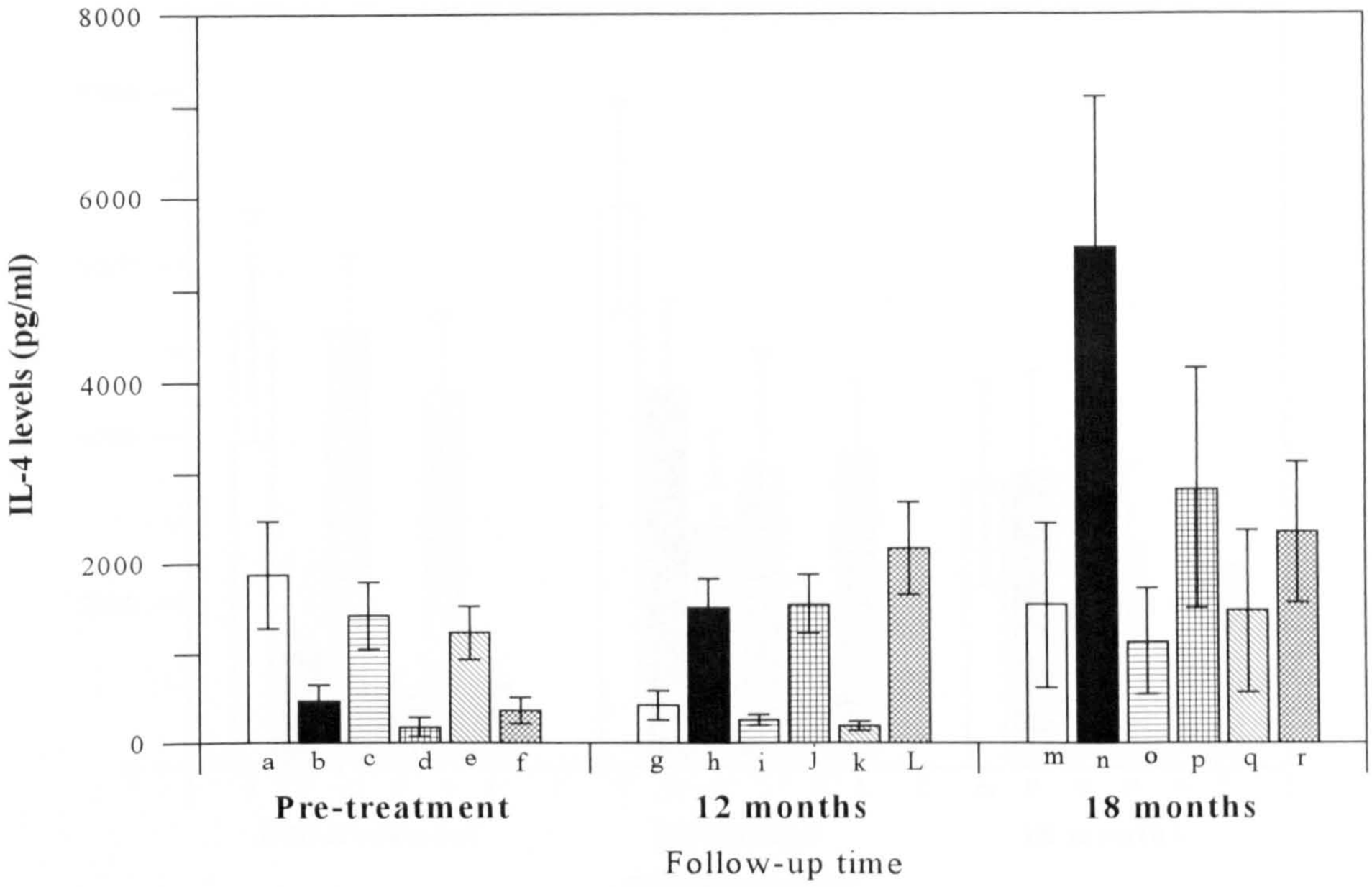


Table 2.7: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-2.0494	0.0404	a vs g	-0.2315	0.8170	j vs p	-1.6432	0.1003
c vs d	-2.9277	**0.0034**	b vs h	-1.3578	0.1745	k vs q	-1.5972	0.1102
e vs f	-2.9277	**0.0034**	c vs i	-0.6944	0.4875	L vs r	-1.0954	0.2733
g vs h	-2.6796	**0.0074**	d vs j	-0.9400	0.3475	m vs a	-1.8516	0.0641
i vs j	-2.8420	**0.0045**	e vs k	-1.2730	0.2030	n vs b	-2.3735	0.0176
k vs L	-2.8420	**0.0045**	f vs L	-0.7311	0.4647	o vs c	-2.7775	**0.0055**
m vs n	-1.2857	0.1985	g vs m	-2.1083	0.0350	p vs d	-2.7386	**0.0062**
o vs p	-2.7143	**0.0066**	h vs n	-1.6432	0.1003	q vs e	-2.1988	0.0279
q vs r	-2.4286	**0.0152**	i vs o	-1.9805	0.0476	r vs f	-2.7386	**0.0062**

Figure 2.24: IL-4 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the High Transmission area with follow-up time following treatment with praziquantel (more values are shown in Appendix Table 6.12).



Stimulant and infection status

PHA infected

PHA uninfected

SWAP infected

SWAP uninfected

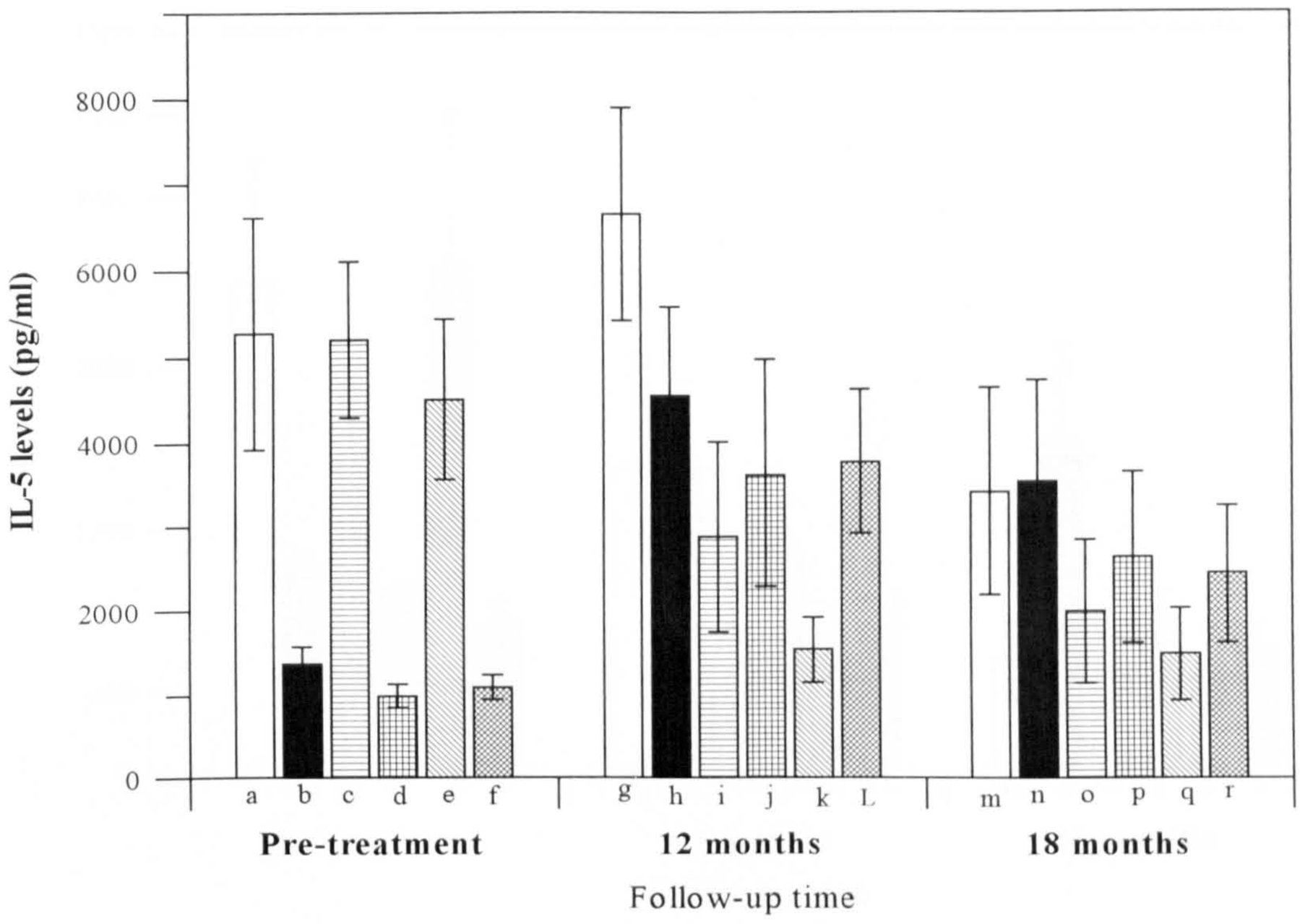
SEA infected

SEA uninfected

Table 2.8: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-2.5476	**0.0108**	a vs g	-2.7175	**0.0066**	j vs p	-1.1547	0.2482
c vs d	-2.5476	**0.0108**	b vs h	-2.1213	0.0339	k vs q	-1.4697	0.1416
e vs f	-2.7175	**0.0066**	c vs i	-2.7175	**0.0066**	L vs r	0.0000	1.0000
g vs h	-2.1213	0.0339	d vs j	-2.1213	0.0339	m vs a	-1.1711	0.2416
i vs j	-2.1213	0.0339	e vs k	-2.7175	**0.0066**	n vs b	-1.8516	0.0641
k vs L	-2.1213	0.0339	f vs L	-2.1213	0.0339	o vs c	-0.7319	0.4642
m vs n	-1.9365	0.0528	g vs m	-0.7348	0.4624	p vs d	-1.8516	0.0641
o vs p	-1.5492	0.1213	h vs n	-1.7321	0.0833	q vs e	-0.7319	0.4642
q vs r	-1.1619	0.2453	i vs o	-1.2247	0.2207	r vs f	-1.8516	0.0641

Figure 2.25: IL-5 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the High Transmission area with follow-up time, following treatment with praziquantel, (more values are shown in Appendix Table 6.13).



Stimulant and infection status

- PHA infected
- PHA uninfected
- SWAP infected
- SWAP uninfected
- SEA infected
- SEA uninfected

Table 2.9: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-2.0059	0.0449	a vs g	-1.1333	0.2571	j vs p	-0.3651	0.7150
c vs d	-2.6232	**0.0087**	b vs h	-2.1320	0.0330	k vs q	-0.7319	0.4642
e vs f	-2.6232	**0.0087**	c vs i	-1.2667	0.2053	L vs r	-1.0954	0.2733
g vs h	-1.4606	0.1441	d vs j	-2.3452	0.0190	m vs a	-1.3472	0.1779
i vs j	-0.5477	0.5839	e vs k	-2.2000	0.0278	n vs b	-1.4697	0.1416
k vs L	-2.0083	0.0446	f vs L	-2.5584	0.0105	o vs c	-2.2132	0.0269
m vs n	-0.2928	0.7697	g vs m	-1.4639	0.1432	p vs d	-1.4497	0.1416
o vs p	-0.5855	0.5582	h vs n	-0.7303	0.4652	q vs e	-2.5019	0.0124
q vs r	-1.0247	0.3055	i vs o	-1.1711	0.2416	r vs f	-1.2247	0.2207

Figure 2.26: IL-10 levels (± S.E. of mean) according to stimulant and schistosome infection status of the children from the High Transmission area with follow-up time, following treatment with praziquantel, (more values are shown in Appendix Table 6.14).

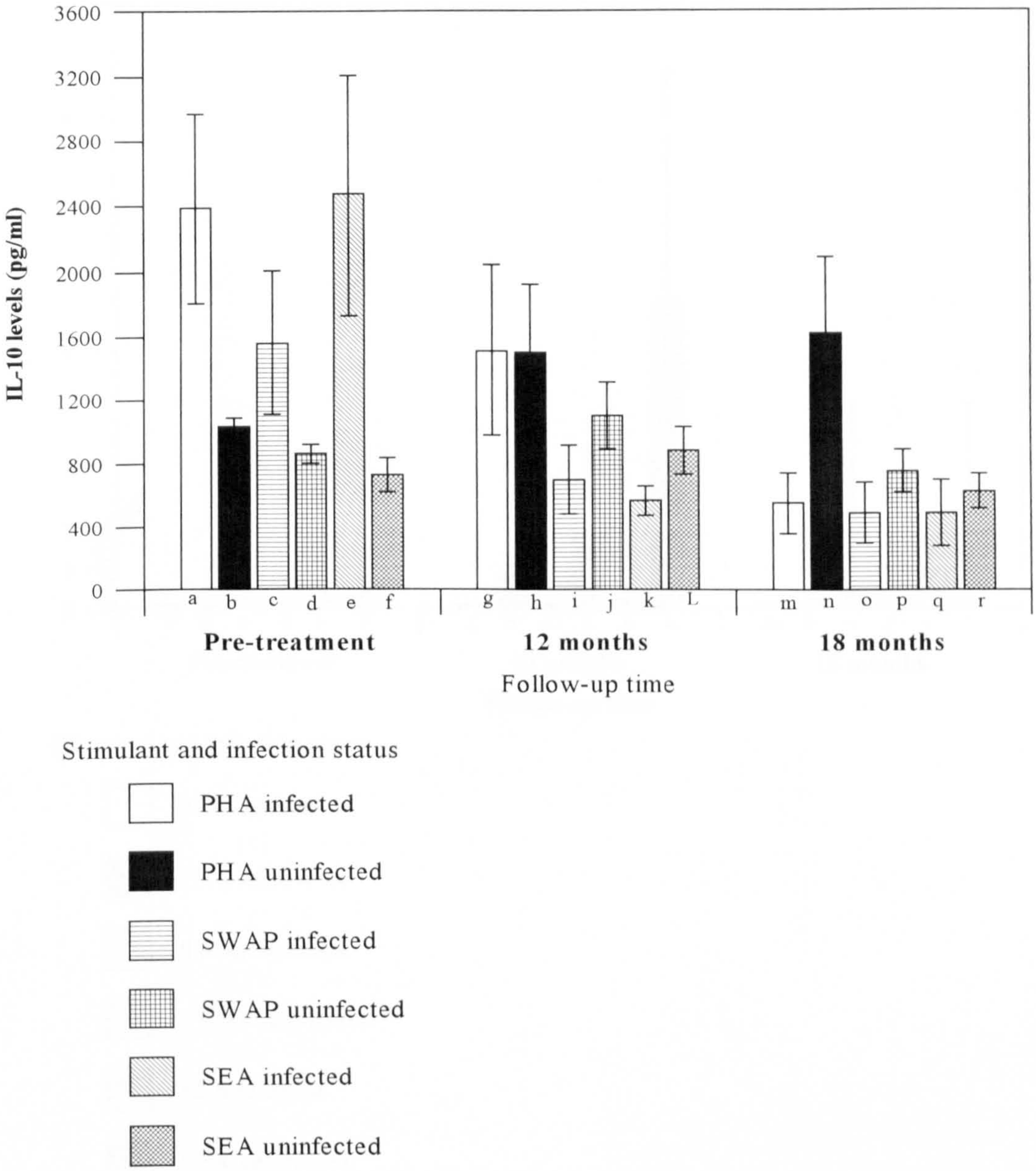


Table 2.10: Showing the significance levels between the comparison of two groups of different infection status or same infection status but at different follow-up time, using the Rank sum test.

Groups	z value	P value	Groups	z value	P value	Groups	z value	P value
a vs b	-1.0801	0.2801	a vs g	-1.0000	0.3175	j vs p	-1.6432	0.1003
c vs d	0.0000	1.0000	b vs h	-0.6396	0.5224	k vs q	-1.0261	0.3048
e vs f	-0.9258	0.3545	c vs i	-1.0000	0.3173	L vs r	-0.9129	0.3613
g vs h	-0.3651	0.7150	d vs j	-0.1069	0.9148	m vs a	-2.6943	**0.0071**
i vs j	-1.0954	0.2733	e vs k	-1.4000	0.1615	n vs b	0.0000	1.0000
k vs L	-1.1894	0.2343	f vs L	0.0000	1.0000	o vs c	-1.7813	0.0749
m vs n	-1.7566	0.0790	g vs m	-1.9030	0.0570	p vs d	-0.9798	0.3272
o vs p	-1.0247	0.3055	h vs n	-0.3651	0.7150	q vs e	-2.4071	0.0161
q vs r	-1.0261	0.3048	i vs o	-1.0247	0.3055	r vs f	-1.1069	0.2683

Figure 2.27: Comparison of IL-4:IFN-γ cytokine ratios (± S.E. of mean) in infected and uninfected children from the Low Transmission area with follow-up time, following treatment with praziquantel.

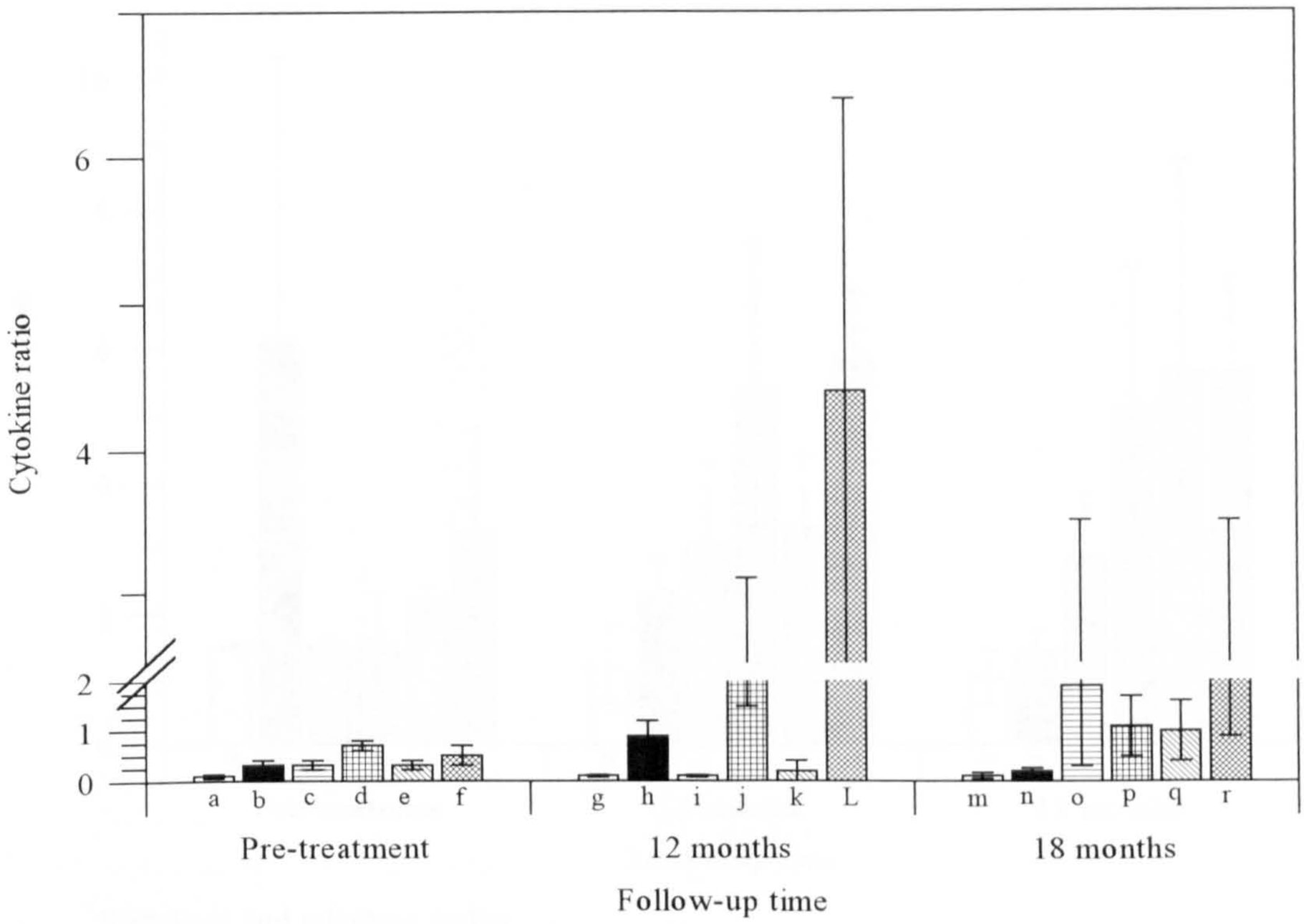


Table 2.11: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.6398	0.1011
c vs d	-1.6398	0.1011
e vs f	-0.4472	0.6547
g vs h	-1.2910	0.1967
i vs j	-0.2593	0.7954
k vs L	-0.7746	0.4386
m vs n	-2.1213	0.0339
o vs p	-2.1213	0.0339
q vs r	-2.1213	0.0339

Figure 2.28: Comparison of IL-5:IFN-γ cytokine ratios (± S.E. of mean) on infected and uninfected children from the Low Transmission area with follow-up time, following treatment with praziquantel.

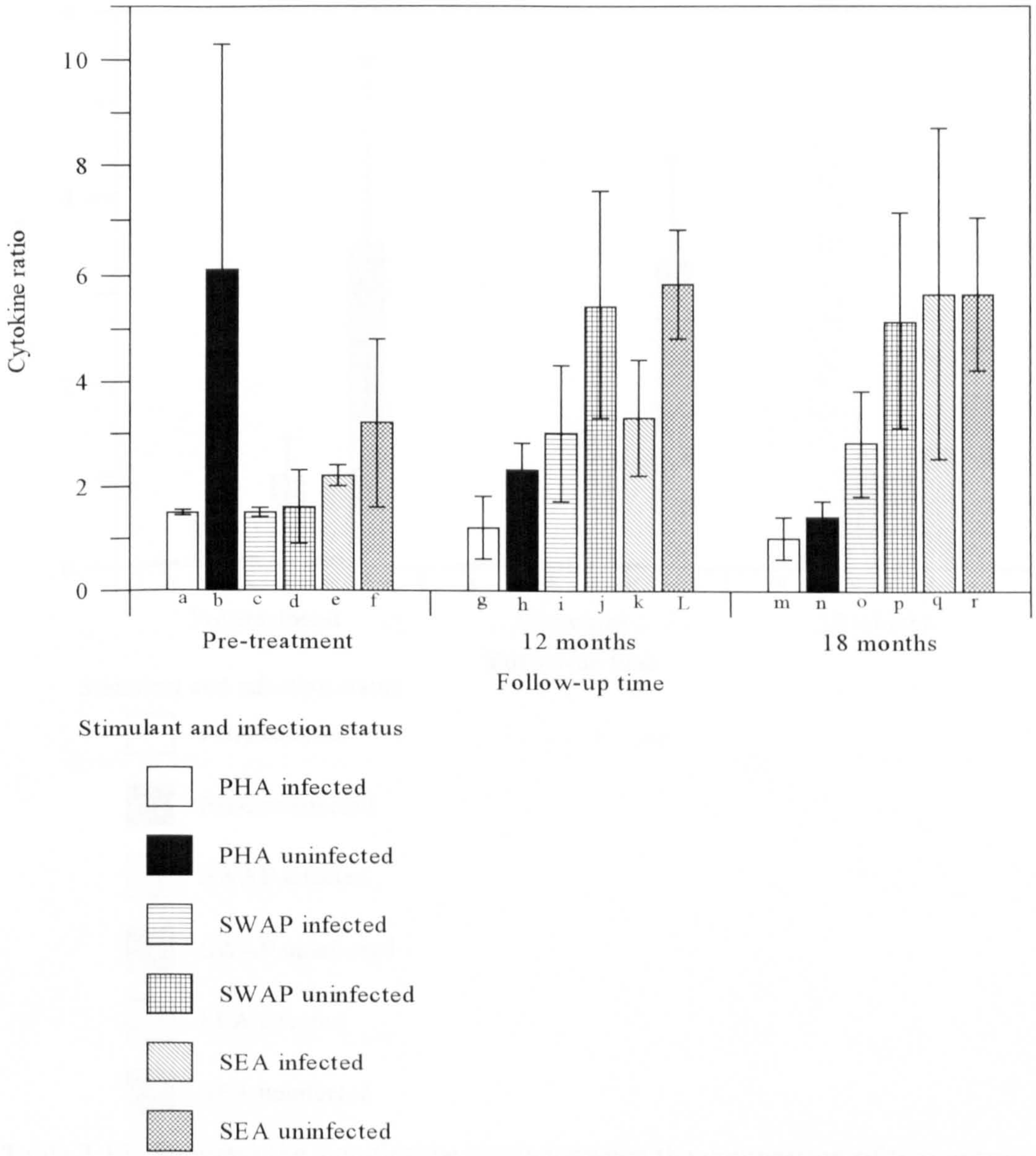


Table 2.12: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.9365	0.0528
c vs d	-1.9365	0.0528
e vs f	-1.5492	0.1213
g vs h	-0.7006	0.4835
i vs j	-0.8563	0.3918
k vs L	-1.9462	0.0516
m vs n	-1.5667	0.1172
o vs p	-2.0889	0.0367
q vs r	-2.0889	0.0367

Figure 2.29: Comparison of IL-10:IFN-γ cytokine ratios (± S.E. of mean) on infected and uninfected children from the Low Transmission area with follow-up time, following treatment with praziquantel.

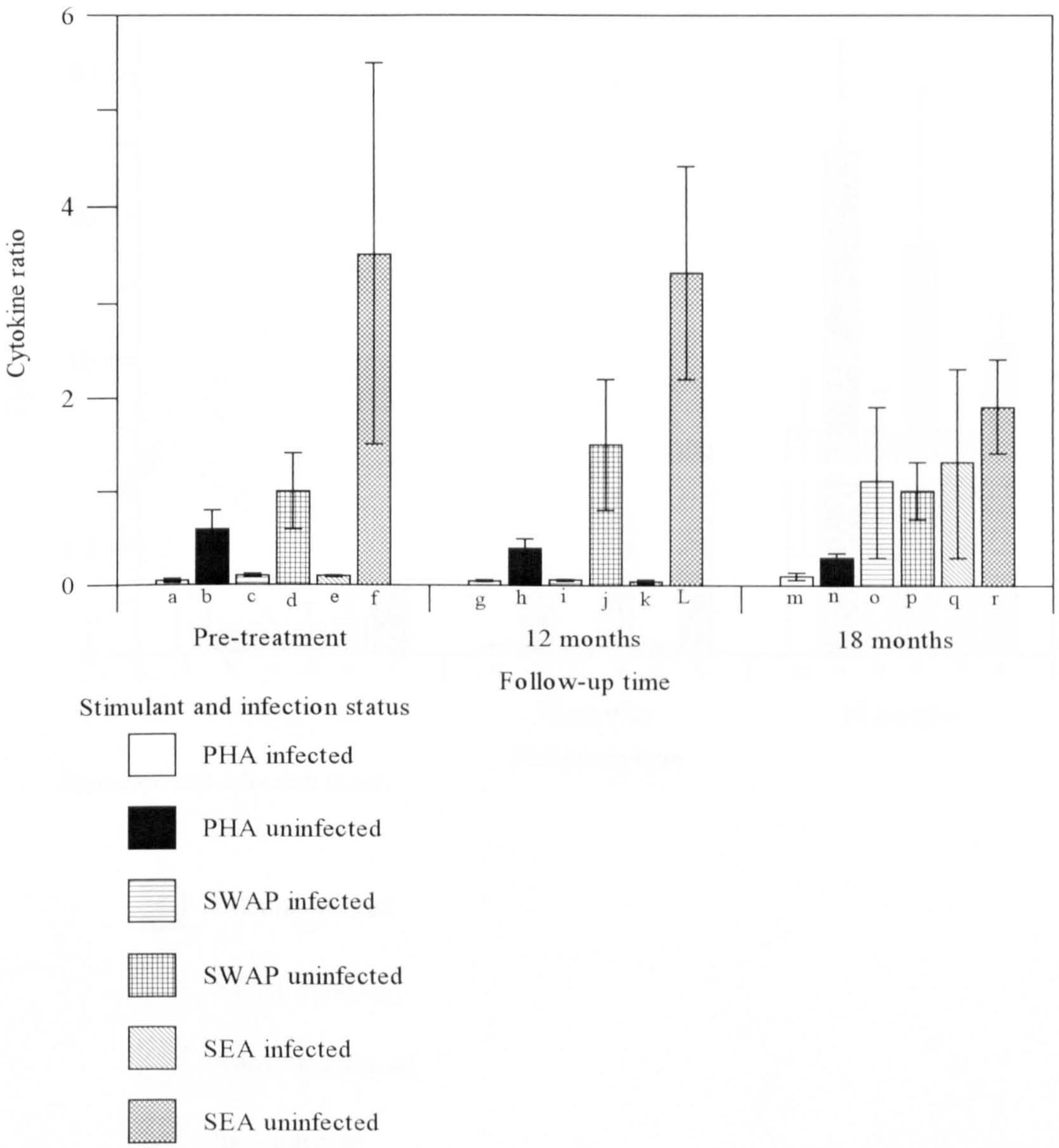


Table 2.13: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.1547	0.2482
c vs d	-0.2887	0.7728
e vs f	-0.2887	0.7728
g vs h	-1.3887	0.1649
i vs j	-0.1543	0.8774
k vs L	-0.3086	0.7576
m vs n	-2.3146	0.0206
o vs p	-1.0801	0.2801
q vs r	-1.3887	0.1649

Figure 2.30: Comparison of IL-4:IFN-γ cytokine ratios (± S.E. of mean) on infected and uninfected children from the High Transmission area with follow-up time, following treatment with praziquantel.

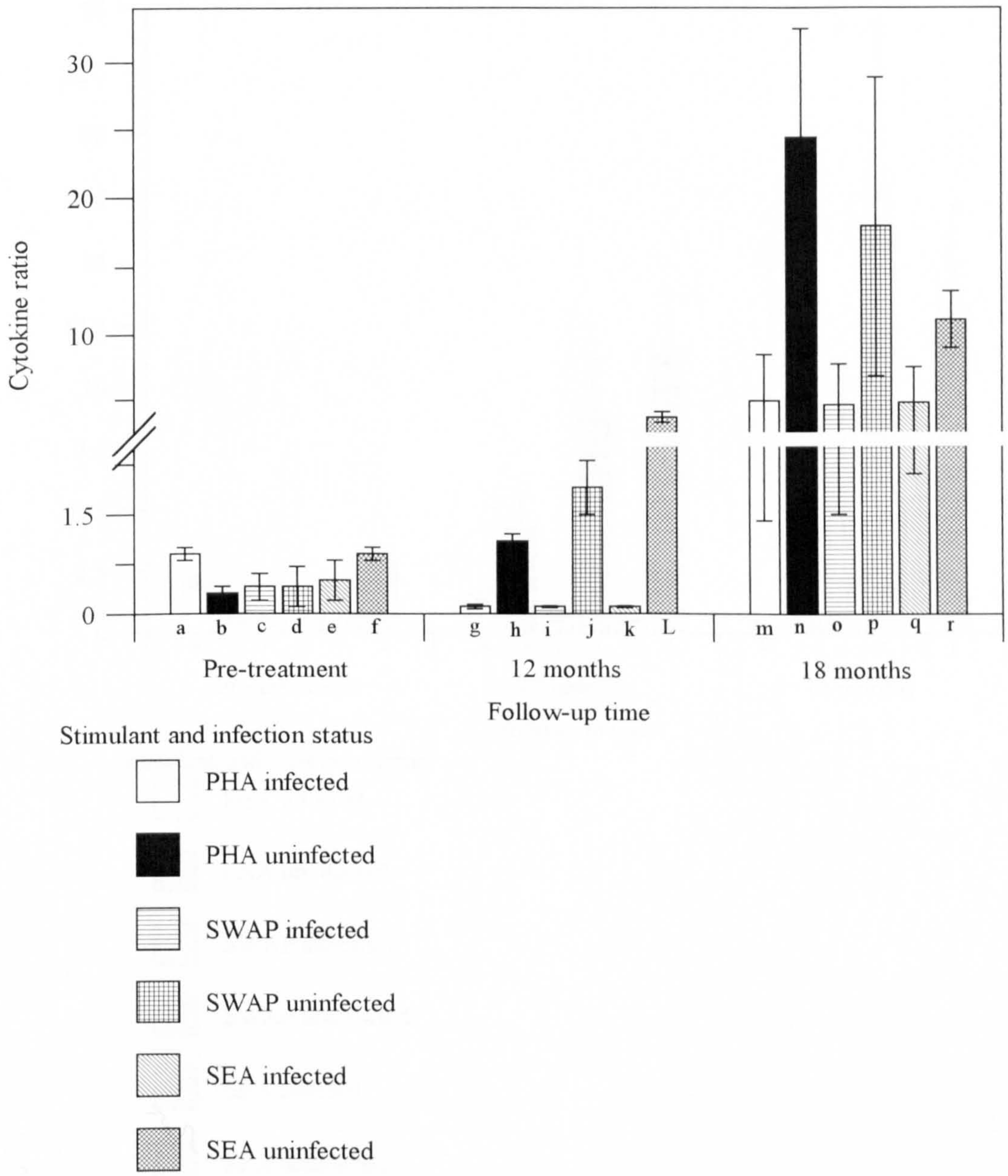


Table 2.14: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.0660	0.2864
c vs d	-0.2132	0.8312
e vs f	-1.0660	0.2864
g vs h	-0.1890	0.8501
i vs j	-1.1339	0.2568
k vs L	-2.4568	**0.0140**
m vs n	-0.5669	0.5708
o vs p	-2.6458	**0.0082**
q vs r	-2.6458	**0.0082**

Figure 2.31: Comparison of IL-5:IFN-γ cytokine ratios (± S.E. of mean) on infected and uninfected children from the High Transmission area with follow-up time, following treatment with praziquantel.

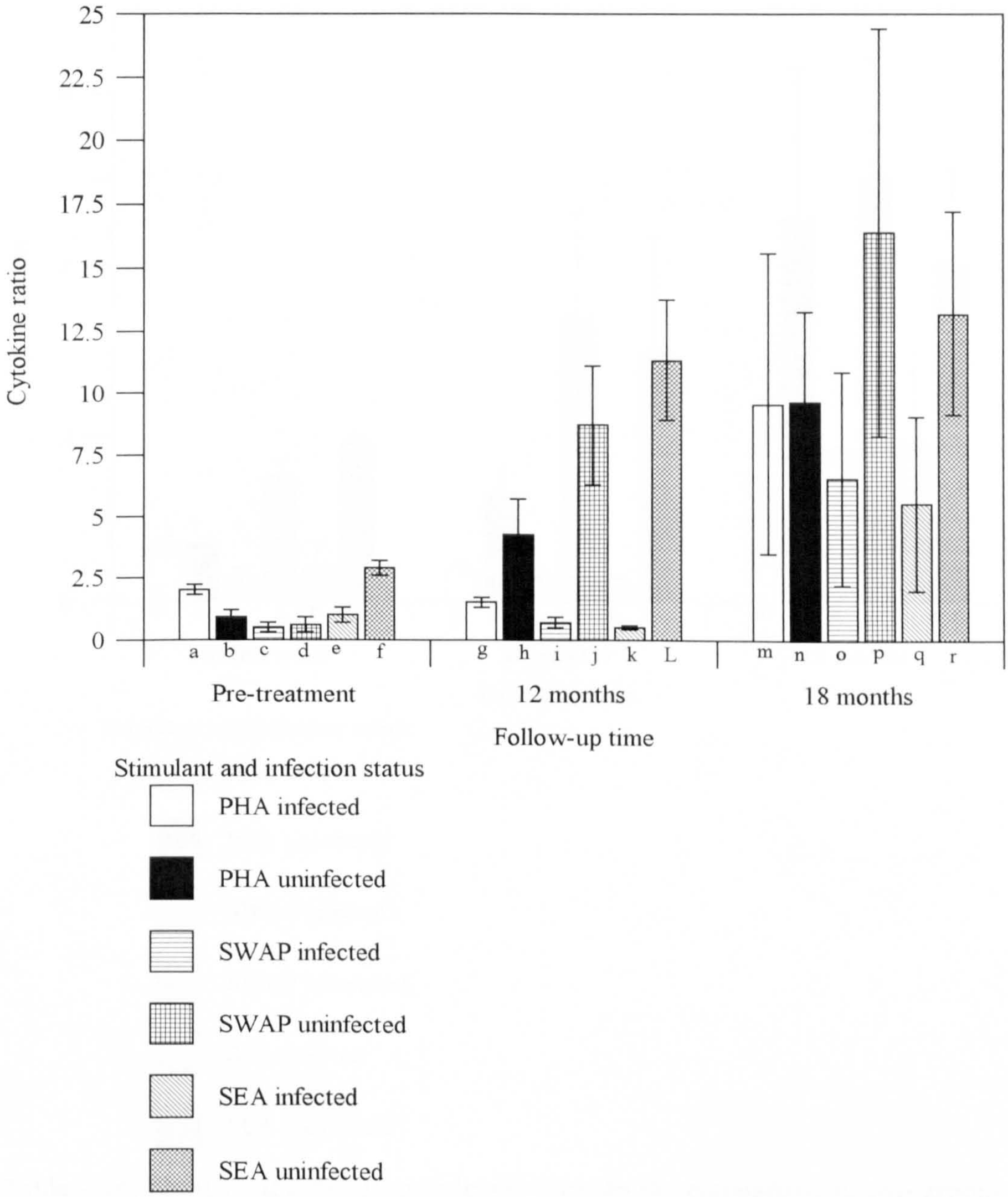


Table 2.15: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.5492	0.1213
c vs d	-1.1619	0.2453
e vs f	-0.7746	0.4386
g vs h	-0.8932	0.3718
i vs j	-1.5428	0.1229
k vs L	-1.5428	0.1229
m vs n	-1.7052	0.0882
o vs p	-1.8676	0.0618
q vs r	-1.5428	0.1229

Figure 2.32: Comparison of IL-10:IFN-γ cytokine ratios (± S.E. of mean) on infected and uninfected children from the High Transmission area with follow-up time, following treatment with praziquantel.

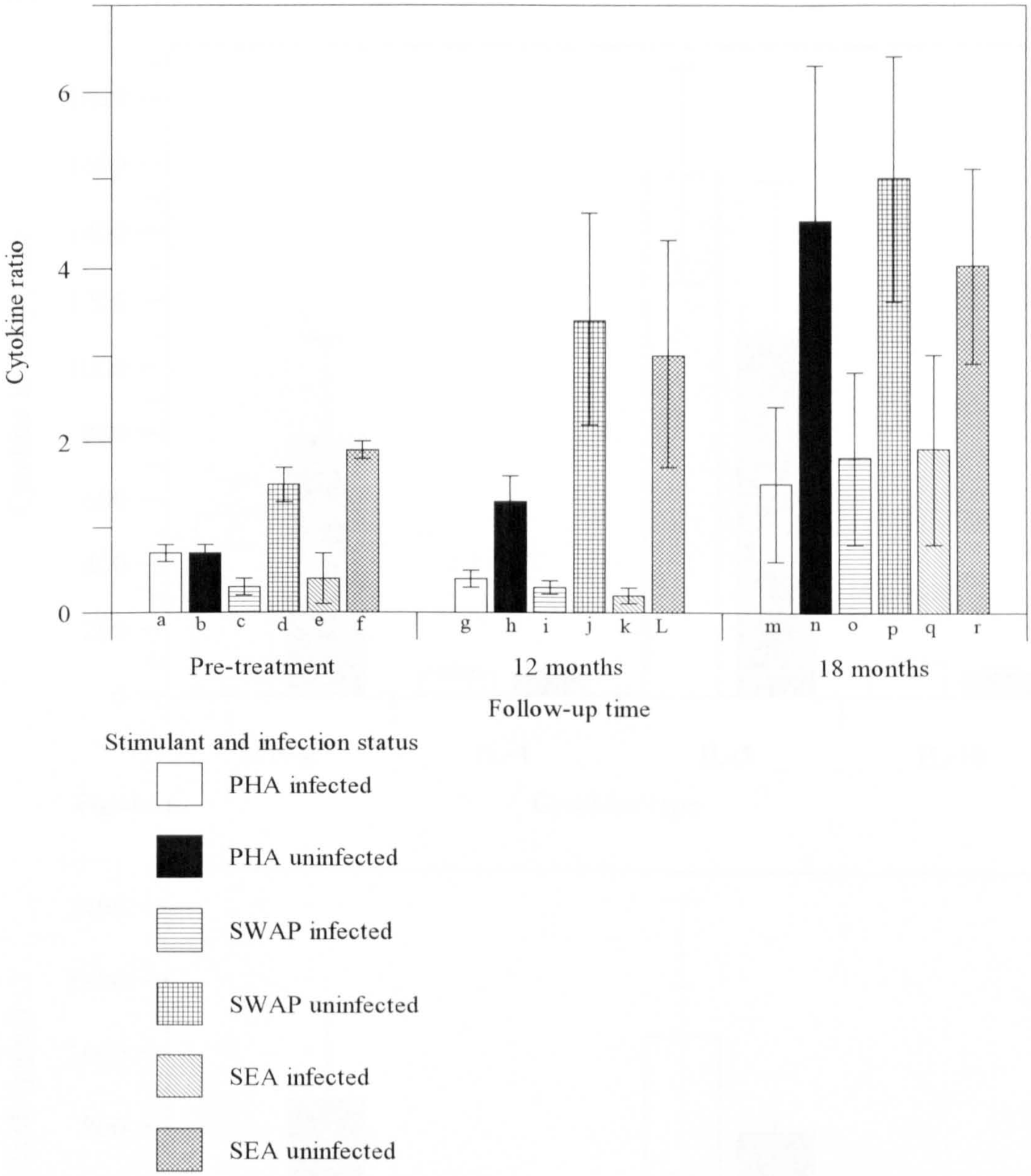


Table 2.16: Showing the significance levels between the comparison of two groups of different infection status at each time point, using the Rank sum test.

Groups	z value	P value
a vs b	-1.8516	0.0641
c vs d	-1.8516	0.0641
e vs f	-1.8516	0.0641
g vs h	-2.6112	**0.0090**
i vs j	-2.6112	**0.0090**
k vs L	-2.6112	**0.0090**
m vs n	-1.9845	0.0472
o vs p	-2.6112	**0.0090**
q vs r	-2.6112	**0.0090**

Figure 2.33: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the Low Transmission area, to PHA stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.15 and 6.16).

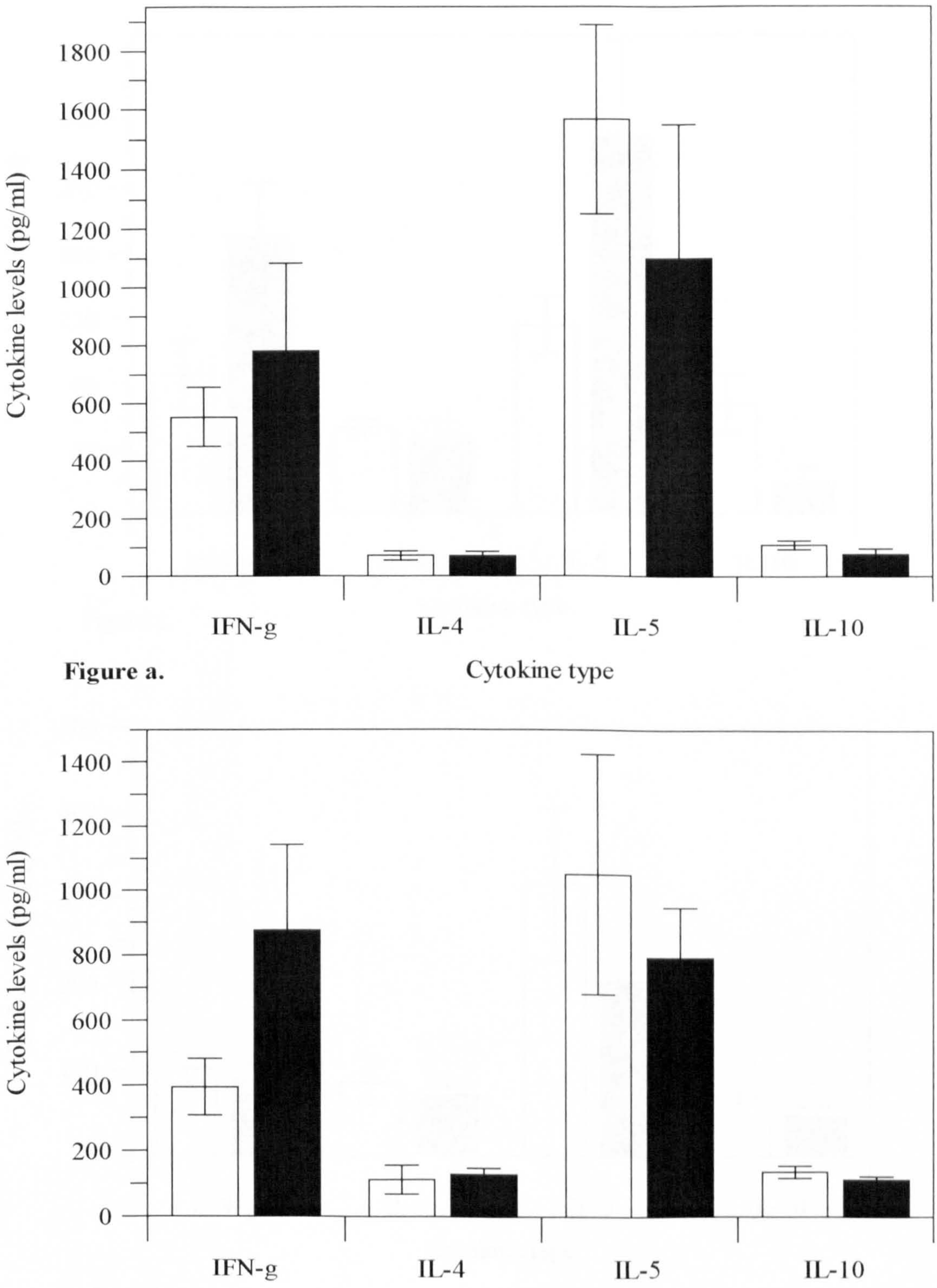


Figure a.

Figure b.

low Moderate/high

Figure 2.34: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the Low Transmission area, to SWAP stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.15 and 6.16).

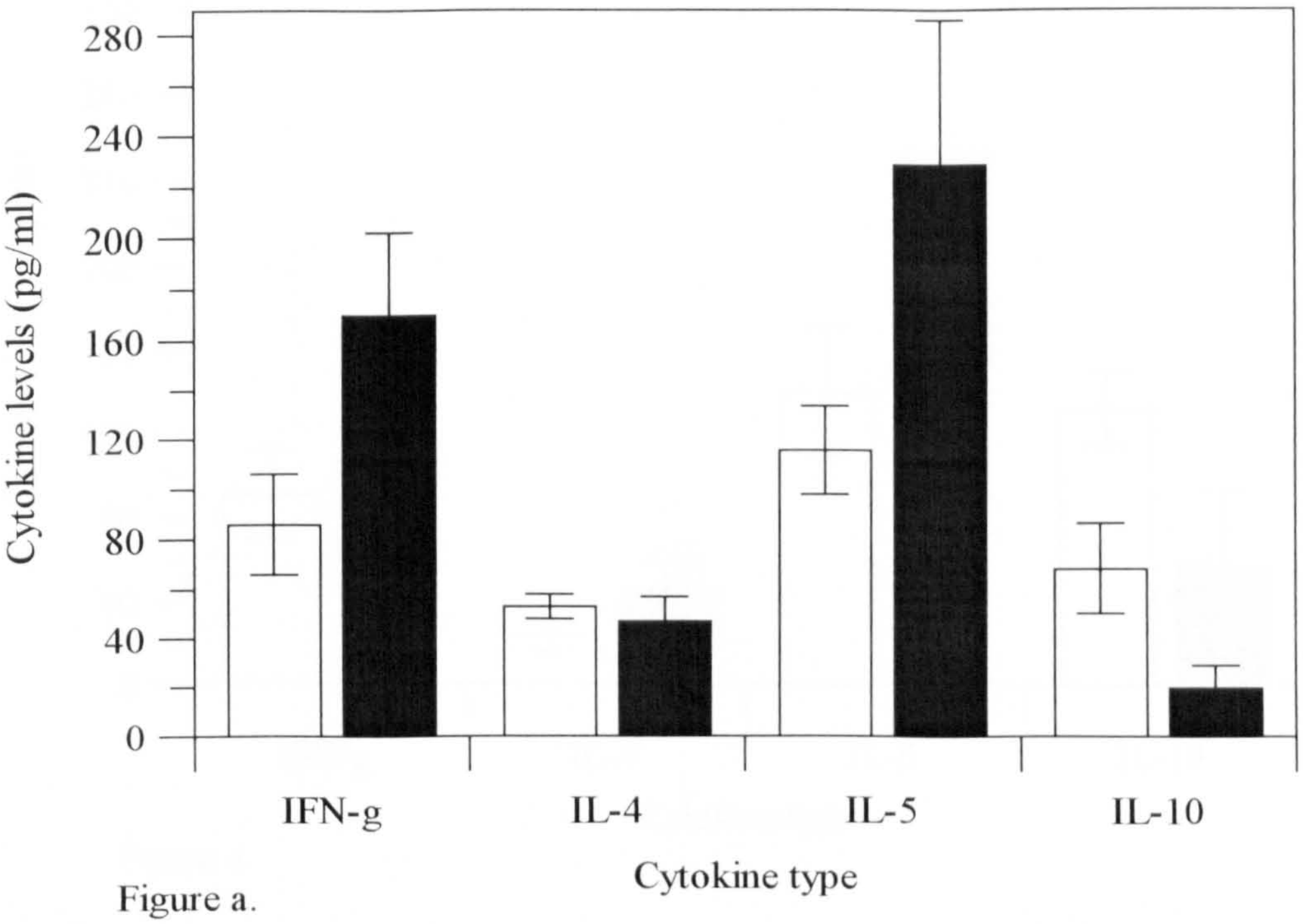


Figure a.

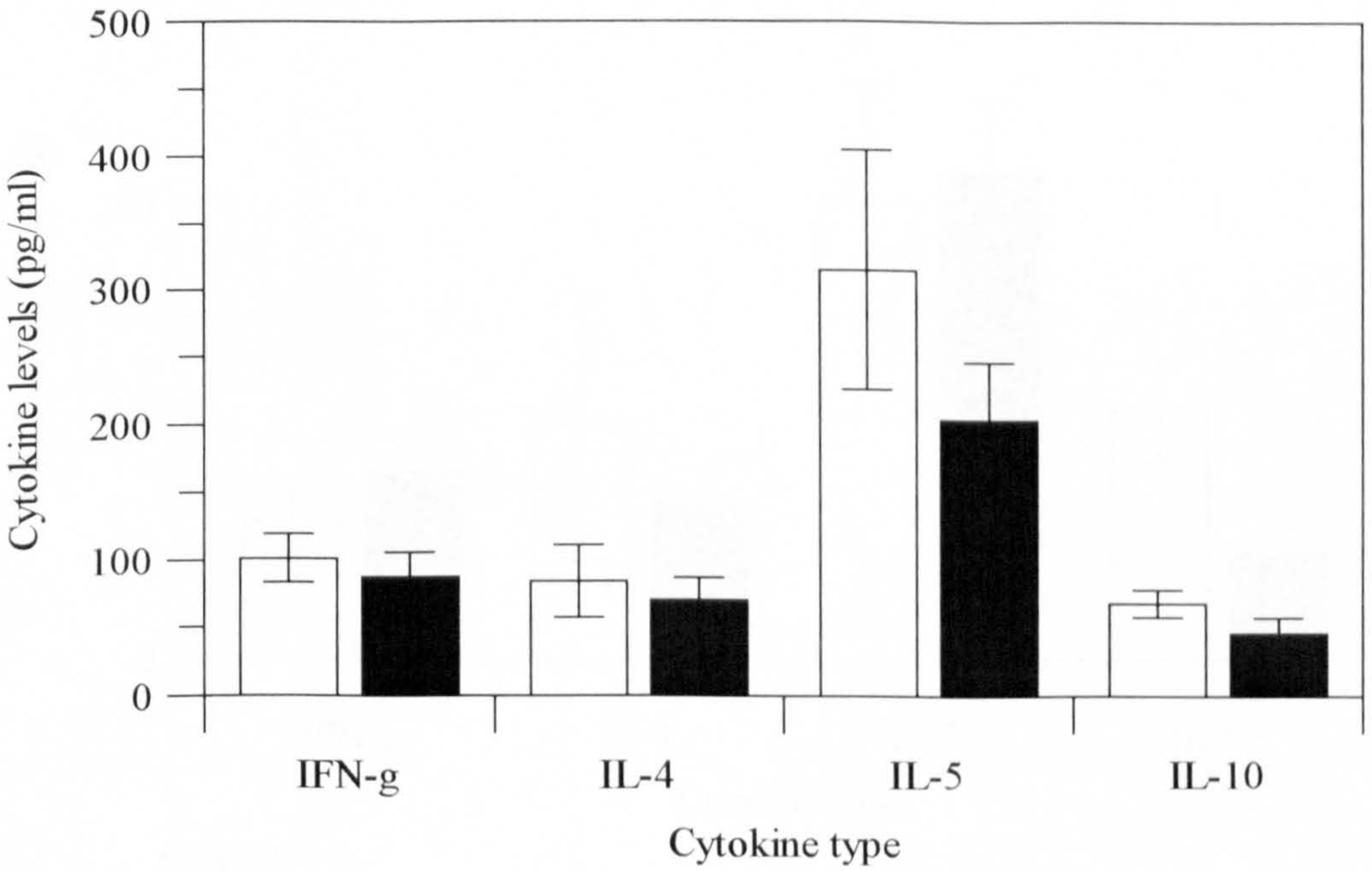


Figure b.

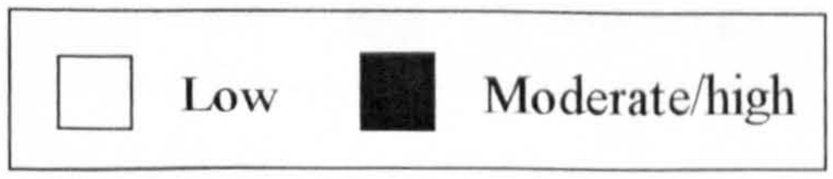


Figure 2.35: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the Low Transmission area, to SEA stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.15 and 6.16).

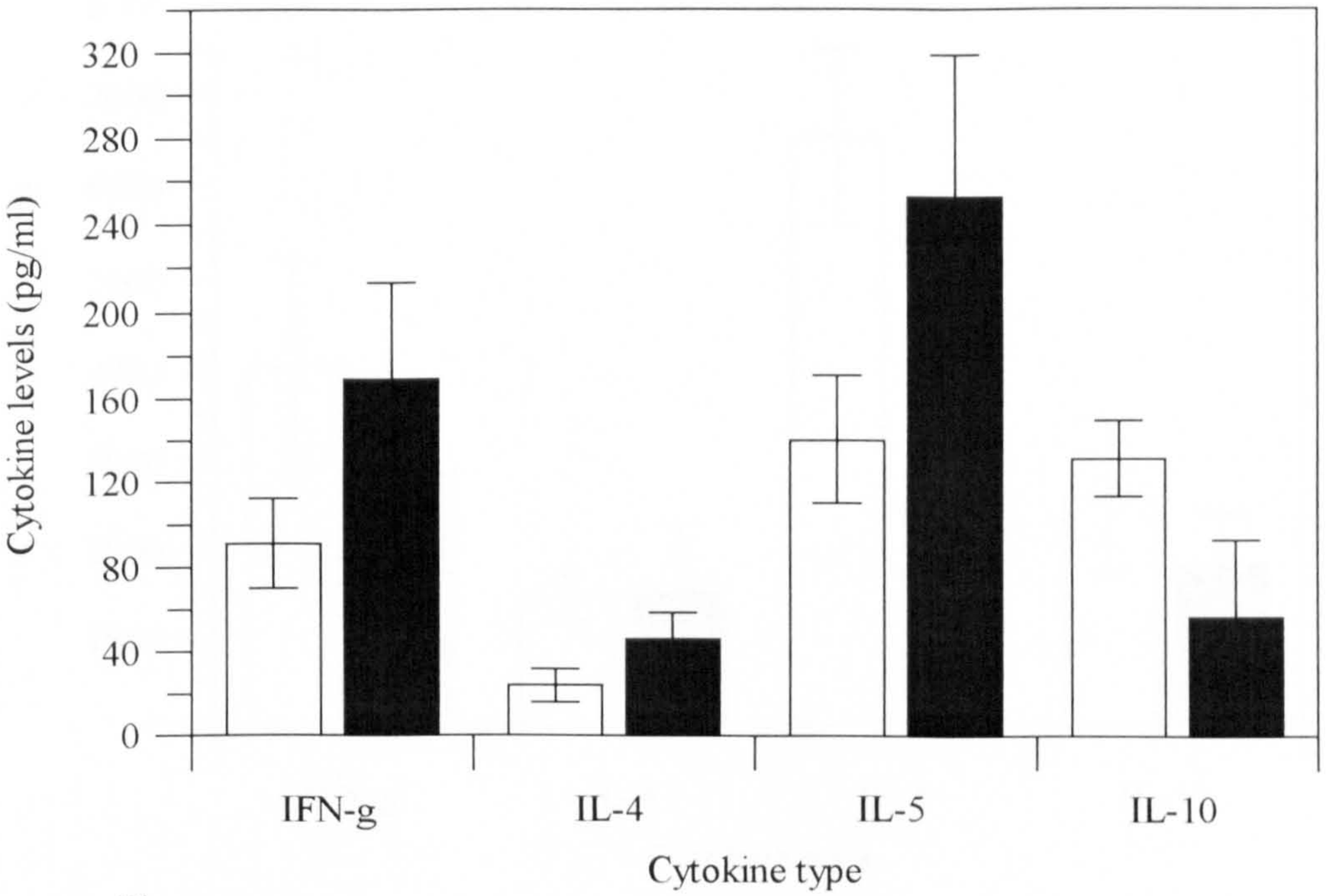


Figure a.

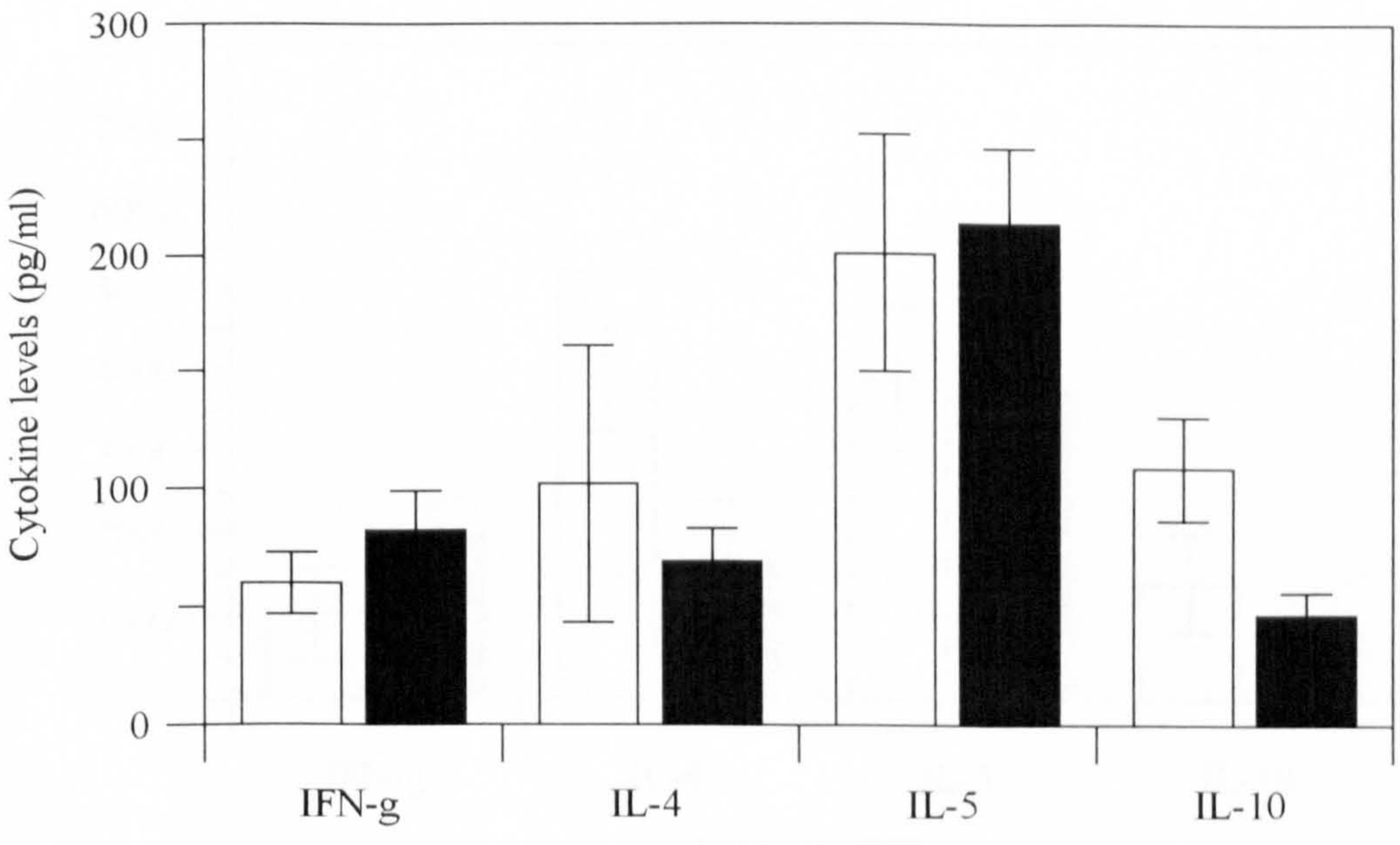


Figure b.

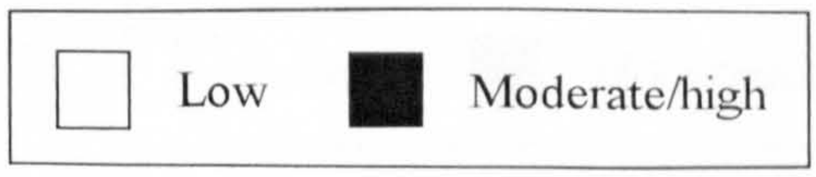
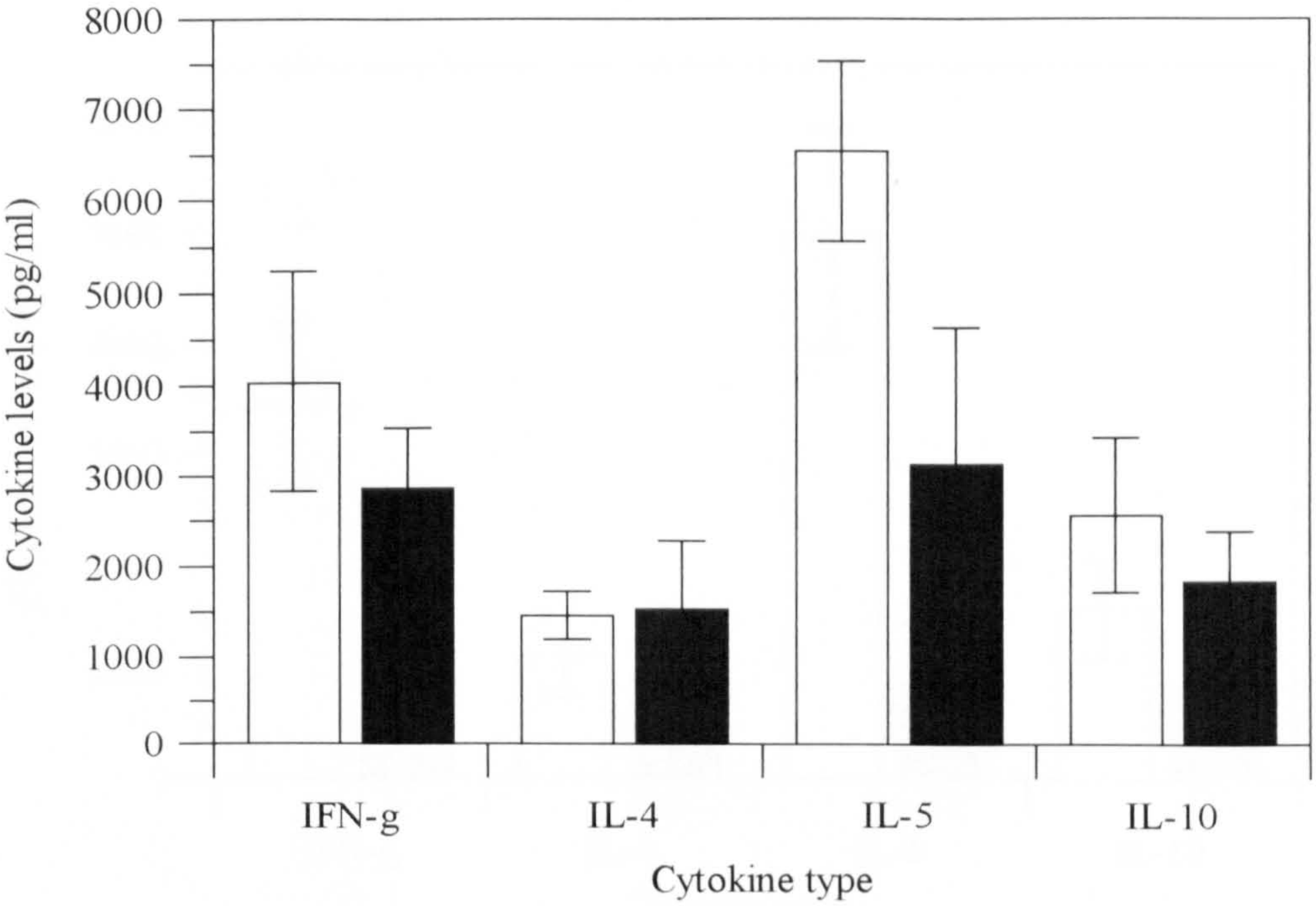
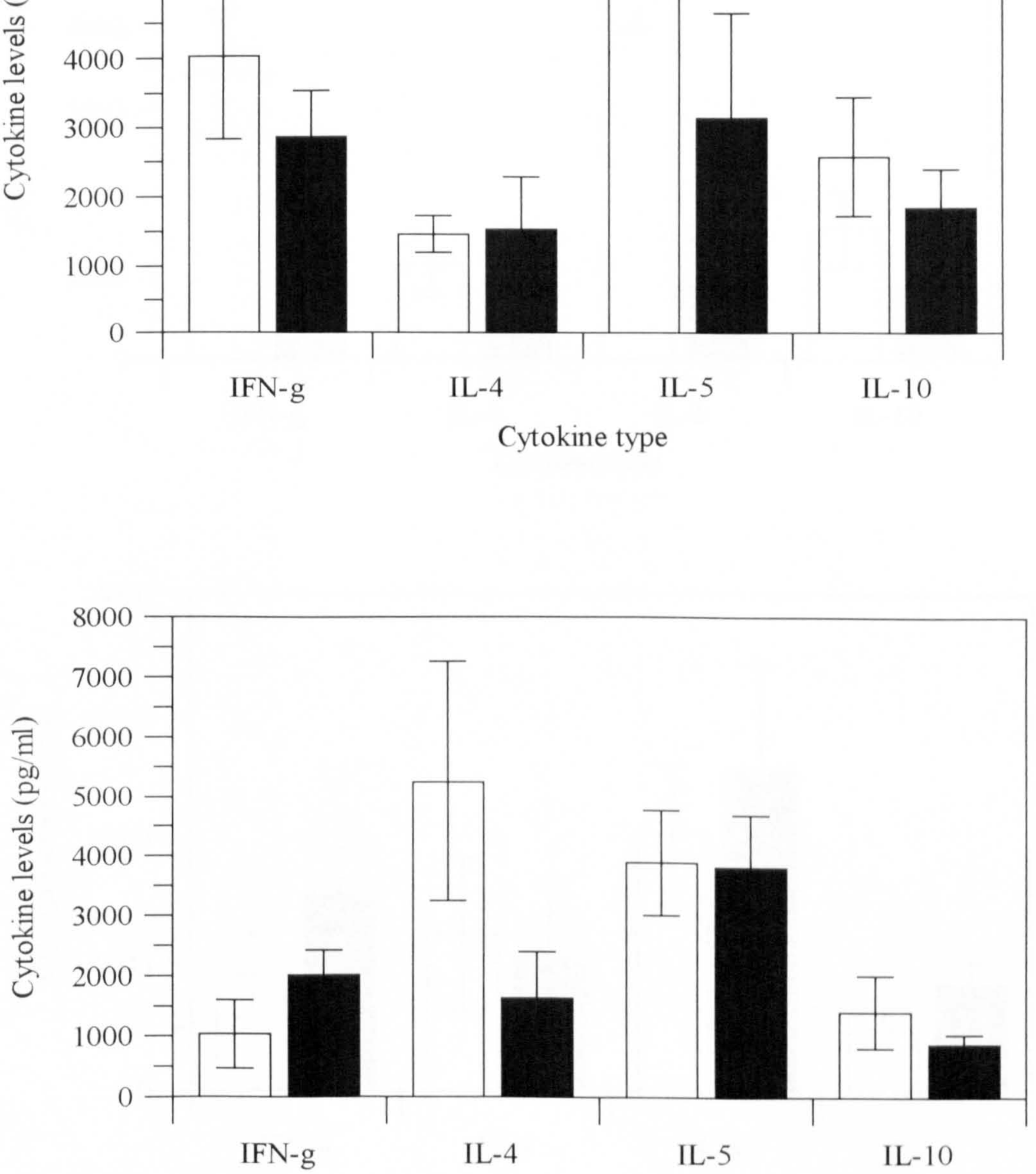


Figure 2.36: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the High Transmission area, to PHA stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.19 and 6.20).





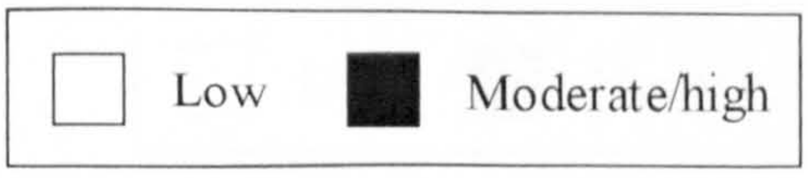


Figure 2.37: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the High Transmission area, to SWAP stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel.(more values are shown in Appendix Table 6.19 and 6.20).

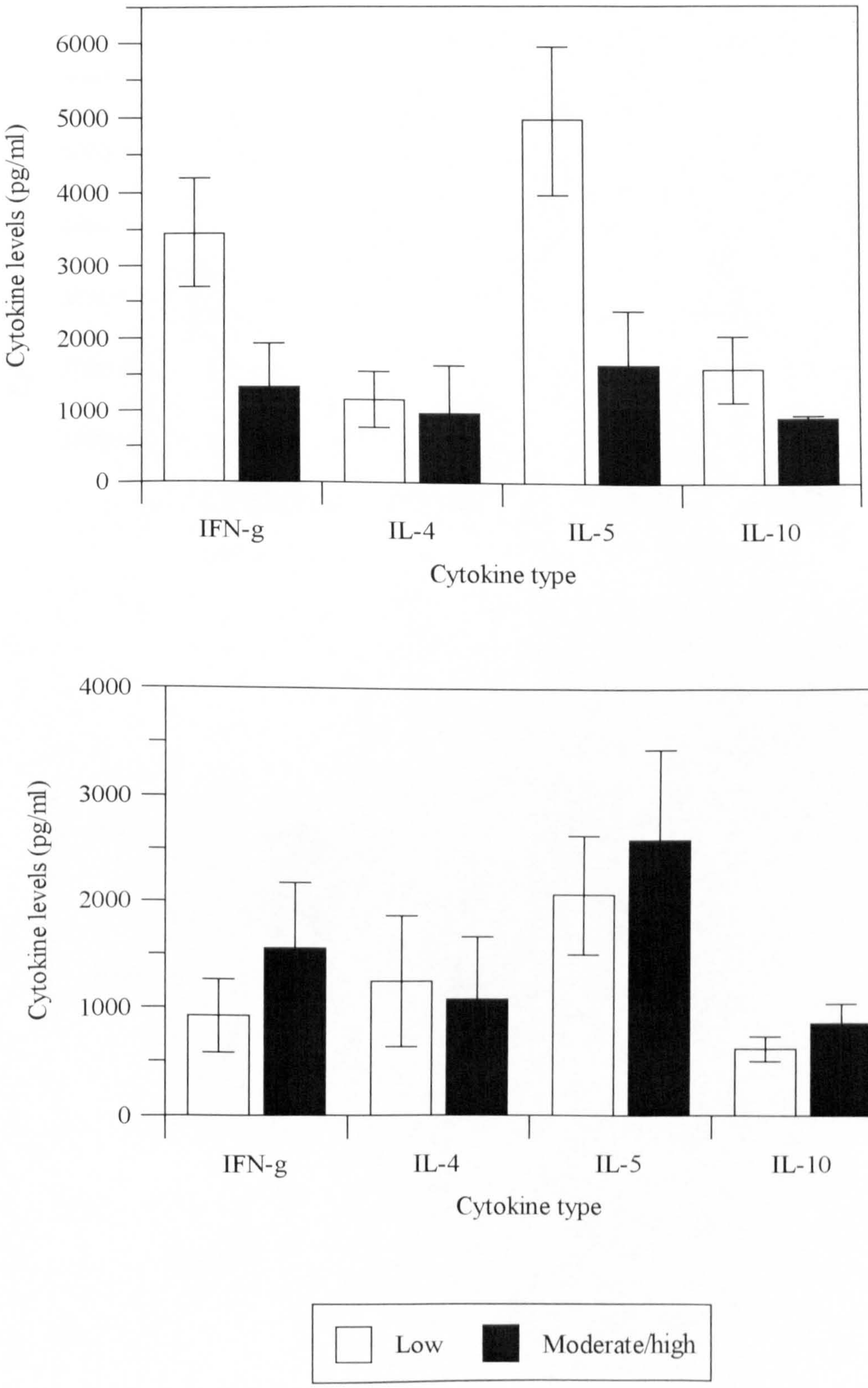


Figure 2.38: Cytokine production levels (± S.E. of mean) by low and moderate/high responding PBMC from the High Transmission area, to SEA stimulation before (Fig.a) and after (Fig. b) treatment with praziquantel, (more values are shown in Appendix Table 6.19 and 6.20).

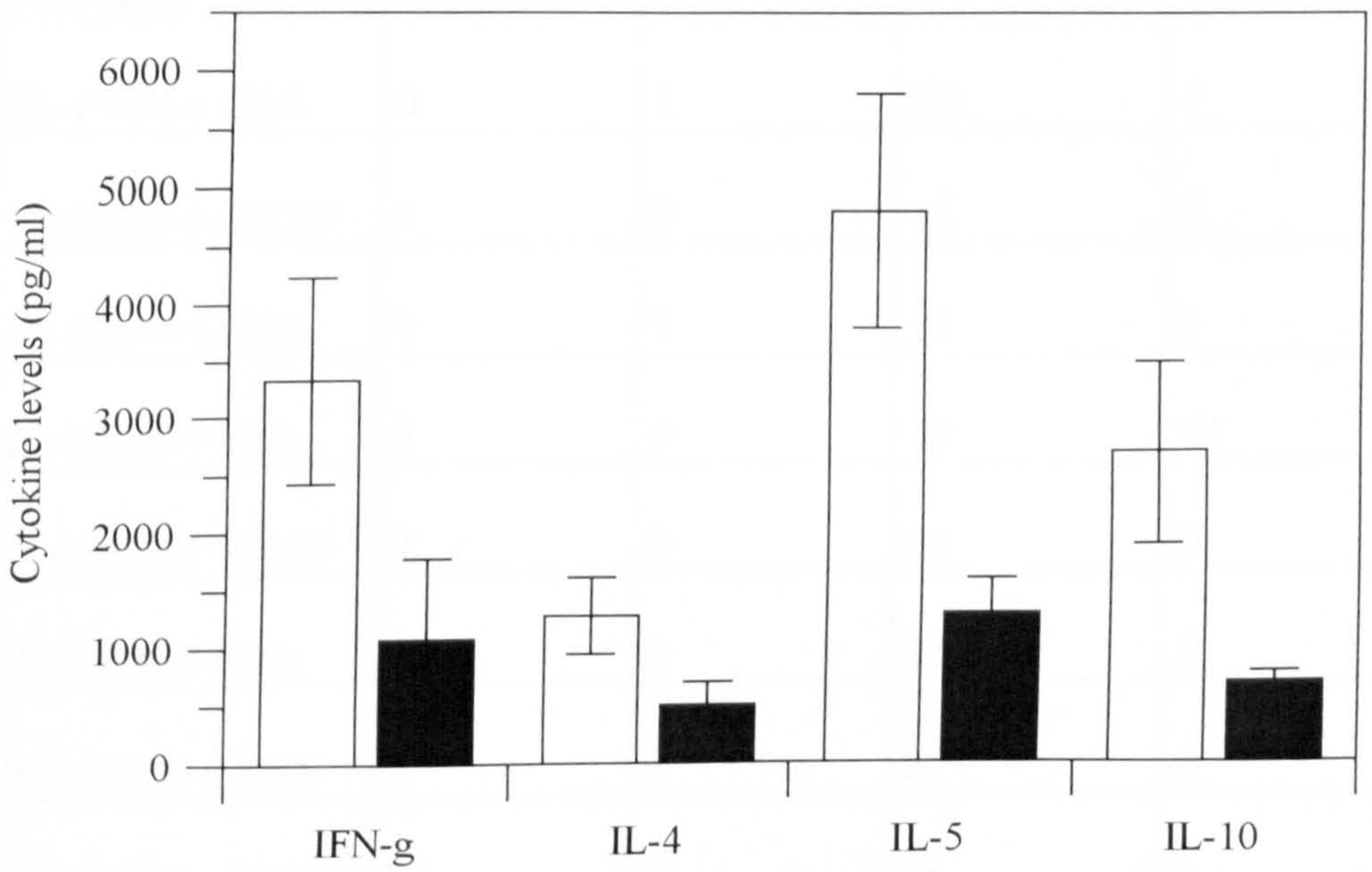


Figure a.

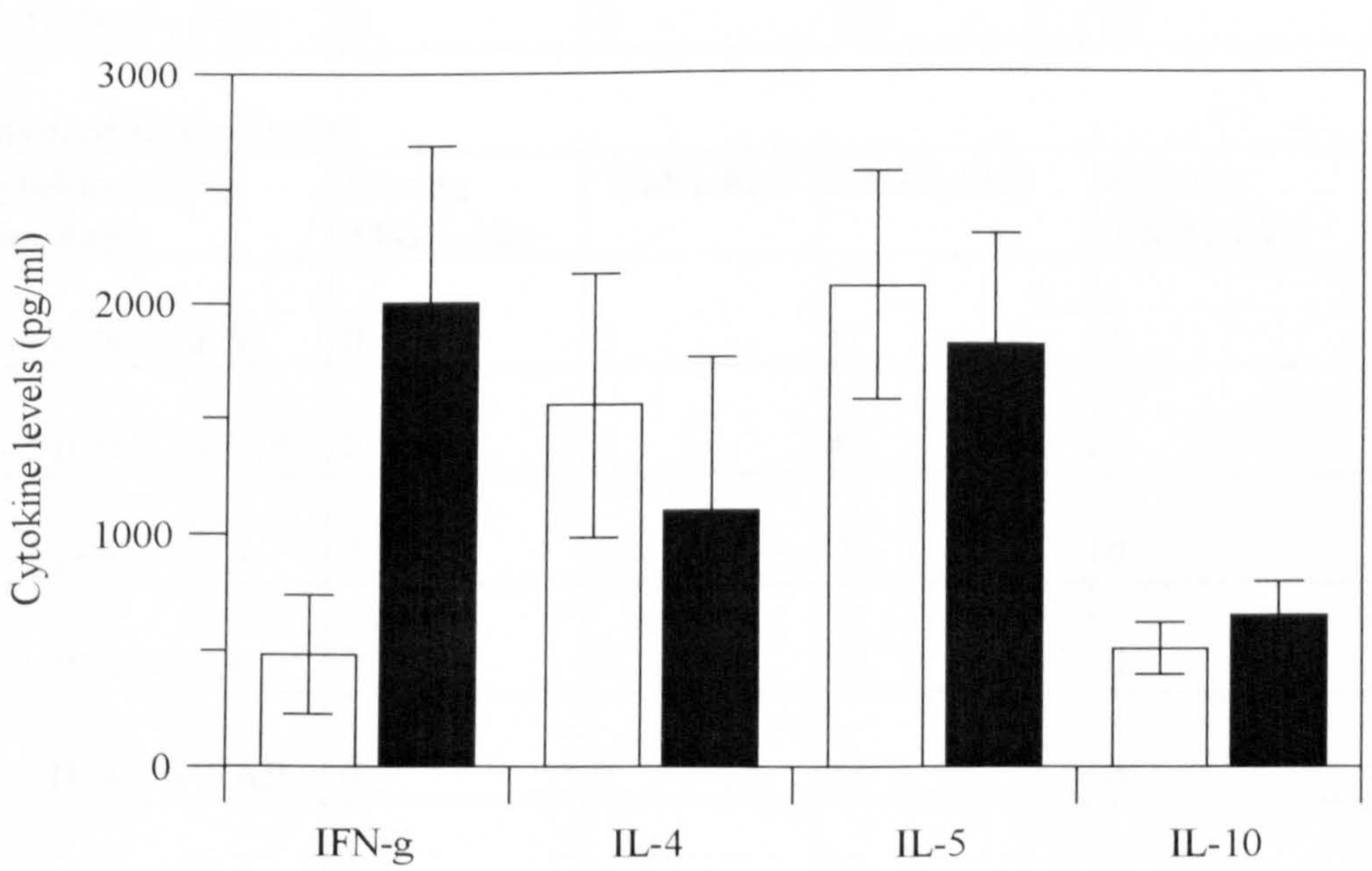


Figure b.

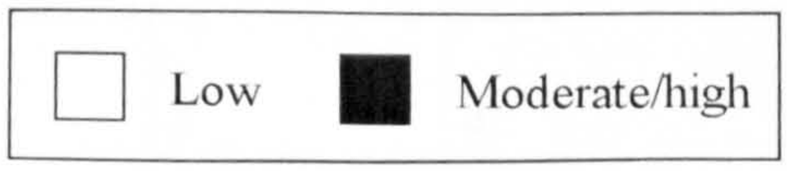


Table 2.17: The ratios of IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ in children from the Low Transmission area, data from all time points combined. The ratios of these 'signature' cytokine are here considered to reflect in the extent to which individuals are Th1- or Th2-like in their responses to PHA, egg or worm antigens.

Uninfected individuals:

Ratio per stimulant	Strong Th0/1-like	Th0/1-like	Th0/2-like	Strong Th0/2-like
IL-4:IFN-γ SEA	0	1	10	1
IL-4:IFN-γ SWAP	0	0	12	0
IL-4:IFN-γ PHA	0	1	11	0
IL-5:IFN-γ SEA	0	0	20	17
IL-5:IFN-γ SWAP	0	0	22	8
IL-5:IFN-γ PHA	0	0	23	1
IL-10:IFN-γ SEA	0	0	21	0
IL-10:IFN-γ SWAP	0	0	21	0
IL-10:IFN-γ PHA	0	0	21	0

Infected individuals:

Cytokine/IFNg stimulant	Strong Th0/1-like	Th0/1-like	Th0/2-like	Strong Th0/2-like
IL-4:IFN-γ SEA	0	3	6	0
IL-4:IFN-γ SWAP	0	1	8	0
IL-4:IFN-γ PHA	2	5	2	0
IL-5:IFN-γ SEA	0	0	11	0
IL-5:IFN-γ SWAP	0	0	11	0
IL-5:IFN-γ PHA	0	2	9	0
IL-10:IFN-γ SEA	0	3	1	0
IL-10:IFN-γ SWAP	0	3	4	0
IL-10:IFN-γ PHA	0	6	4	0

Table 2.18: The ratios of IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ in children from the High Transmission area, data from all time points combined. The ratios of these 'signature' cytokine are here considered to reflect in the extent to which individuals are Th1- or Th2-like in their responses to PHA, egg or worm antigens.

Uninfected individuals:

Ratio per stimulant	Strong Th0/1-like	Th0/1-like	Th0/2-like	Strong Th0/2-like
IL-4:IFN-γ SEA	0	0	8	2
IL-4:IFN-γ SWAP	0	0	8	2
IL-4:IFN-γ PHA	0	1	6	3
IL-5:IFN-γ SEA	0	0	10	6
IL-5:IFN-γ SWAP	0	0	11	5
IL-5:IFN-γ PHA	0	0	12	4
IL-10:IFN-γ SEA	0	0	16	0
IL-10:IFN-γ SWAp	0	0	16	0
IL-10:IFN-γ PHA	0	0	16	0

Infected individuals:

Cytokine per stimulant	Strong Th0/1-like	Th0/1-like	Th0/2-like	Strong Th0/2-like
IL-4:IFN-γ SEA	1	4	7	0
IL-4:IFN-γ SWAP	1	4	7	0
IL-4:IFN-γ PHA	1	2	9	0
IL-5:IFN-γ SEA	0	1	15	0
IL-5:IFN-γ SWAP	0	1	15	0
IL-5:IFN-γ PHA	0	1	15	0
IL-10:IFN-γ SEA	0	5	11	0
IL-10:IFN-γ SWAP	0	4	12	0
IL-10:IFN-γ PHA	0	5	11	0

CHAPTER 3

T CELL CLONING OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM FOUR CHILDREN FROM A HIGH TRANSMISSION AREA FOR *S.haematobium* IN THE BURMA VALLEY.

3.1 INTRODUCTION

Evidence has been accumulated which has underlined the existence of functionally distinct T helper (Th) cell subsets. These originate from $CD4^+$ T cell precursors and are defined by the cytokines they produce (Mosmann *et al.*, 1988; Mosmann *et al.*, 1989; Mosmann and Coffman, 1986, 1989; Romagnani, 1991, 1994; Sher and Coffman, 1992; Abbas, Murphy and Sher, 1996). These subsets have been shown to have a central role in the control and regulation of many immune responses including those directed against parasitic infections such as *Leishmania major* and *Schistosoma mansoni*. In murine models of *S.mansoni* infection, a well defined T helper subset dichotomy has been reported in which T helper1 (Th1) produce interferon gamma (IFN-γ) and tumour necrosis factor-β (TNF-β), which activate macrophages as part of a delayed type hypersensitivity (DTH) reaction. In mice this has been reported to provide protection against *S.mansoni* infection (Sher *et al.*, 1994). Th2 lymphocytes are known to produce IL-4, IL-5, IL-10 and IL-13, which are essential for the production by B cells of IgE, IgG1, IgA and IgM isotypes, and eosinophil and mast cell activation. Th2 type cells are believed to be essential in human protection from schistosome infections but have been shown to play a role in pathology which accompanies the deposition of eggs in the tissues of *S.mansoni* infected mice (Sher and Coffman, 1992).

The patterns of cytokines produced by mouse T cell clones seems adequately defined by the Th1 and Th2 labels (Mosmann and Coffman, 1989). Initially the same classification was applied to human T cell clones but the results of studies of T cell clones derived from patients with schistosomiasis showed that they lacked the distinct Th1/Th2 polarisation observed in the murine models. Instead responses ranged from Th0, Th1, Th2 to Th3 (Romagnani, 1991; Couissinier and Dessein, 1995). Many groups have

reported PBMC cultures from schistosomiasis infected individuals producing IL-2, IL-4, IL-5, IL-6 , IL-10 and IFN-γ (Roberts *et al.*, 1993; Viana *et al.*, 1994; Williams *et al.*, 1994; Araujo *et al.*, 1996; Grogan *et al.*, 1996). This lack of confirmation of the Th1/Th2 concept in human schistosomiasis suggests that the observed differences in the cytokine production in the mouse are more likely to reflect differences between the murine models and humans (Couissinier and Dessein, 1995). Additional studies are required on human schistosomiasis from different transmission areas in endemic zones and on different schistosome species.

Depending on the distinct cytokine profiles produced by the T helper cells involved in activation, expansion and differentiation of B cell responses, the antibody isotype production and isotype regulation of B cell responses to most antigens may be finely regulated. Coffman and colleagues (1988) found that Th2 supernatants enhanced IgE, IgG1 and IgA production, while Th1 supernatants inhibited IL-4 mediated effects. IFN-γ at relatively low concentrations completely inhibited IL-4 enhanced IgE production and reduced IgE levels in mice (Sher *et al.*, 1992). IgE production was regulated by the opposing effects of two cytokines, IL-4 and IFN-γ, which may be considered to be made by two different subsets of the T helper cell subsets. The model of Coffman and colleagues (1988) predicts that Th1 cells may be acting as helper cells for the response of other isotypes. Given appropriate conditions, the majority of both Th1 and Th2 clones can produce cytokines which can provide help to B cells but the immunoglobulin isotypes produced by these B cells varies in accordance with the quality and quantity of the cytokines produced, as discussed in Section 1.10.3.

Most groups which have attempted to identify the T helper cell subsets involved in schistosomiasis susceptibility and protection have extrapolated their findings from cytokine profiles as seen in PBMC cultures and not from single cell culture studies. The use of PBMC has helped in identifying and observing the overall changes in response to schistosome infections especially with *S.mansoni* which has received much attention, in contrast to *S.haematobium* infection. The observed changes in cytokine profiles from PBMC work has been used as evidence to identify the T helper cell subset as Th1 or Th2 type. This includes work on *S.haematobium* by Grogan and colleagues (1996), who reported an increased production of IL-4 by PBMC after treatment with praziquantel, and unchanged production of IL-5 in response to adult worm antigen stimulation. IFN-γ and IL-2 production were low before treatment and unchanged after treatment. Other studies on *S.mansoni* by Viana *et al.*, (1994); Williams *et al.*, (1994) and Araujo *et al.*, (1996) on Brazilian patients, observed the cytokine response of PBMC when stimulated with worm antigen preparations. Williams and colleagues (1994) reported that patients with acute and hepatosplenic infections produced higher levels of IL-4 and IL-5 than uninfected persons. According to this study, the responses of PBMC to mitogen and egg antigen stimulations revealed a Th2 pattern of cytokine production, while the response to worm antigen was said to result in a Th0 cytokine pattern. Araujo and colleagues (1996) reported evidence of a Th2 response in patients with *S.mansoni* infection. The patients showed no lymphoproliferation and IFN-γ production following stimulation with SWAP, in contrast, IL-5 and IL-10 were detected after stimulation with SWAP. Another group, Roberts and colleagues, (1993) working on *S.mansoni* infection in Kenya reported elevated levels of IL-5 and IFN-γ in individuals considered to be resistant to *S.mansoni* infection.

The studies cited above have associated the dominance of the particular sets of cytokines produced by PBMC to a particular T helper cell subset and are therefore of limited value in determining the precise details of the T helper cell dichotomy of the immune response to schistosome infection. In contrast to studies using PBMC, the use of T cell clones provides an opportunity to examine responses under well-defined culture conditions. The present study was designed to develop T cell clones from the PBMC from *S.haematobium* infected and uninfected children living in an area of high transmission of *S.haematobium*. Studies of the cytokine production of such T cell clones would shed some light on the immune processes triggered by natural repeated exposures to *S.haematobium* infection.

3.2 MATERIALS AND METHODS:

3.2.1 REAGENTS

Cell culture reagents were similar to those listed in Section 2.2.1.

Additional reagents were required specifically for T cell cloning:

Monoclonal antibodies, mouse antihuman CD8 (Immunotech ref no. 0178-lyophilised)

Monoclonal antibodies, mouse antihuman CD4 (Immunotech-10b40 ref-1374)

Lysis reagent and Fixing reagent (FixDenat™, Immunotech).

PMA, Phorbol-12-Myristate 13-Acetate P-8139 (SIGMA)

5-Bromo-2'-deoxyUridine (BrdU-Boehringer Mannheim)

Anti-BrdU Peroxidase Dismutase (antiBrdU POD)

FITC (Immunotech)

3.2.2 STUDY AREA AND POPULATION.

The study population was selected from a farming community of The Burma Valley Area, Eastern Highlands of Manicaland Province in Zimbabwe. The farming activities were based on irrigation and on seasonal rains. In the area there is a permanent flowing river with a dam and several seasonal river tributaries. The community has low *S.mansoni* prevalence and subjects were selected from Valhalla primary school having a high *S.haematobium* infection intensity.

Four school children, aged 10, 11, 11 and 12 years were selected for a detailed study of their cellular immune response by cloning T cells derived from their PBMC prior to treatment with praziquantel. Two of the children were infected, one moderately and the other heavily with *S.haematobium* as determined by egg counts on urine specimens. Neither of these children had received treatment in the two years before the study. The other two children were uninfected. The aim of this part of the study was to examine whether the presence or absence of *S.haematobium* infection could be related to cytokine responses which could be classified as Th1, Th2 or another subset type.

3.2.3 PARASITOLOGY

Urine and stool samples for parasitological examination for *S.haematobium* and *S.mansoni* infections were collected, processed and classified as described in Section 2.2.3.

3.2.4 COLLECTION OF VENOUS BLOOD AND PBMC PREPARATION

Approximately 20 mls of blood was collected from each child using a 21G cannulae and sterile heparinized syringes. Blood was transported to the laboratory in a cooler box and PBMC were separated from the blood within 2 hours of collection. The PBMC were then gradient frozen in liquid nitrogen as described in Section 2.2.6.

3.2.5 TREATMENT

All children were treated with praziquantel, at a dose of 40 mg/kg body weight. Children were finally retreated at the end of the two year follow-up study period.

3.2.6 PBMC PROLIFERATION ASSAYS USING TRITIATED THYMIDINE:

The cryopreserved PBMC were washed, resuspended, cell concentration determined and the cells cultured using the method described previously (Section 2.2.7). The stimulants used were PHA and the *S.haematobium* antigens: SEA and SWAP at 10 μg/ml (the concentration being predetermined from early experiments, data not shown).

The amount of tritiated thymidine incorporation induced by antigen or mitogen was determined with a Liquid Scintillation Analyser (TRICAB™, Model 1600 CA Packard USA). Data were expressed as stimulation index of mean counts per minute (SI) calculated as follows:

$$\text{SI of mean cpm} = \frac{\text{Mean counts from stimulated cells}}{\text{Mean counts from control (unstimulated) cells.}}$$

Response to each stimulant was classified as low response-when SI was less than 2 and moderate or high response when SI was ≥2.

3.2.7 T CELL LINES FOR T CELL CLONE DERIVATION AND T CELL CLONES.

Short term T cell lines (TCL) were established from the PBMC of subjects numbers 309, 311, 329 and 350, all from the High Transmission area. The frozen PBMC were retrieved from liquid nitrogen and rapidly thawed with constant agitation, until a small ice crystal remained, they were then transferred to incomplete media with 5% FCS and 50μg/ml gentamycin. After washing twice, the cells were resuspended in complete media containing 10% hAB serum and seeded at 1 x 10^6 cells/ml in the presence of 20 μg/ml SEA, SWAP or PPD. After 6 days at 37°C in 5% CO_2 in humidified air, cells were washed and resuspended in complete media containing 10% human AB serum supplemented with 10 IU/ml human IL-2, and cultured for 5-10 days. After the first stimulation the TCL were directly cloned by the limiting dilution method (Lefkovits and Waldmann, 1984). Cells were seeded at 0.3 or 1 cell/well in the presence of irradiated (3500 rads) heterologous PBMC as feeder cells (5 -7.5 x 10^5 cells/ml), PHA-P at 2 μg/ml and hIL-2 (20 IU/ml). The clones were left to proliferate and expand for 2-4 weeks, with repeated observation. Plates with less than 30% of the wells with growing clones were selected for further study and the clones were then expanded first in 96 well round bottomed plates then 48 well plates and finally in 24 well plates (1 ml/well) by re-stimulation every 15 days with irradiated feeder cells at 7.5 x 10^5 cells/ml, with 2 μg/ml PHA-P and 20 IU/ml hIL-2. T cell clones (TCC) were maintained in complete media with 10% FCS supplemented with 20 IU/ml hIL-2 between each stimulation.

3.2.8 ANTIGEN SPECIFICITY TEST OF CLONES.

T cell clones were tested for their proliferative response to parasite antigen 10-15 days after the last stimulation. After two washes in RPMI supplemented with 50 μg/ml

gentamycin, the cells were suspended in complete media with 10% FCS and seeded at 2 x 10^4 cells per well in flat bottomed microtitre plates containing 5 x 10^4 (cells/ml) irradiated (3500 rad) autologous PBMC. After addition of parasite antigen or (non-specific) mitogen (PHA-P), cultures were incubated for 3 days at 37°C in 5% CO_2 in an humidified air. Cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU; Boehringer Mannheim) during the last 16-18 hours. After aspirating the labelling media, Cells were briefly further cultured in complete media for 2 hours at 37°C in 5% CO_2 humidified incubator. The cells were then washed and the plates dried before fixing the cells with FixDenat™ solution and incubating for 30 minutes at room temperature. The FixDenat was removed by washing 3x, the anti-BrdU labelled with Peroxidase dismutase (POD) added and incubated for 3 hours at room temperature. The plates were then washed 3x before adding substrate and the absorbance measured using an ELISA reader set at 405 nm with 630 nm as the reference wavelength.

3.2.9 STIMULATION OF T CELL CLONES FOR CYTOKINE PRODUCTION

T cell clones were stimulated for assay of lymphokine secretion 10-15 days after the stimulation with feeder cells and PHA-P. The feeder cells have a limited life-span and having been irradiated were considered to be no longer active, and the T cell clones produced no lymphokine spontaneously. The T cell clones were washed twice and seeded at 10^5 cells/100µl in flat bottomed microtitre plates in complete media with 10% FCS. The cells were then incubated for 24 hours at 37°C, in media alone or in the presence of mitogenic stimuli: anti-CD3 Ab (20 ng/ml) plus PMA (10 ng/ml) and PHA-P (2 µg/ml) plus PMA (10 ng/ml). Cell free supernatants were collected 24 hours later under sterile conditions and were stored at -70°C before assaying for cytokines. All the supernatants derived from T cell clones were tested for IL-4, IL-5 and INF-γ.

3.2.10 CYTOKINE TITRATION BY ELISA.

Cytokine detection was performed on the supernatants harvested after 24 hours mitogenic stimulation. The Nunc-Immunoplates Maxisorp™ (InterMed) were coated with anti-human monoclonal antibodies (mAb) at 0.5 μg/ml for IFN-γ and at 1 μg/ml for IL-4 and IL-5 in carbonate/bicarbonate buffer at pH 9.6 at 4°C on a shaker. After the overnight incubation, the plates were washed twice with PBS containing 0.05% Tween 20 at pH 7.2 and then blocked with complete RPMI for an hour at room temperature. The RPMI was aspirated and the plates washed once with PBS containing 0.05% Tween 20. Standards for each cytokine were added in duplicate for each concentration within the range 15-2000 pg/ml. Then various dilutions of clonal supernatants were added in duplicate and diluted in complete RPMI at 1/4 for the control, 1/5, 1/25 and 1/125 for the samples and incubated over night at 4°C on a shaker. After three washes with PBS+0.05% Tween 20, the plates were incubated for 4 hours at room temperature with a second anti-human biotinylated monoclonal antibody specific for each cytokine, at 0.5 μg/ml for IFN-γ, 1 μg/ml for IL-4 and IL-5. After washing 3x with PBS+0.05% Tween 20, streptavidin-alkaline phosphatase conjugate or an alkaline phosphatase-conjugated mAb at a dilution of 1/1000 for IFN-γ and IL-4, and at a dilution of 1/2500 for IL-5 then incubated for 2 hours at room temperature. Enzymatic activity was determined by incubating the plates with p-nitrophenyl phosphate (SIGMA FAST)™. Absorbance was read at 405 nm with 630 nm as the reference wavelength on a DIAS Multiscan reader (Dynatech Laboratories). The standard curves were calculated for each plate according to the sigmoid curve equation:

$$Y = D+(A-D)/(1+(X/C)^B)$$

Where X is the cytokine concentration and Y is the absorbance at 405 nm

A, B, C and D are constants.

Examples of the standard curves for each cytokine are shown in Figure 2.1

3.2.11: $CD4^+$ AND $CD8^+$ SURFACE PHENOTYPING

Approximately 10^5 cells/well of each T cell clone were placed into each of three wells for assessment of CD4 and CD8 numbers, with the third well serving as the control. The T cell clones were then centrifuged and the culture medium supernatant aspirated. The cells were washed with 200 µl PBS buffer by centrifugation at 400xg for 5 minutes. The supernatant was discarded and the cells resuspended in a solution containing mouse anti-human IgG primary monoclonal antibodies (diluted in PBS with 0.1% BSA) specific for CD4 and CD8 surface markers. The T cell clones were then incubated at room temperature for 45-60 minutes, after which the cells were washed twice in 150 µl of PBS by centrifugation at 400xg for 5 minutes. Then 50 µl of FITC-conjugated anti-mouse IgG diluted 50x in PBS with 0.1% BSA was added and incubated at room temperature for 45-60 minutes. The cells were washed twice and then re-suspended in PBS + 0.1% BSA + EDTA. Cells were mounted in PBS + 50% glycerol (1:1 cell suspension:PBS/glycerol) and observed by fluorescence microscopy.

3.2.12 STATISTICAL ANALYSIS

The levels of IFN-γ, IL-4 and IL-5 were expressed as pg/ml. The data sets were mostly not normally distributed hence non-parametric tests were used for the analyses. Wilcoxon matched pairs sign ranks test was used for comparison between two cytokine phenotypes of each clone. Kruskal-Wallis one-way ANOVA was used to compare the cytokine levels of all the clones. The Mann-Whitney Rank sum test was used to compare the responses of the antigen specific and non-specific clones.

To reduce the risk of type 1 errors arising from making multiple pairwise comparisons, Bonferroni's method was applied which uses the function $p=\alpha/m$, where α is a p constant for statistical comparison and m is the number of comparisons. When comparisons were made of levels of cytokines produced by T cell clones from an individual, the revised significance level was; $p=0.05/3 = 0.017$; because three comparisons (PPD, SWAP and SEA) were made at each time point.

Each clone was categorised as a T helper class using a ratio obtained from the measured classes of cytokine IFN-γ, IL-4 and IL-5. The ratio was obtained using the following expression:

x = IL-4:IFN-γ or IL-5:IFN-γ

Based on the ratio, the phenotype of each clone was reported as Th1, Th0/1, Th0/2 or Th2 as shown below:

$x \leq 0.1$	**then the clone is Th1**
$0.1 < x \leq 1$	**then the clone is Th0/1**
$1 < x \leq 10$	**then the clone is Th0/2**
$x > 10$	**then the clone is Th2**

A similar classification has been proposed for classification of human T cell clones during Leishmaniasis and schistosomiasis mansoni although it remains to be tested in further studies (Couissinier-Paris *et al.*, personal communication).

3.3 RESULTS

3.3.1 PARASITOLOGY

Of the four children selected from the High Transmission area, two were infected with *S.haematobium* and the other two were not infected as indicated by the urine examinations. Parasitology results for the four children at various times during the study are shown in Table 3.1. One of the children, 309, was heavily infected with mean infection intensity of 53 eggs per 10 ml of urine. The other child, 350, was moderately infected with an egg output of 20 eggs per 10 ml of urine. The heavily infected child became heavily reinfected by one year post treatment (115 ep10ml) and was re-treated, while the other initially moderately infected child, 350, remained uninfected during the two year follow-up. The two children initially uninfected were found to be infected when examined at six months post-treatment (child 329) or one year post treatment (child 311). The intensities of infection in these children were heavy and light, respectively (Table 3.1).

3.3.2 PROLIFERATION

The PBMC from each of the children were examined for proliferative responses to mitogen and worm and egg antigens. The proliferative responses of PBMC from children 329 (uninfected), 309 (infected) and 350 (infected) were not statistically significantly different from one another but there was a clear biological trend of the PBMC from child 329 (uninfected) having higher levels of proliferation to schistosome antigen. The proliferative responses of PBMC from child 311 (uninfected) were higher than the responses of the PBMC from the other three children (Figure 3.1).

3.3.3: T CELL CLONES: ANTIGEN SPECIFICITY AND SURFACE PHENOTYPE.

After expanding the T cell clones, each clone was tested for antigen specificity using the antigen used in their derivation. The PBMC from the heavily infected child had few T cell clones testing positive to the worm and egg antigens. The other children had high numbers of T cell clones testing positive to the worm and egg antigens used to derive them. Total number of T cell clones derived from each child according to the deriving antigen and the number of clones which were antigen specific are shown in Table 3.2. All the tested T cell clones were $CD4^+$ (positive).

3.3.4 CYTOKINE PROFILES

The cytokine profiles produced by T cell clones derived from the schistosome antigen stimulation of PBMC from each individual differed markedly from each other. The results are summarised in Figures 3.2-3.7 and additional results are given in Appendix 6.23, 6.24 and 6.25; corresponding statistical results are shown in Tables 3.3-3.6.

Child 309 (heavily infected)

T cell clones derived from worm antigen stimulation of PBMC from the heavily infected child, 309, produced higher levels of IL-4 than IFN-γ (Figure 3.2 and Table 3.3). The T cell clones derived from egg antigen stimulation of PBMC from child 309, produced similar levels of IL-4 and IFN-γ (Figure 3.2 and Table 3.3). Comparison of the other cytokines produced by the T cell clones derived from worm and egg antigens stimulation of PBMC from child 309 showed that IL-5 levels were higher than the levels of IFN-γ and IL-4 (Figure 3.2 and Table 3.3). The PPD derived T cell clones produced higher levels of IFN-γ and IL-5 than IL-4 (Figure 3.2 and Table 3.3). The levels of IL-5

production were higher than the other measured cytokines produced by the T cell clones derived from worm and egg antigens stimulation of PBMC from child 309, except PPD derived T cell clones which produced similar levels of IL-5 and IFN-γ (Figure 3.2 and Table 3.3).

When considering the biological trends of the antigen specific T cell clones, the worm antigen specific T cell clones which were derived from worm antigen stimulation of PBMC from child 309, produced higher levels of IL-4 and IL-5 than IFN-γ (Figure 3.3 and Table 3.4). The T cell clones that were egg antigen specific, and derived from the egg antigen stimulation of PBMC from child 309, produced similar levels of IL-4 and IFN-γ, but higher levels of IL-5 (Figure 3.3 and Table 3.4).

Child 350 (moderately infected)

T cell clones derived from worm antigen stimulation of PBMC from the moderately infected child, 350, produced higher levels of IFN-γ and IL-5 than IL-4 (Figure 3.2 and Table 3.3). These T cell clones also produced higher levels of IL-5 than IFN-γ (Table 3.3). The T cell clones from child 350 derived from egg antigen stimulation of PBMC, produced similar levels of IL-4 and IFN-γ, but low levels of IL-5 (Table 3.3). The PPD derived T cell clones produced similar levels of IL-4 and IFN-γ, but the levels of these cytokines were higher than the levels of IL-5 (Figures 3.2 and Table 3.3).

When considering only the antigen specific T cell clones, the T cell clones that were worm antigen specific, and derived from worm antigen stimulation of PBMC from child 350, produced similar levels of IL-4 and IFN-γ, but higher levels of IL-5 than IL-4

(Figure 3.3 and Table 3.4). Only one T cell clone from child 350 responded specifically to egg antigen stimulation.

Child 329 (uninfected)

The T cell clones derived from worm antigen stimulation of PBMC from uninfected child, 329, produced higher levels of IFN-γ than IL-4 and IL-5 (Figure 3.4 and Table 3.5). These T cell clones also produced higher levels of IL-5 than IL-4 (Table 3.5). The T cell clones derived from egg antigen stimulation of PBMC from child 329, produced similar levels of IL-4 and IFN-γ. However, the T cell clones produced lower levels of IL-4 and IFN-γ than the levels of IL-5 (Table 3.5). The PPD derived T cell clones produced similar levels of IL-4, IL-5 and IFN-γ (Figure 3.4 and Table 3.5).

When considering only the antigen specific T cell clones, the T cell clones derived from worm antigen stimulation of PBMC from child 329, that were specific to worm antigen stimulation produced higher levels of IFN-γ and IL-5 than levels of IL-4 (Table 3.6). The T cell clones derived from egg antigen stimulation of PBMC from child 329, that were specific to egg antigen, produced a similar pattern to the clones that were specific to worm antigen stimulation (Figure 3.5 and Table 3.6).

Child 311 (uninfected)

The worm antigen derived T cell clones from PBMC from the uninfected child, 311, produced similar levels of IL-4 and IFN-γ, and higher levels of IL-5 than IL-4 and IFN-γ (Figure 3.4 and Table 3.5). The T cell clones derived from egg antigen stimulation produced similar levels of IL-4 and IFN-γ, but these cytokine levels were lower than IL-5 levels (Table 3.5). The PPD derived T cell clones produced higher levels of IL-4 than

IFN-γ (Figure 3.4), while the levels of IL-5 were higher than IL-4 and IFN-γ levels (Table 3.5). The levels of IL-5 production were higher than all the other cytokines produced by the T cell clones from the PBMC from child 311 (Figure 3.4 and Table 3.5).

When considering only the antigen specific T cell clones, the T cell clones derived from worm antigen stimulation of PBMC from child 311, that were specific to worm antigen stimulation produced higher levels of IL-4 than IFN-γ (Figure 3.5). The T cell clones that were derived from egg antigen stimulation of PBMC from child 311 and were egg antigen specific, produced higher levels of IL-4 and IL-5 than IFN-γ (Table 3.6), while IL-5 levels were higher than IL-4 levels (Figure 3.5 and Table 3.6).

3.3.5 CYTOKINE RATIOS

In addition to investigating the changes in absolute levels of cytokines, described above, the changes in ratios of 'signature' cytokines were also analysed. This analysis is based on the premise that Th1 and Th2 cytokines cross-regulate the other T cell subset and changes in the relative abundance of Th1 and Th2 cytokines may be more sensitive indices of the *in vivo* T helper cell environment with the 'signature' cytokine acting as an indicator of the T helper cell subset dominating during an immune response to schistosome infection.

The T cell clones from the heavily infected child, 309, gave a high IL-5:IFN-γ ratio for the clones derived from worm and egg antigen stimulation (Figure 3.6a). The moderately infected child, 350, had T cell clones derived from worm antigen stimulation of PBMC giving a high IL-5:IFN-γ ratio, while the egg antigen derived T cell clones showed a high

IL-4:IFN-γ ratio. The PPD derived T cell clones from children 309 and 350 both showed a low ratio for both comparison of IL-4:IFN-γ and IL-5:IFN-γ (Figure 3.6a).

When considering only the T cell clones that were antigen specific, the T cell clones from infected children showed an IL-5:IFN-γ ratio to be higher than IL-4:IFN-γ for the T cell clones derived from egg and worm antigen stimulation of PBMC. The T cell clones derived from worm antigen stimulation of PBMC from infected children, 309 and 350, showed an IL-5:IFN-γ ratio of approximately 10, indicating the levels of IL-5 being 10 times the levels of IFN-γ (Figure 3.6b), indicating the cytokine balance of the response tipping towards a Th2 type.

The mean cytokine ratios from T cell clones from the uninfected children, 329 and 311, showed marked differences in the patterns of the cytokine ratios between them. Child 329 showed a high IL-5:IFN-γ ratio from the egg antigen derived clones only, with the other ratios being low (Figure 3.7a). Child 311 showed higher IL-5:IFN-γ ratios than IL-4:IFN-γ for all the stimulants (Figure 3.7a). The IL-4:IFN-γ ratio showed IL-4 to be between approximately eight and 13 times higher than IFN-γ levels from worm and PPD stimulants, respectively.

When considering only the T cell clones that were antigen specific, the T cell clones from uninfected children showed the IL-5:IFN-γ ratio to be higher than the IL-4:IFN-γ for the T cell clones derived from egg and worm antigen stimulation of PBMC. The T cell clones derived from worm and egg antigen stimulation of PBMC from the uninfected child, 329, showed that the IL-5:IFN-γ ratio was slightly raised, while IL-4:IFN-γ ratio was very low (Figure 3.7b), indicating a mixed cytokine environment of the response in

this child, with neither T helper subset dominating. The child 311, showed the IL-4:IFN-γ and IL-5:IFN-γ ratios being high, showing the levels of IL-4 and IL-5 to be approximately 10 times the levels of IFN-γ (Figure 3.7b), showing the cytokine environment tipping towards a strong Th0/2 type.

3.3.6 T HELPER SUBSETS

The T helper subset classification is described in Section 3.2.13, and is repeated here for convenience. Each clone was categorised as one of four possible T helper subsets using a ratio obtained from the following expression:

x = IL-4:IFN-γ or IL-5:IFN-γ

Based on the ratio, the phenotype of each clone was reported as Th1, Th0/1, Th0/2 or Th2 type as shown below:

$x \leq 0.1$ then the clone is Th1

$0.1 < x \leq 1$ then the clone is Th0/1

$1 < x \leq 10$ then the clone is Th0/2

$x > 10$ then the clone is Th2

The classifications of all derived T cell clones according to their stimulant source and according to the IL-4:IFN-γ ratios are shown in Table 3.7a and according to the IL-5:IFN-γ ratios are shown in Table 3.7b. The percentage distribution of the T cell clones according to subject and stimulant for all the T cell clones is shown in Figure 3.8 and for worm and egg antigen specific T cell clones shown in Figure 3.9. The T cell clones derived from the PBMC from children living in high transmission area of *S.haematobium* proved to be predominantly classified as Th0/2.

Child 309 (heavily infected)

T cell clones derived from either worm or egg antigen stimulation of PBMC from the heavily infected child, 309, using IL-4:IFN-γ ratio, proved to be predominantly classified as Th0/2 subset and a small number of T cell clones being classified as Th0/1, Th1 and Th2 type. Using the IL-5:IFN-γ ratio, some of the T cell clones were classified as Th2 type. However, the PPD derived T cell clones produced cytokines that led to their being classified as Th0/2 type with a small number of T cell clones as Th0/1 and Th1 type (Table 3.7 and Figure 3.8).

When considering only the T cell clones that were antigen specific, the cytokines produced led to the T cell clones being classified as Th0/2 type, with a small percentage of T cell clones being classified as Th0/1 and Th2 types (Figure 3.9).

Child 350 (moderately infected)

T cell clones derived from either worm or egg antigen stimulation of PBMC from the moderately infected child, 350, using IL-4:IFN-γ ratio, proved to be predominantly classified as Th0/2 type (Table 3.7a). A small number of the T cell clones were classified as Th0/1 and Th2 type. When using the IL-5:IFN-γ ratio, a similar distribution of the T cell clones as for IL-4:IFN-γ was observed (Table 3.7b and Figure 3.8). The PPD derived T cell clones produced cytokines that led to their being classified as predominantly Th0/2 with a small number of T cell clones as Th0/1 and Th1 type (Table 3.7b and Figures 3.8).

When considering only the T cell clones that were antigen specific, the cytokines produced led to the T cell clones being classified as Th0/2 type, with a small percentage of T cell clones being classified as Th0/1 and Th2 types (Figure 3.9).

Child 329 (uninfected)

T cell clones derived from either worm or egg antigen stimulation of PBMC from the uninfected child, 329, using IL-4:IFN-γ ratio, proved to be predominantly classified as Th0/2 type (Table 3.7a). A small number of T cell clones were classified as Th0/1 and Th2 type. Using the IL-5:IFN-γ ratio, most of the T cell clones were classified as Th0/2 with a small number of T cell clones being classified as Th0/1 and Th1 types (Table 3.7b and Figure 3.8). The PPD derived T cell clones produced cytokines that led to their being classified as predominantly Th0/2 with a small number of T cell clones as Th0/1, Th1 and Th2 type (Table 3.7b and Figure 3.8b).

When considering only the T cell clones that were antigen specific, the cytokines produced led to the T cell clones being classified as predominantly Th0/2 type, with a small percentage of T cell clones being classified as Th0/1 and Th1 types (Figure 3.9).

Child 311 (uninfected)

T cell clones derived from worm and egg antigen stimulation of PBMC from uninfected child, 311, using IL-4:IFN-γ ratio, proved to be predominantly classified as Th0/2 type. A small number of T cell clones were classified as Th0/1 and Th2 type (Table 3.7a). Using the IL-5:IFN-γ ratio, similar distribution of the T cell clones as for IL-4:IFN-γ was observed with the T cell clones predominantly being classified as Th2 type (Table 3.7b

and Figure 3.8). The PPD derived T cell clones showed similar classification patterns as for the schistosome antigen derived T cell clones (Table 3.7 and Figure 3.8).

When considering only the T cell clones that were antigen specific, the cytokines produced led to the T cell clones being classified as Th0/2 type, with an increased percentage of T cell clones being classified as Th2 types (Figure 3.9).

3.4 DISCUSSION

At the start of the study the children showed different levels of *S.haematobium* infection, which might be interpreted as supporting the idea reviewed by Hagan, (1987) that individuals living in endemic areas have different immune responses, making them either resistant or susceptible to infection. Parasitology follow-up results for two years after isolation of T cells and treatment with praziquantel, supported the view that immune responses may be playing a role in making some individuals resistant and others susceptible to schistosome infection. The heavily infected individual, 309, became heavily reinfected by one year after treatment, and one of the individuals who was uninfected, 311, became lightly infected after one year. The individual who was moderately infected, 350, before treatment surprisingly remained uninfected throughout the two years follow-up. The other uninfected individual, 329, became heavily infected early, about six months after treatment. Such results are of major interest and studies involving detailed analysis of individual's T lymphocyte responses may offer one possible explanation for the above results. The immunological responses can be elucidated further by studying the cytokines produced by peripheral blood mononuclear cells or by cloned T lymphocytes that may

contribute to determining whether an individual is either susceptible or resistant to further *S.haematobium* infection.

T lymphocytes play a significant and important role in immune responses to infection with schistosomes through the different members of the cytokine classes they produce. During heavy schistosomiasis infection, it is usually observed that the PBMC do not give raised levels of proliferation after stimulation with schistosome-specific antigens. This characteristic has been reported in adults chronically infected with *S.mansoni* (Ottesen *et al.*, 1977, 1978, 1979; Colley *et al.*, 1977a,b, 1986; Roberts *et al.*, 1993). The individuals in the present study were children of an average age of 11 years old living in a highly endemic area for *S.haematobium*. The heavily infected child gave very low levels of proliferation to schistosome-specific antigens, while the moderately infected gave slightly raised levels of proliferation. In a related study by Grogan *et al.*, (1996) and King *et al.*, (1996) on *S.haematobium* infected patients, unresponsiveness was seen prior to treatment, but after treatment was reported to be increased several fold high. Such unresponsiveness is thought to be the way the immune response is regulated during heavy infection when the parasite establishes a favourable environment which is beneficial to itself. The heavily infected child may be considered as having had infection for a long duration. As heavy infections normally take some time to accumulate in an individual, the heavily infected child in this study probably acquired their first infections at an early age and might now be considered to have a chronic infection. In contrast, for the non-infected individuals, levels of proliferation to the schistosome antigen stimulation were observed to be high.

During an acute schistosomiasis infection the immune response is characterised by raised levels of proliferation by PBMC on stimulation with schistosome antigen, but during heavy or chronic infection some immune evasion mechanism that includes immunomodulation by the worms renders the host unresponsive to any further stimulation. Studies by Colley *et al.*, (1977b); Ottesen and Pointdexter, (1980) and Gazzinelli *et al.*, (1985) reported the presence of some suppressive factors in serum of subjects who had a long duration of infection, which suggested that immunomodulation is a characteristic of those individuals who are heavily or chronically infected, whereas individuals in acute stages of infection have strong cellular responses to specific schistosome antigens. However, in another study by Colley *et al.*, (1986) an association was reported between levels of response to antigens and levels of reinfection. People who were reinfected had lower responses to schistosome antigens than people who remained uninfected. Similar proliferation results were obtained in this study from children 311 and 329, who were uninfected, but child 329 gave lower levels of proliferation to schistosome antigen stimulation than child 311 and became infected earlier.

The levels of proliferation to schistosome-specific antigen may be related to the number of T cell clones derived from the stimulation that would react specifically to the antigen after expanding the clones. The heavily infected child who displayed low levels of proliferation had a small number of T cell clones testing positive to the antigen used to derive them. However, considerable number of T cell clones (specific and non-specific) were derived from each child's PBMC after stimulation with schistosome-specific antigen and PPD. Further investigations are required to understand the generation of T cell lines and T cell clones growing well that become non-specific to the antigen used in

their derivation after expansion. However, the T cell clones were derived from the T cell lines generated from PPD and schistosome worm and egg antigen stimulation of PBMC from the individuals, analyses of the cytokine profiles and the dominating T helper cell subsets were carried out on both the few antigen-specific T cell clones and all the T cell clones as per stimulant source.

The T cell clones from infected children, 309 and 350, and one of the uninfected children, 329 (who became heavily infected early), produced either high levels of IFN-γ and IL-5 but low levels of IL-4 or similar levels of IFN-γ and IL-4. This may indicate that during schistosomiasis infection and susceptibility (as in child 329) either IFN-γ is found dominating or the two contrasting cytokines (IFN-γ and IL-4) are produced at similar levels, though the sample size limits confidence in this interpretation. The egg antigen derived T cell clones produced similar levels of IFN-γ and IL-4, while the worm antigen derived T cell clones produced predominantly higher levels of IFN-γ than IL-4. This may indicate differences in antigenicity of the schistosome antigens according to life cycle stages. T cell clones derived by stimulation with egg antigens had the ability to stimulate production of IFN-γ and IL-4 at similar levels in individuals who were moderately infected and those susceptible to infection. The cytokine levels from the other uninfected child, 311, showed high levels of IL-4 and IL-5, but low levels of IFN-γ. This could be interpreted as indicating that high levels of IL-4 production may be associated with absence of infection or resistance to infection. In contrast, high levels of IFN-γ production may be an indicator of ongoing infection or susceptibility to infection, as seen in the infected children 309 and 350. However, child 329 was initially uninfected, but by the end of six months post treatment became heavily infected. The presence of IFN-γ may not denote susceptibility but instead a raised level of IFN-γ may indicate a

developmental stage in acquisition of protective immunity with the IFN-γ mobilising the cells that may be involved in later protective responses. The presence of IFN-γ may mark an immune state which is not sufficient to give complete protection. The dominance of IFN-γ may also mark the presence of T helper subsets that develop through a Th0 phase then into Th1, during early infection and become further polarised as the infection progresses.

The role played by cytokines in the control of *S.haematobium* infection was extrapolated from comparing cytokines and infection status at the time of collecting the PBMC and the parasitological status that was determined from examining urine samples collected during the follow-up time points. Raised levels of IL-4 and IL-5 (Th2 type cytokines) were clearly related to a lack of infection as shown by the parasitological status of uninfected child, 311. The T cell clones from this child were classified as predominantly Th0/2 and Th2, which indicates that a cytokine balance that is dominated by IL-4 and IL-5 over IFN-γ is protective. IL-4 has been reported to be involved in switching B cells to secrete specific antibody isotypes (notably IgE, IgG1 and IgA) and also IL-4 is involved in activation, proliferation, differentiation and enhanced production by B cells of antibodies which may be involved in ADCC reactions during schistosome infection (as reviewed by Romagnani, 1991, 1994; Abbas, Murphy and Sher, 1996; Mosmann and Sad, 1997).

The moderately infected child, 350, and the uninfected child, 329, produced higher levels of IFN-γ than the heavily infected child 309. High IFN-γ levels may be a feature of moderately infected individuals or individuals who, though uninfected may be at least partially susceptible to infection. During heavy or chronic infection (as in child 309)

much of the T cell responsiveness may be downregulated, effectively "switched off" and this is reflected in low levels of IFN-γ. While this may minimise the pathological consequences of infection and therefore be of benefit to the host once infection has reached this stage, prior to this stage some element of T cell responsiveness is desirable and the raised IFN-γ levels of the infected child, 350, and uninfected child, 329, may be indicative of continuing T cell reactivity in these children. The presence of significant levels of IL-4 and IL-5 in these children may indicate that this is a crucial stage in the development of their responses to infection when IL-4 or IL-5 are beginning to dominate the response with humoral responses becoming significant. It may be possible to speculate that at this stage the immune responses may continue to develop giving rise to protection or may instead result in susceptibility to infection. This of course will, in part, be dependent upon the cytokine profile which dominates.

Antigen dosage studies suggest that it is the initial response event that determines cytokine-producing phenotype and this may be under the influence of factors present at the time of primary stimulation. Some striking correlation of antigen dose and development of immune responses dominated by cellular immunity or by antibody production have been reported (Parish and Liew, 1972). In these experiments low doses of antigen favoured cellular immunity; intermediate doses, antibody formation and high doses both cellular immunity and antibody formation. Assuming that cellular immunity represents IFN-γ-dominated responses and the antibody production, IL-4-dominated responses, commitment to IFN-γ or IL-4 production may be determined in part by the concentration of antigen and the time the antigen persists in the body. The data might be used to support the idea that the moderately infected individual (350) and the individual showing the cytokine phenotype tipping towards susceptibility (329) show elevated IFN-

γ and presence of low levels of IL-4 could be considered to downregulate IFN-γ and it's likely damaging effects. At the same time IFN-γ may be inducing macrophages to express MHC class II for antigen presentation during light infection helping curb the invading parasite and limiting the antigen load by driving the immune response toward killing of the invading parasite. When such an immune response takes place, the events lead to expression of both T helper-like responses that resultantly downregulate each other. The cytokine phenotypes lead to the T cell clones being classified as Th0/2 with some clones being classified as Th0/1 and Th1 for the moderately infected and the susceptible child with some T cell clones as Th2, Th0/1 and Th1.

Th1/Th2 polarisation is thought to increase with the chronicity of the immune responses which in turn depends on intensity of exposure to infection and the consequences of the exposure. Some reports have described that mixed cytokine patterns exist early in lymphocyte activation with a clearer Th1 and Th2 subset definition in chronic disease states (Chikunguwo *et al.*, 1991, 1992; Seder *et al.*, 1993; Abbas, Murphy and Sher, 1996). Although the children in this study were relatively young and had a limited number of years of exposure to infection (children of one year old are exposed to infection in these communities) chronicity could not be ruled out but, based on the mixed cytokine patterns recorded, any chronicity of infection had not yet been translated into distinct polarisation of T cell subset responses based on the murine classification. Instead Th0/1 and Th0/2 subsets were isolated and the possibility remains that these could have further differentiated into Th1 and Th2 if exposure and infection had continued.

The children were residents in a *S.haematobium* endemic area and showed high levels of IL-5. IL-5 is the principal eosinophil-activating cytokine and eosinophils have been reported to be elevated in individuals with parasitic infections (Hagan *et al.*, 1985a,b; Butterworth *et al.*, 1992). However both IL-5 and IL-4 are normally expressed together although at different levels. The co-expression of the two cytokines by the same T cell subsets may indicate their involvement in a common effector mechanism. Several other cytokines produced by Th2 cells play an anti-inflammatory role. For example, IL-4 and IL-10 antagonise the macrophage-activating action of IFN-γ (Fiorentino *et al.*, 1991; Del Prete *et al.*, 1993). IL-10 also suppresses macrophage responses resulting in an anti-proliferative response of cells (King *et al.*, 1996). Thus, Th2 type cytokine responses may act as inhibitors of acute and chronic inflammation and as a result act as regulators of cellular immune responses and not necessarily as effectors in the same manner as Th1 cells. Th1 may appear early in an immune response, generating a Th1 cell-mediated protective immune response, while Th2 type comes late to control the damaging effects potentially caused by Th1-mediated response and is believed to accumulate as the response progresses (Pearce *et al.*, 1991, Sher *et al.*, 1991).

Both subsets are believed to originate from the same T cell precursor whose differentiation is influenced by the manner and environment in which the precursor is stimulated (Swain *et al.*, 1990; Janeway and Bottomley, 1994). The production of the 'signature' cytokines may indicate the developmental processes to originate from the same precursor cell. During schistosome infection, events may take place in stages with the initial stage being the development of Th1 type of response which has been revealed at a clonal level by the dominance of IFN-γ. The progression of the disease seems to lead

into Th2 type response shown by raised levels of IL-4, this status having the ability to suppress the earlier events dominated by IFN-γ of the Th1 response.

In this study the major 'signature' cytokines marking Th1 or Th2 subsets type were found to be co-expressed by the same T cell clones. In a study by Couissinier and Dessein, (1995) the 'signature' cytokines marking each subset were found to be co-expressed and this resulted in difficulties in classifying the subsets in terms of the Th1 and Th2 subsets reported from murine models. This may mark T cell clones lacking distinct cytokine profiles for Th1/Th2 polarisation in human schistosomiasis unlike the distinct polarised cytokine profiles marking the T helper cell subsets in mice.

In addition to their role in effector immune responses cytokines are also responsible for activating the development of T cells into particular subset "lines". Normally, cytokines produced by a T cell subset serve to activate and increase production of cell lineages which will result in increased frequencies of the same T cell subset and in turn production of the same cytokines (Coffman *et al.*, 1991; Vella and Pearce, 1992; Bradley *et al.*, 1993; Coffman and von der Weid, 1997). When a cytokine reaches a threshold level then that T cell subset is initiated and the cytokine production increases progressively. With repeated stimulation taking place in individuals in areas of high transmission, the high exposure and continuous stimulation may drive the response in the direction of the first selected response or along the dominating cytokine response. Other factors important in determining the balance between Th1 and Th2 subsets in immune responses are the dose or concentration of the antigen and this may influence the types of responses encountered when infections become chronic. A low antigen concentration and low infection load tends, it seems, to induce Th1 response whereas a high dose induces Th2

development. At low antigen concentration the APCs crucial to the response have been reported to be dendritic cells and macrophages which produce the cytokines that drive the development of the Th1 response. At high antigen concentration the APC may not produce the Th1 favouring cytokines, thus IL-4 is produced and further drives the response towards the Th2 type (Seder *et al.*, 1991, 1992; Paul and Seder, 1994; Sprent, 1994). Also at high antigen concentration there is repeated T cell stimulation that increases IL-4 production and Th2 development and sometimes induces a state of immunological unresponsiveness or tolerance which switches off Th1 development (Swain *et al.*, 1990, 1991; Pearce *et al.*, 1991). However in this state, as shown by heavily infected child 309, both subset cytokine markers may be elevated and the levels of proliferation are low.

The data presented in this part of the thesis have related the cytokine profiles and phenotypes of T cell clones from PBMC of children living in a High Transmission area of an endemic region. The cytokine profiles of the T cell clones from PBMC obtained before treatment were also related to parasitological data from a two year follow-up period. The T cell clones were classified into T helper subsets and the frequency of each subset from the total number of T cell clones was determined. The overall immune response of the individuals in terms of the T helper subsets appeared to be related to putative susceptibility or resistance. The T cell clones from the heavily infected child showed low levels of antigen specificity, while the uninfected children had more T cell clones responding specifically to the antigen used to derive the T cell clone.

A ratio of 'signature' cytokines, used to identify T cell subsets in mice, was used to classify the human T cell clones produced in this study. The co-expression of 'signature' cytokines by a single human T cell clone demanded that the standard Th1/Th2 classification from murine studies be modified. As a consequence a revised classification, based on the ratio of cytokines expressed was derived, allowing T cell clones to be assigned to Th1, Th0/1, Th0/2 and Th2 categories. In this respect the T cell clones derived from PBMC of *S.haematobium* infected or exposed humans appear to be similar to those identified in earlier studies of *S.mansoni* infected humans from Brazil (Couissinier and Dessein, 1995). In that study the Th0/2 subset was identified as a marker of resistance while the Th0/1 subset was associated with susceptibility or the absence of protection. In the present study as judged by the changes in infection status with time, the T cell responses appeared to be less stable and more dynamic, suggesting that small quantitative changes in the balance of the cytokines response could result in either susceptibility or resistant to *S.haematobium* infection.

Table 3.1: Results of *S.haematobium* parasitological examination (eggs per 10 ml of urine) over a two year follow-up period of the children whose PBMC were cloned. The egg counts were determined by filtration of 10 ml of urine collected on three consecutive days. (ND=not done)

Subject (age)	Aug'94 pre-treat	Nov'94 post1	Feb'95 post2	May'95 post3	July'95 post4	1 year post5	1.5 years post6	2 years post7
Infected								
309 (11)	53	0	0	0	0	ND	115	70
350 (12)	20	0	ND	0	0	0	0	0
Uninfected								
329 (10)	0	0	0	55	82	27	11	ND
311 (11)	0	0	0	0	0	7	7	4

Figure 3.1: The levels of proliferation to PHA and schistosome antigen stimulation of PBMC from children before deriving T cell lines for T cell cloning. Proliferation levels were determined by tritiated thymidine incorporation.

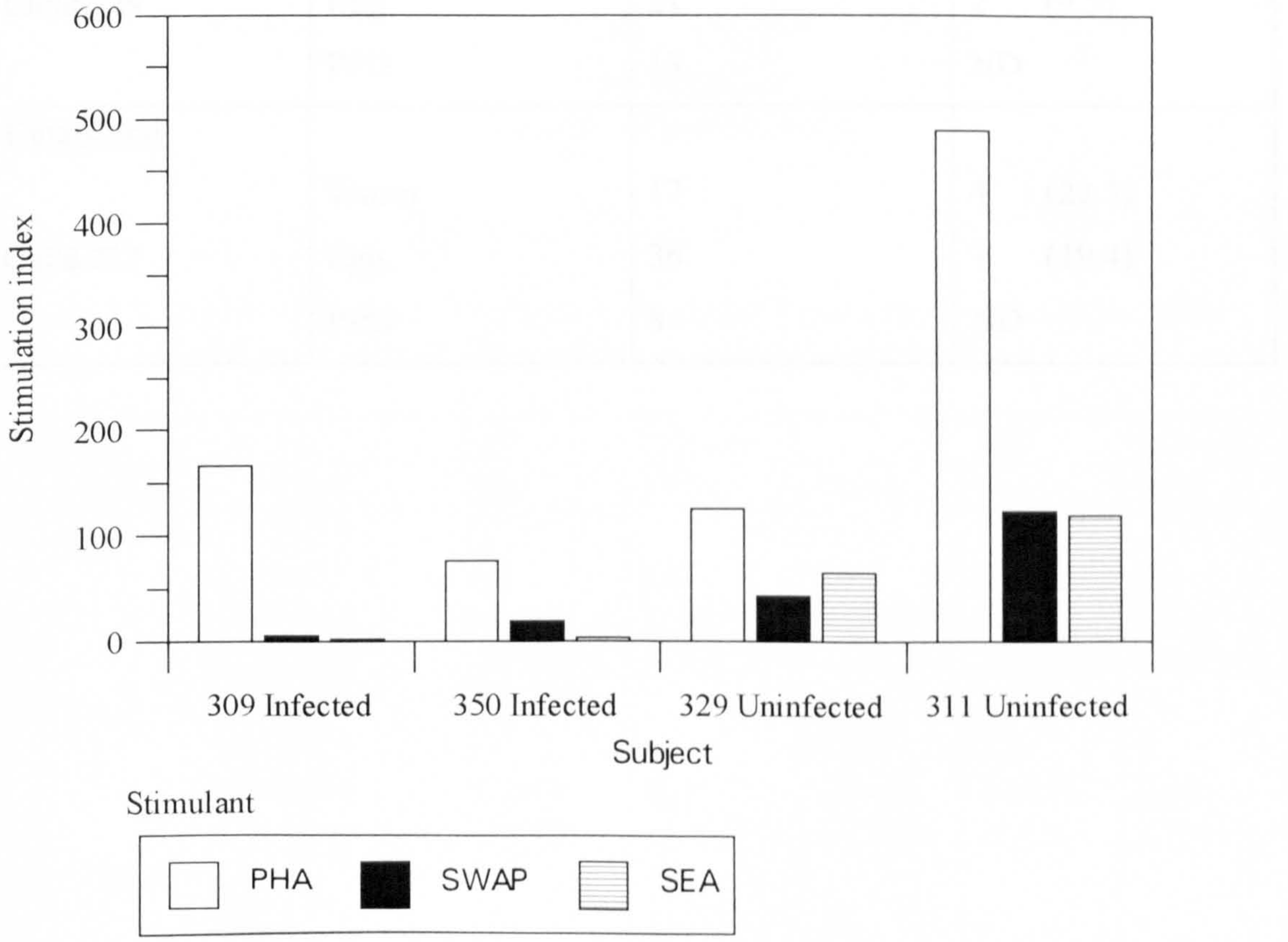


Table 3.2: The total number of T cell clones obtained per subject, per stimulant and the number of T cell clones that were antigen specific per stimulant.
ND= not done

Infection status Subject	Stimulus used for clone derivation	Total number of clones derived	Number of antigen specific clones (%)
<u>Heavily Infected</u>			
	Worm	19	3 (15.8)
Child 309	Egg	14	2 (14.3)
	PPD	15	ND
<u>Infected</u>			
	Worm	68	18 (26.5)
Child 350	Egg	12	1 (8.3)
	PPD	9	ND
<u>Uninfected</u>			
	Worm	43	11 (25.6)
Child 329	Egg	21	2 (9.5)
	PPD	13	ND
<u>Uninfected</u>			
	Worm	17	4 (23.5)
Child 311	Egg	36	7 (19.4)
	PPD	8	ND

Figure 3.2: *S.haematobium* infected subjects:- 309 and 350. Mean cytokine levels (mean ±SE) produced by all clones according to subject and stimulation with worm and egg antigen and PPD. The number of clones tested is shown in brackets.

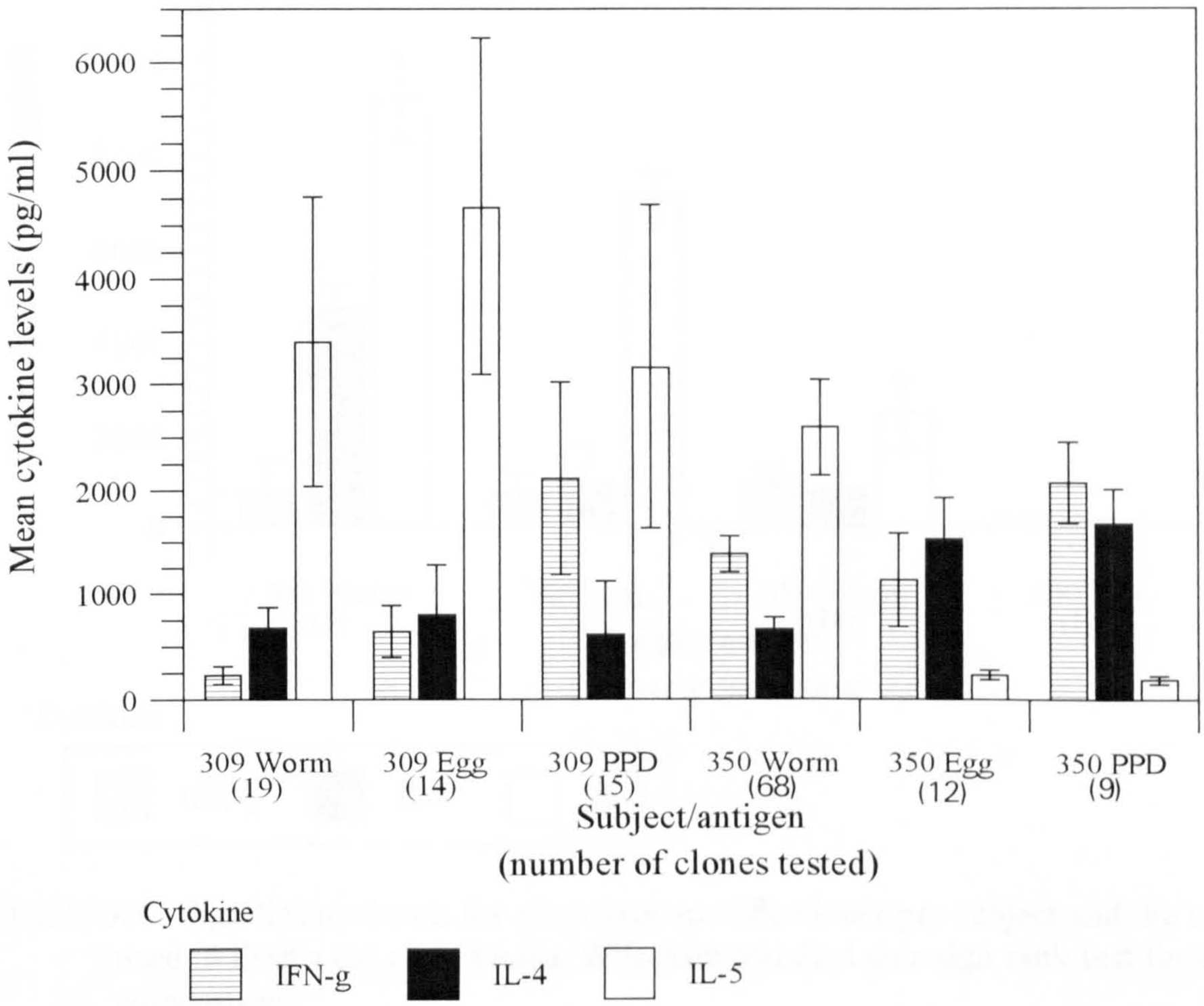


Table 3.3: Significance levels for all the derived clones per subject and antigen using Wilcoxon matched pair sign rank test for cytokine comparison.

Subject		**309**	infected		**350**	infected
Comparison	Worm	Egg	PPD	Worm	Egg	PPD
IL-4 vs IFN-γ						
n	19	14	15	68	12	9
p	**0.0218**	**0.4326**	**0.0007**	**0.0003**	**0.3882**	**0.5147**
z	-2.294	-0.7847	-3.4078	-3.6540	-0.8629	-0.6516
IL-4 vs IL-5						
n	19	14	15	49	12	9
p	**0.0001**	**0.0010**	**0.0007**	**0.0000**	**0.0047**	**0.0077**
z	-3.823	-3.2958	-3.4078	-4.5310	-2.8241	-2.6656
IFN-γ vs IL-5						
n	19	14	15	49	12	9
p	**0.0003**	**0.0043**	**0.1398**	**0.0150**	**0.0229**	**0.0077**
z	-3.5816	-2.8563	-1.4767	-2.4321	-2.2749	-2.2749

Figure 3.3: *S.haematobium* infected subjects:- 309 and 350. Mean cytokine levels (mean ±SE) produced by antigen specific clones according to subject and stimulation with worm and egg antigen. The number of clones tested is shown in brackets.

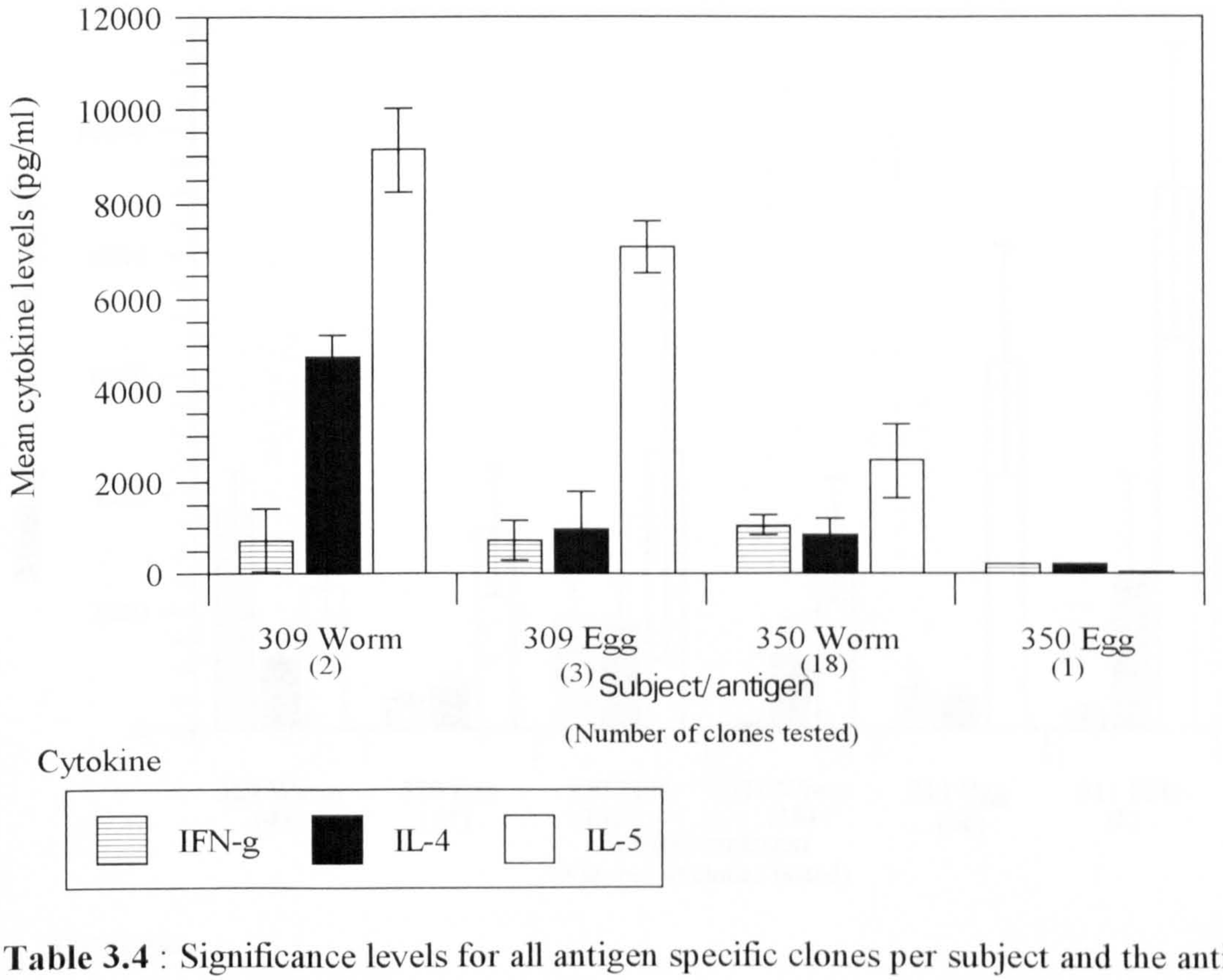


Table 3.4 : Significance levels for all antigen specific clones per subject and the antigen used to derive the clones using Wilcoxon matched pair sign rank test for cytokine comparison.

Subject	**309** (heavily infected)		**350** (moderately infected)	
Comparison	Worm	Egg	Worm	Egg
IL-4 vs IFN-γ				
n	2	3	18	1
p	**0.6547**	**1.000**	**0.0707**	-
z	-0.447	0.000	-1.807	-
IL-4 vs IL-5				
n	2	3	17	1
p	**0.1797**	**0.109**	**0.0004**	-
z	-1.342	-1.60	-3.527	-
IFN-γ vs IL-5				
n	2	3	17	1
p	**0.1797**	**0.285**	**0.2659**	-
z	-1.342	-1.07	-1.112	-

Figure 3.4 Uninfected subjects:- 329 and 311. Mean cytokine levels (mean ±SE) produced by all clones according to subject and stimulation with worm and egg antigen and PPD. The number of clones tested is shown in brackets.

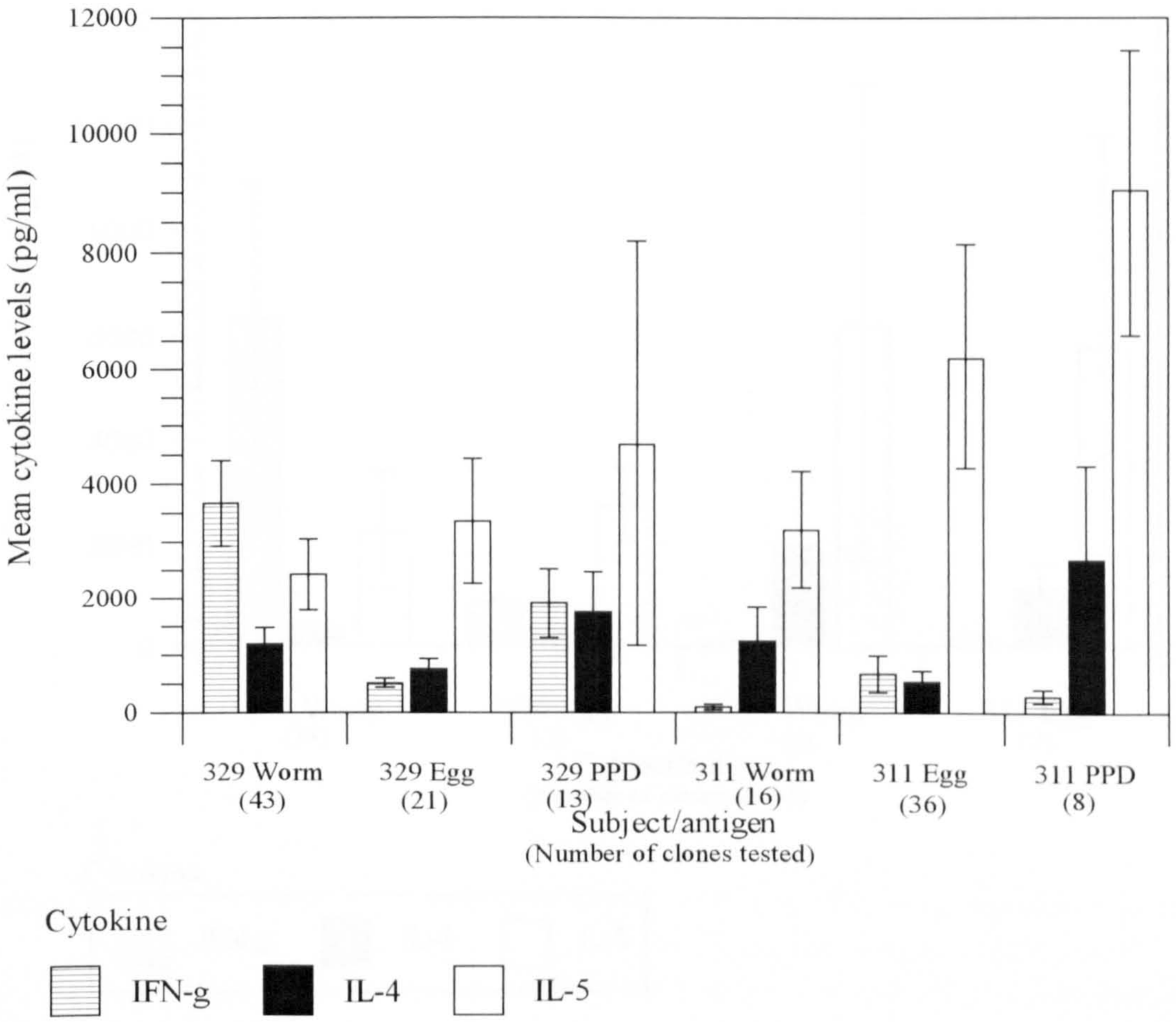


Table 3.5 : Significance levels for all the derived clones per subject and antigen using Wilcoxon matched pair sign rank test for cytokine comparison.

Subject	**329**			**311**		
Comparison	Worm	Egg	PPD	Worm	Egg	PPD
IL-4 vs IFN-γ						
n	43	21	13	16	36	8
p	**0.0001**	**0.7413**	**0.8068**	**0.0557**	**0.7894**	**0.7794**
z	-3.9485	-0.3302	-0.2446	-1.9132	-0.2671	-0.2801
IL-4 vs IL-5						
n	39	18	13	17	36	8
p	**0.0057**	**0.0074**	**0.8068**	**0.0003**	**0.0000**	**0.0117**
z	-2.7631	-2.6783	-0.2446	-3.6214	-5.1216	-2.5205
IFN-γ vs IL-5						
n	39	18	13	16	36	8
p	**0.0491**	**0.0018**	**0.4216**	**0.0005**	**0.0001**	**0.0251**
z	-1.9677	-3.1139	-0.8037	-3.4645	-3.9591	-2.2404

Figure 3.5: Uninfected subjects, 329 and 311: Mean cytokine levels (mean ±SE) produced by antigen specific clones according to subject and stimulation with worm and egg antigen. The number of clones tested is shown in brackets.

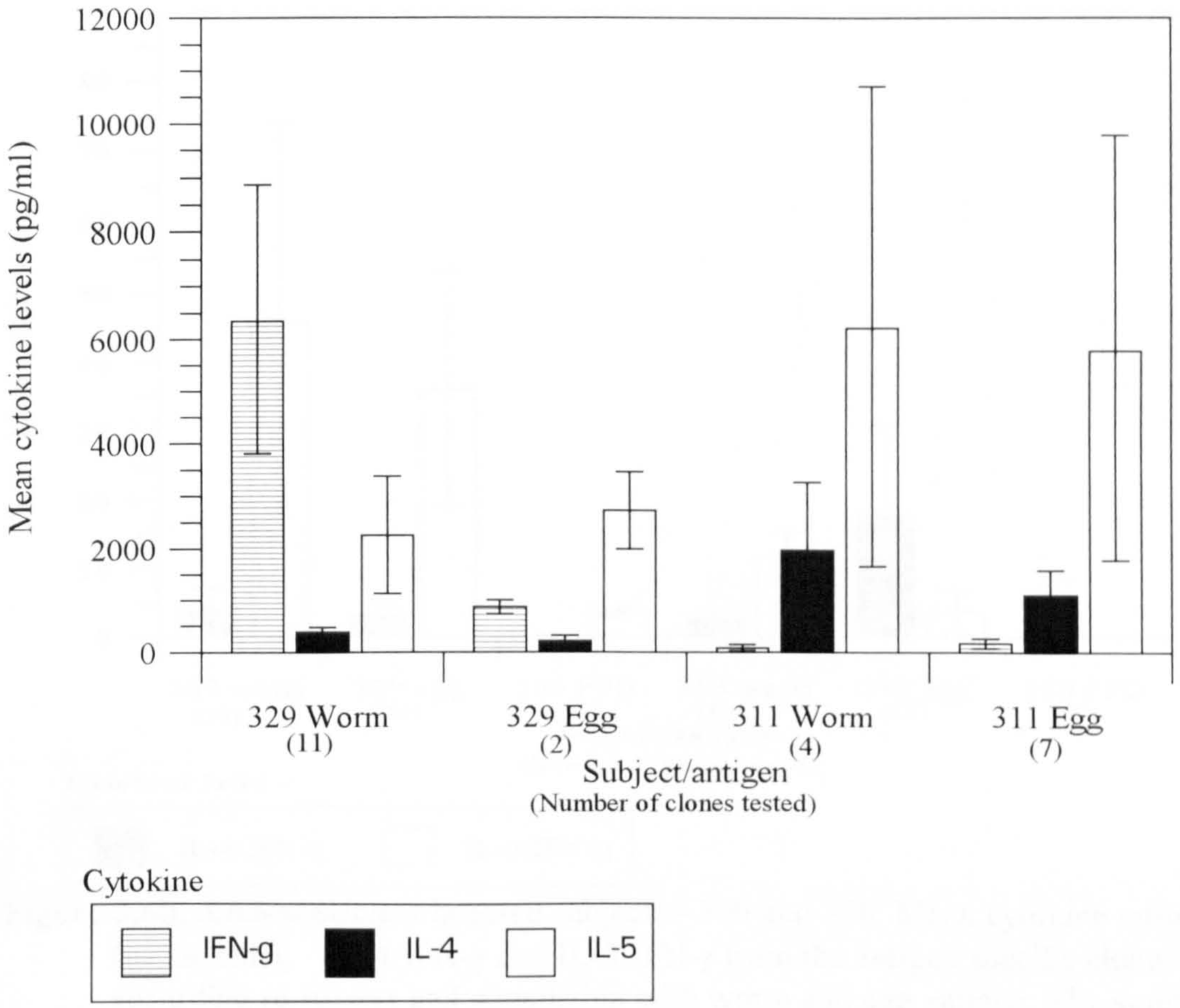


Table 3.6 : Significance levels for all antigen specific clones per subject and the antigen used to derive the clones using Wilcoxon matched pair sign rank test for cytokine comparison.

Subject	**329** uninfected		**311** uninfected	
Comparison	Worm	Egg	Worm	Egg
IL-4 vs IFN-γ				
n	11	2	4	7
p	**0.0044**	**0.1797**	**0.0678**	**0.0180**
z	-2.845	-1.3416	-1.8257	-2.3664
IL-4 vs IL-5				
n	11	2	4	7
p	**0.0033**	**0.6547**	**0.0679**	**0.0425**
z	-2.934	-0.4472	-1.8257	-2.0284
IFN-γ vs IL-5				
n	11	2	4	7
p	**0.0754**	**0.6547**	**0.0679**	**0.0180**
z	-1.778	-0.447	-1.8257	-2.3664

Figure 3.6a: *S.haematobium* infected subjects:- 309 and 350. Mean cytokine ratios (mean ±SE), IL-4:IFN-γ and IL-5:IFN-γ from all the clones according to subject and stimulation with worm and egg antigen and PPD. The number of clones tested is shown in brackets.

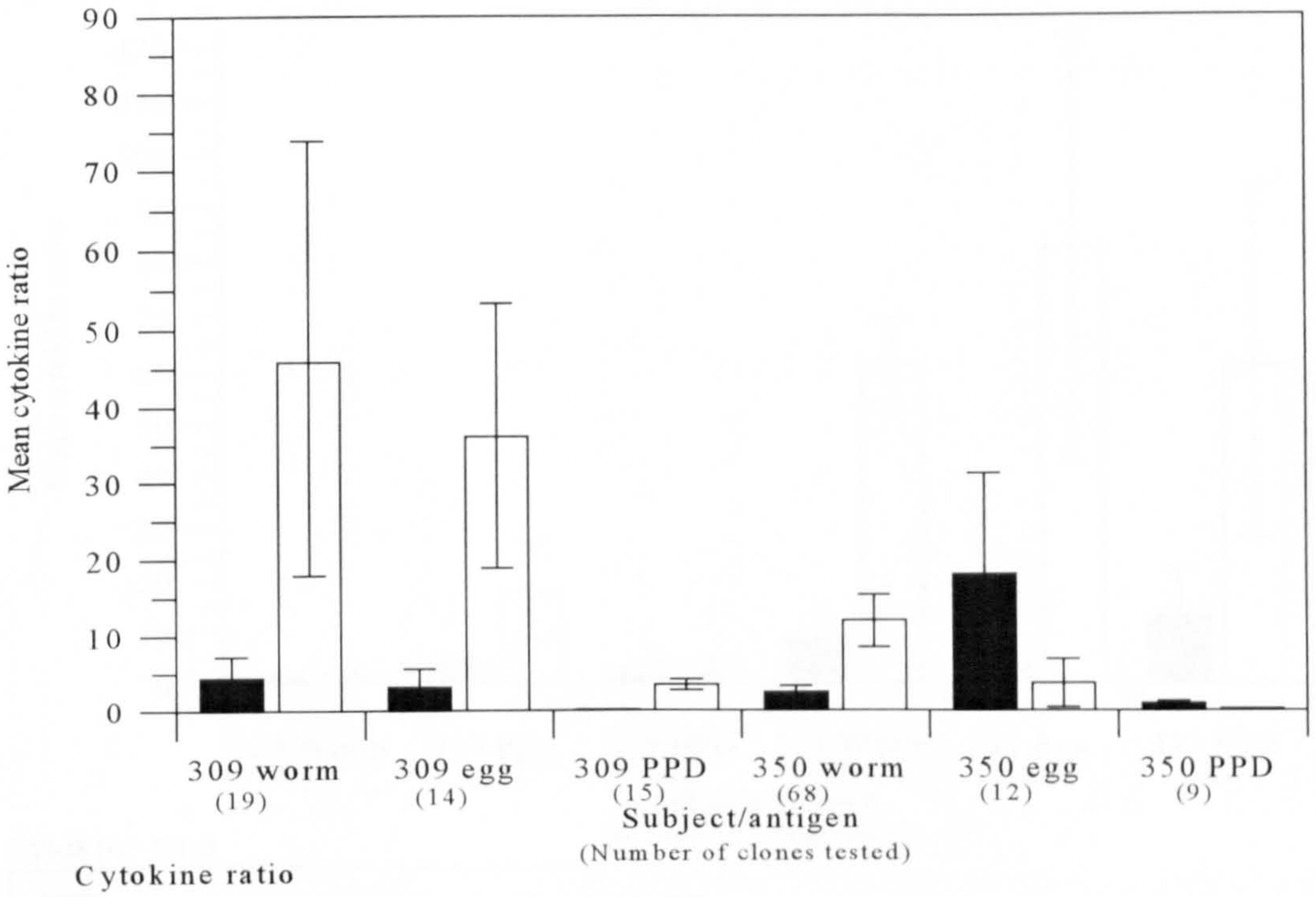


Figure 3.6b: *S.haematobium* infected subjects:- 309 and 350. Mean cytokine ratios (mean ±SE), IL-4:IFN-γ and IL-5:IFN-γ from the antigen specific clones according to subject and stimulation with worm and egg antigen. The number of clones tested is shown in brackets.

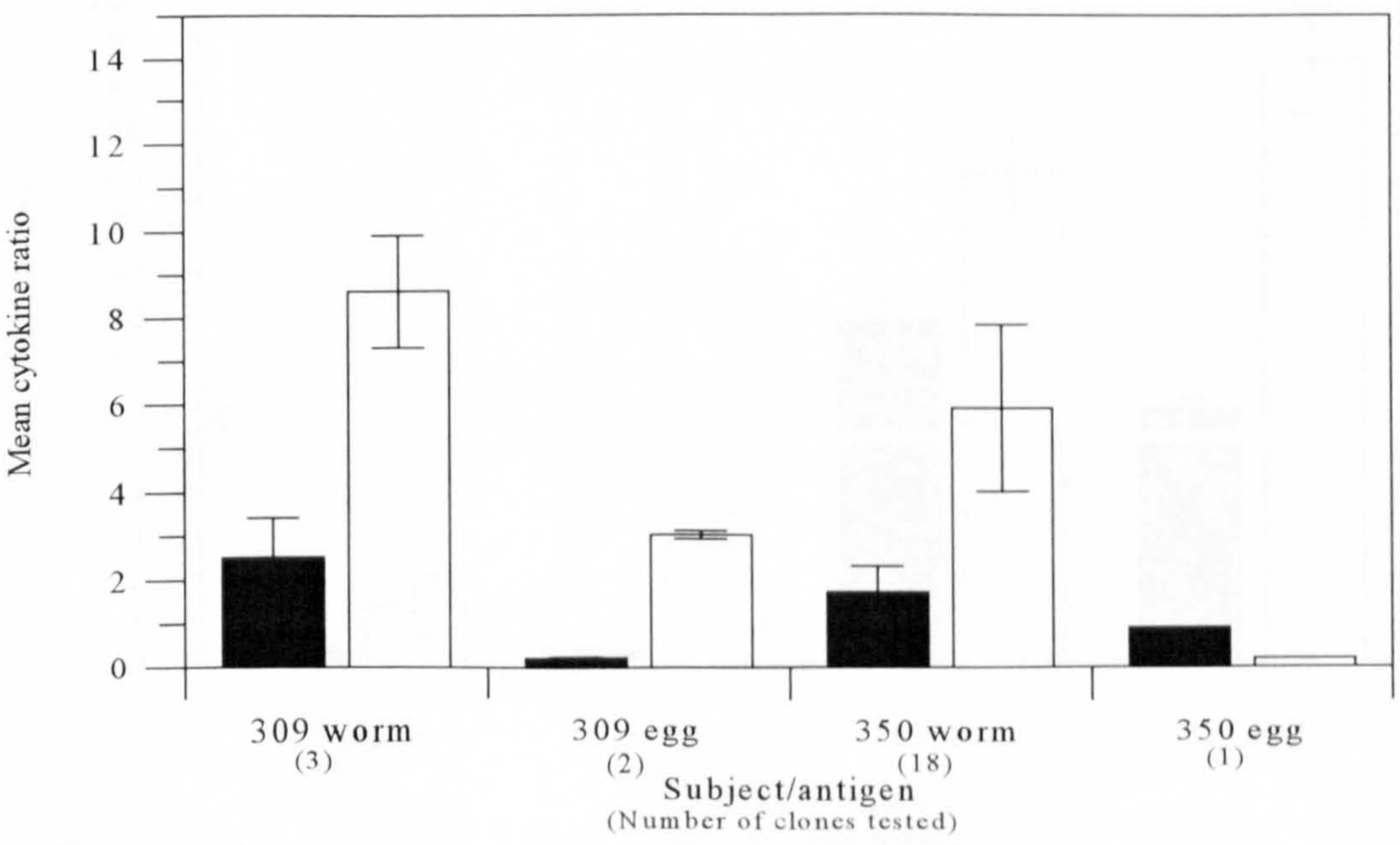


Figure 3.7a: Uninfected subjects:- 329 and 311. Mean cytokine ratios (mean ±SE), IL-4:IFN-γ and IL-5:IFN-γ from all the clones according to subject and stimulation with worm and egg antigen and PPD. The number of clones tested is shown in brackets.

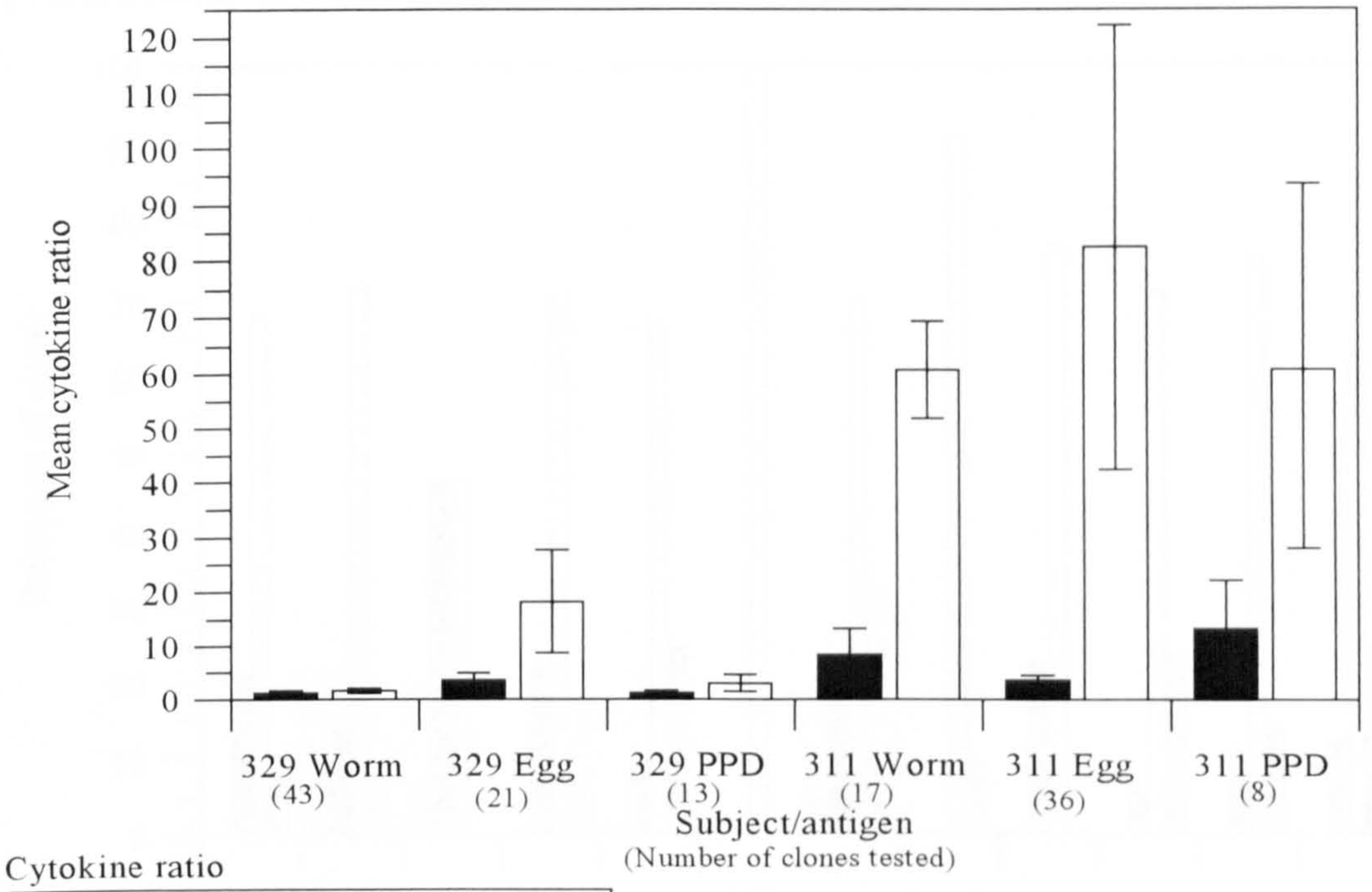


Figure 3.7b: Uninfected subjects:- 329 and 311. Mean cytokine ratios (mean ±SE), IL-4:IFN-γ and IL-5:IFN-γ from the antigen specific clones according to subject and stimulation with worm and egg antigens. The number of clones tested is shown in brackets.

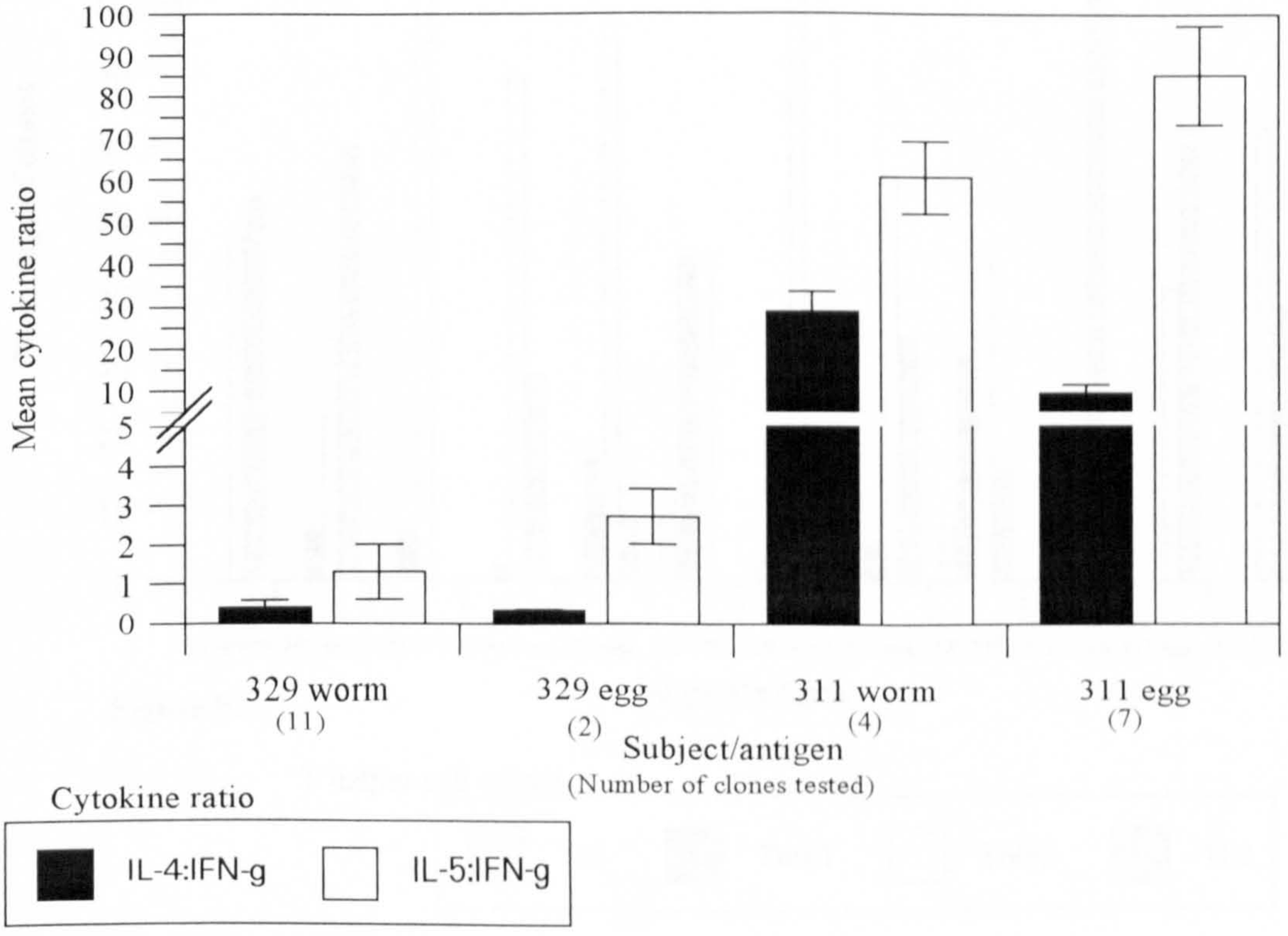


Figure 3.8: T helper cell dichotomy showing the percentage of clones in each subset according to the antigen used to stimulate the PBMC from each child for T cell clone derivation: **Figure a**. showing the number of T cell clones from the IL-4:IFN-γ ratio and **Figure b**. showing the number of T cell clones from IL-5:IFN-γ ratio.
(Total number of clones tested are shown in Table 3.2)

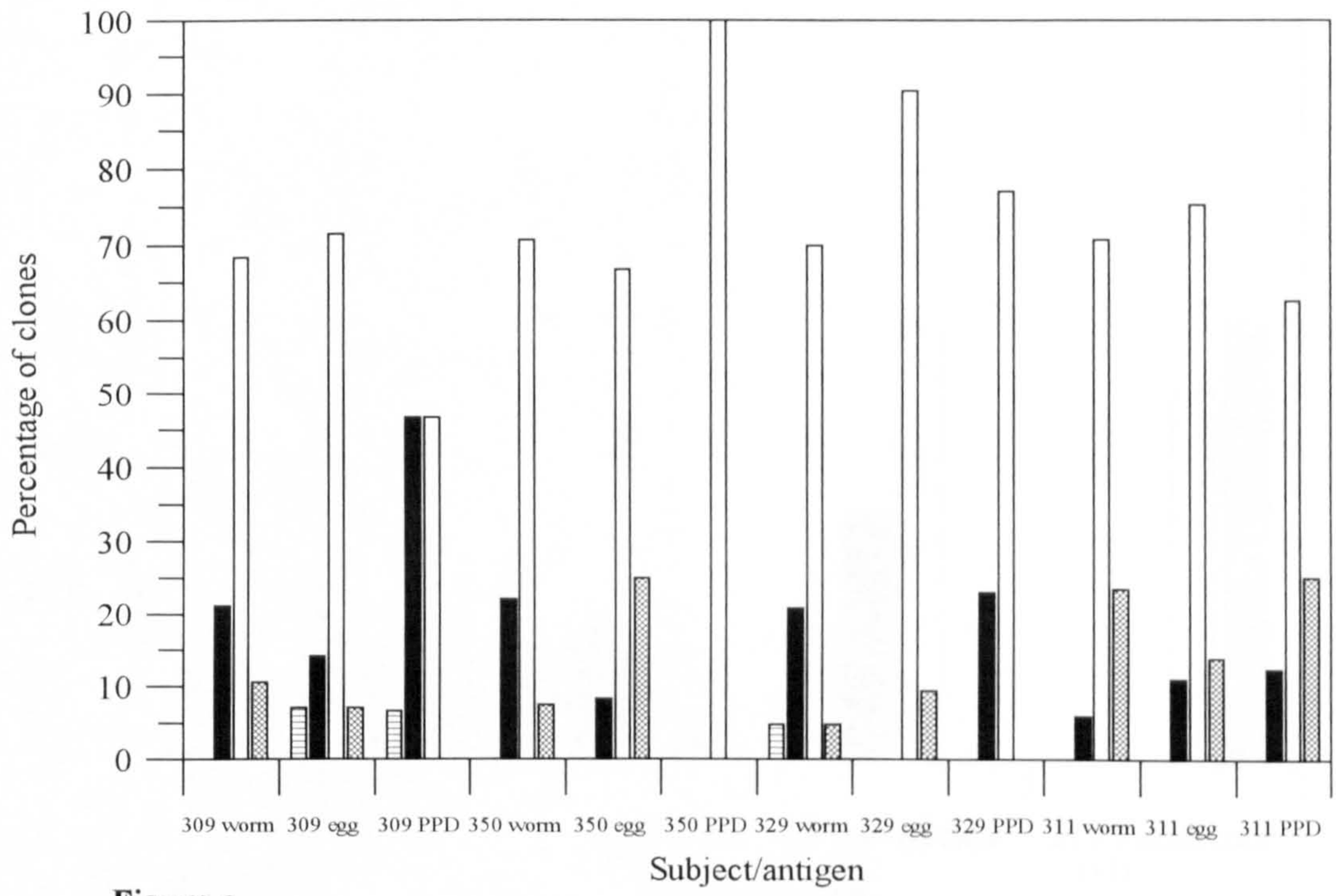


Figure a.

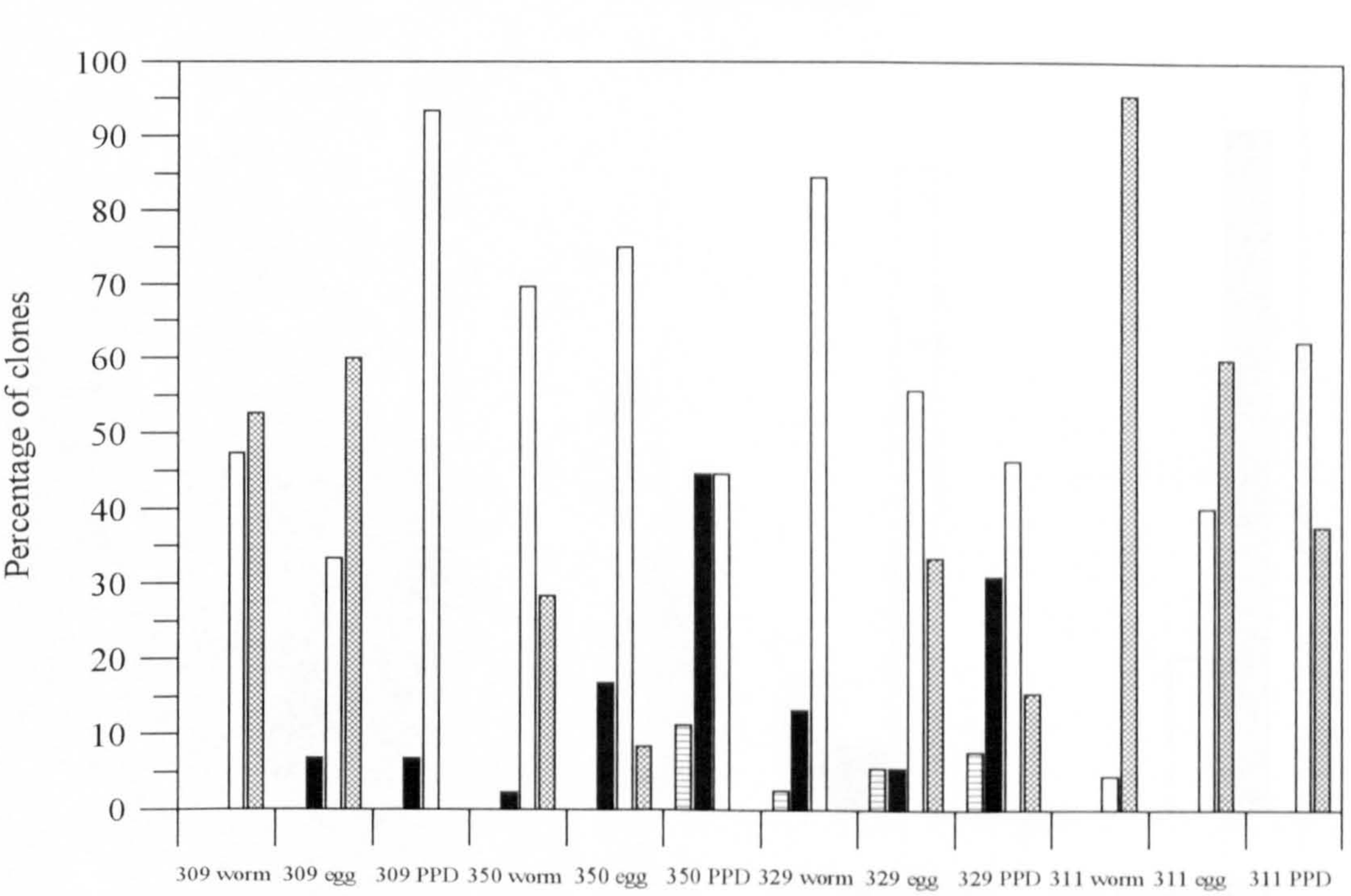


Figure b.

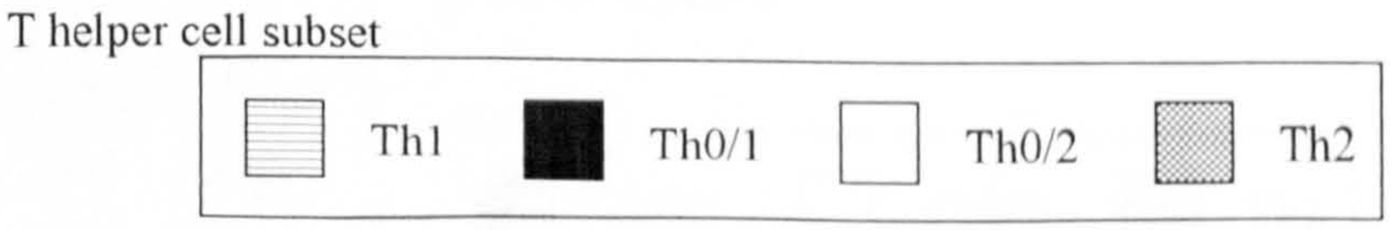


Figure 3.9: T helper cell dichotomy showing the percentage of clones in each subset according to subject source of the PBMC for T cell clones that were specific to the antigen used for their derivation: **Figure a**. showing the percentage of T cell clones from the IL-4:IFN-γ ratio and **Figure b**. showing the percentage of T cell clones from IL-5:IFN-γ ratio. The number of clones tested is shown in brackets.

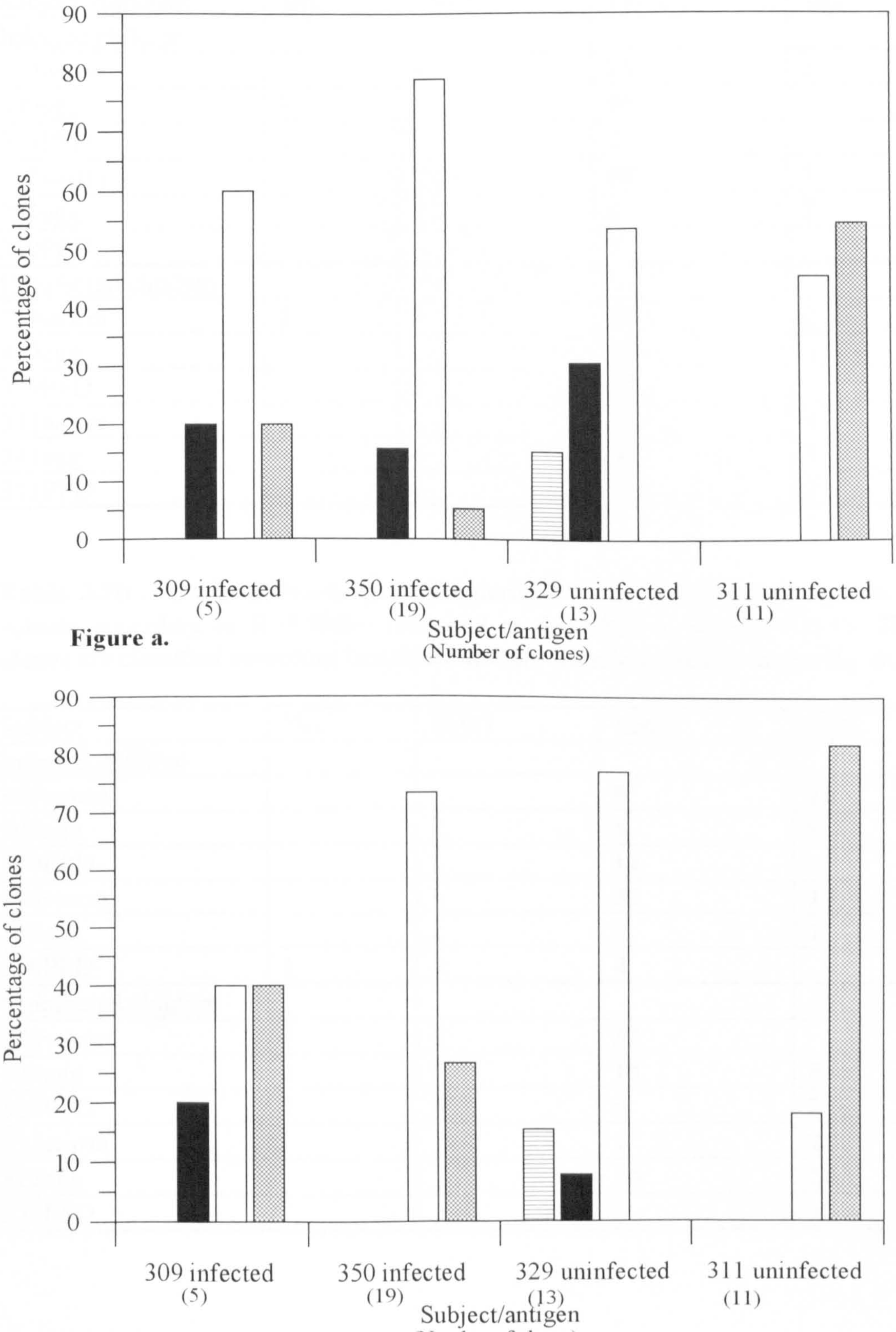


Figure a.

Figure b.

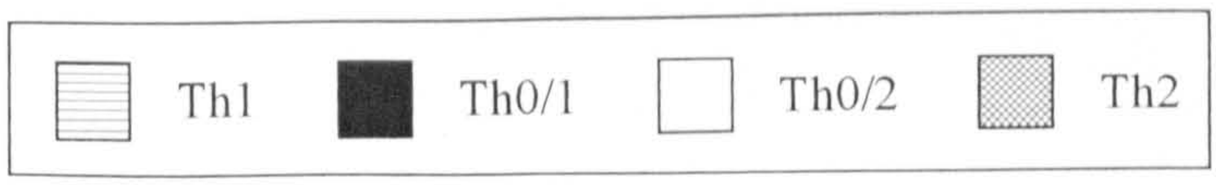


Table 3.7a : The distribution of all the derived T cell clones (number) into T helper subsets according to IL-4:IFN-γ ratio and as described in Section 3.2.13. The T cell clones are classified according to subject and the stimulant used to derive the clones.

Subject/stimulant	Th1	Th0/1	Th0/2	Th2
Infected children				
309worm		4	**13**	2
309egg	1	2	**10**	1
309PPD	1	**7**	**7**	
350worm		15	**48**	5
350egg		1	**8**	3
350PPD			**9**	
Uninfected children				
329worm	2	9	**30**	2
329egg			**19**	2
329PPD		3	**10**	
311worm		1	**12**	4
311egg		4	**27**	5
311PPD		1	**5**	2

Table 3.7b : The distribution of all the derived T cell clones (number) into T helper subsets according to IL-5:IFN-γ ratio and as described in Section 3.2.13. The T cell clones are classified according to subject and the stimulant used to derive the clones.

Subject	Th1	Th0/1	Th0/2	Th2
Infected children				
309worm			**9**	**10**
309egg		1	5	**9**
309PPD		1	**14**	
350worm		1	**32**	13
350egg		2	**9**	1
350PPD	1	**4**	**4**	
Uninfected children				
329worm	1	5	**32**	
329egg	1	1	**10**	6
329PPD	1	4	**6**	2
311worm			1	**21**
311egg			16	**24**
311PPD			**5**	3

CHAPTER 4

REPEATED PRAZIQUANTEL TREATMENT OF SCHISTOSOMIASIS IN CHILDREN UNDER SIX YEARS OLD LIVING IN MADZIWA; AN ENDEMIC AREA FOR *Schistosoma haematobium* INFECTION.

4.1 INTRODUCTION

Information on schistosomiasis infection from a number of different geographical settings indicates that infection intensities and the development of immunological resistance to infection are related, in some way, to age (Barsoum *et al.*, 1982; Butterworth *et al.*, 1984, 1985, 1991, 1992; Hagan *et al.*, 1987, 1991; Wilkins *et al.*, 1987; Dessein *et al.*, 1992 and Roberts *et al.*, 1993). Infection levels are normally highest in children and following chemotherapeutic cure, children are more rapidly reinfected and at higher rates than older children and adults (Wilkins *et al.*, 1987; Butterworth *et al.*, 1992). These higher rates of reinfection are in part related to higher levels of water contact by children, a feature characteristic of the majority of endemic settings (Barbour, 1985). While the severe clinical forms of schistosomiasis infection may take many years to develop, there is general agreement that targetting treatment to children can make a considerable impact on the development of pathology (Kloetzel, 1967; Sturrock, 1987). The use of chemotherapy to control schistosomiasis is usually based on annual rounds of screening and treatment. In many endemic settings this has been successful but in areas of intense transmission more frequent interventions could ensure a greater impact on infection levels.

In the past, schistosomiasis intervention programmes aimed at controlling schistosomiasis with chemotherapy were hampered by the treatment regimes which, with the drugs available at that time, necessitated several doses at intervals for a single treatment. One of the drugs used extensively for treating *S.haematobium*, for example, was metrifonate that is given in three doses at weekly intervals. In the recent past the prohibitive cost of praziquantel restricted its use, but now that the drug is being produced on a wider scale, and by a variety of manufacturers the cost has been reduced

to a level at which more frequent use can be contemplated. Frequent repeated parasite elimination with praziquantel may possibly reduce the pathology which normally acccompanies the repeated cycles of infection and reinfection in normal endemic foci. It could also conceivably influence the development of protective immune responses to infection.

Chemotherapy-induced changes in responses to infection have been reported on a number of occasions (Barsoum *et al.*, 1982; Ellner *et al.*, 1982; Sturrock *et al.*, 1983; Gazzinelli *et al.*, 1985; Colley *et al.*, 1986; Dunne *et al.*, 1992; Butterworth *et al.*, 1991; Demeure *et al.*, 1993; Roberts *et al.*, 1993; Grogan *et al.*, 1995, 1996), often as an increased lymphoproliferative ability of PBMC collected after treatment. The impact of frequent repeated treatments on immunological responses to sschistosome infection have, however, never been reported. Whether repeated treatments block the development of protective immune responses to infection or accelerate the development of protective immune responses has yet to be established. Studies of the effects of chemotherapy on other (intestinal) helminths indicate that the drug treatment can facilitate the development of resistance to reinfection (as reviewed by Wilson, 1993). Even if chemotherapy slows the development of immunological responses, this may be counterbalanced by the benefits to be gained from the reduction in levels of pathology arising from infection.

A similar dilemma has been addressed in studies designed to reduce the impact of malaria infection on pregnant women and on children (Picard *et al.*, 1993; Greenwood *et al*, 1994; Menendez *et al.*, 1994 and as reviewed by Garner and Brabin, 1994; Cot *et al.*, 1995). A positive effect of chemoprophylaxis on mean birth weight in primigravidae and

in grand multigravidae has been demonstrated in The Gambia, but the extent to which it can be generalised to other parts of the world is unclear. Evidence has been presented that chloroquine, proguanil and mefloquine prophylaxis was essential in reducing antenatal illness and malaria in children. In addition Cot *et al.*, (1995) in a study of randomised chloroquine chemoprophylaxis trials during pregnancy reported a reduction in malaria parasites in the placenta, even in conditions of chloroquine resistant malaria.

A child born in an area where schistosomiasis is endemic can be expected to harbour worms for most of his or her life owing to repeated exposure to infection, although some protective immunity is likely to develop, depending on exposure to the infectious agent (Woolhouse *et al.*, 1991). Schistosomes are known to survive in the host for long periods, about 3 - 8 years with some reports stating survival of up to 30 years (Warren, 1972, 1978; Vermund, Bradley and Ruiz-Tiben, 1983; Harris, Russel and Charters, 1984), despite the development of concomitant immunity. A key characteristic of schistosomiasis promoting this logevity is the development of several mechanisms by which the parasites evade or modulate the host's immunological attack. Since parasites and hosts may co-exist for lengthy periods, this might indicate that some degree of immunological tolerance or anergy become induced (Ottesen, 1979; Hofstetter, Fasano and Ottesen, 1983; Maizels *et al.*, 1993). It is because of the longevity of infection that the disease causes serious chronic morbidity rather than acute mortality, with severity related to worm burdens to which is related egg-laying (Warren, 1972; Weinstock, 1992; Weist, 1996; Gryseels and de Vlas, 1996). In addition to the longevity of individual infections, there is also persistence of infection due to repeated re-infection throughout the host life (Bradley and McCullough, 1973; Arap Siongok *et al.*, 1976).

It has been reported that most stages of schistosome infection may be identified by circulating antibodies. Some of these antibodies are known to be protective while others are believed to block the activities of the protective isotypes. The antibody isotypes of IgE and IgG4 have been reported to be produced in high levels during infections (Hagan *et al.*, 1991; Rihet *et al.*, 1991) and are affected differently by chemotherapy (Grogan *et al.*, 1995), but protection may be modulated by the IgG4 to IgE ratio which tends to be high in heavily infected individuals. Antibodies of the IgM and IgG2 isotypes, directed against carbohydrate determinants, also block responses to ADCC responses in schistosomiasis (Khalife *et al.*, 1986, 1989; Butterworth *et al.*, 1988). The presence of blocking antibodies is correlated with susceptibility to re-infection following chemotherapy of human population, whereas, expression of IgG1 and IgG3 antibodies to some epitopes correlated with resistance (Butterworth *et al.*, 1992). Understanding immune mechanisms, both protective and blocking, may assist in the development of other methods of control such as vaccination.

Effective vaccines are a long way from being developed, even when good candidates have been identified, they may take many years to pass through pre-clinical trials (Bergquist, 1994). In the absence of a vaccine, the most effective current method to combat the scourge of schistosomiasis is through targeted chemoprophylaxis of children (Hagan *et al.*, 1994). Even after mass chemotherapy, as reported from several programmes, the children become reinfected with heavy intensities such that there is a need for improved early diagnosis and treatment of young children in endemic areas who seem to lack developed immune systems against the disease (Sturrock *et al.*, 1983; Mahmoud *et al.*, 1983; Wilkins *et al.*, 1984a,b). A possible method to combat this

problem would be to keep the children under staggered treatment until they are of an age where they are capable of developing their own protective immunity.

This current study was designed such that transmission of infection would have continued. Older, and usually more heavily infected, children who normally have high levels of water contact were not part of the study and they can reasonably be assumed to be responsible for most of the environmental contamination which results in transmission of infection. As a result, the children in this study would have continued to have exposure to infection and therefore be exposed to repeated parasite challenges. The timing of the chemotherapy was designed to remove any adult worms which may have developed after the preceding treatment therefore minimising the number of eggs which may have been produced and thus reducing exposure of the children to egg antigens. The hypothesis on which this study was based was that the continued exposure to the worm antigens released by the praziquantel treatment, would result in the development of protective immunological responses, but the prevention of deposition of large numbers of eggs would ablate development of immunopathology. To monitor anti-schistosome responses, the children were examined for development of potentially protective, schistosome-specific antibody isotypes.

4.2 MATERIALS AND METHODS

4.2.1 STUDY AREA SELECTION AND STUDY POPULATION

The study was carried out in the Madziwa area in the high veldt in north eastern Zimbabwe. The Madziwa area is a rural setting based on seasonal small-scale commercial and subsistence farming. The living conditions of the people in the area are reasonably comfortable but sanitation facilities and water supplies for domestic use are not well

developed. The area was chosen because there was no evidence of infection with other helminths, a low *S.mansoni* prevalence and a high *S.haematobium* prevalence (Table 4.1). The villagers have unlimited use of river water. The area has several river tributaries which are seasonal and a major perennial river running through the settlements.

Madziwa lies about 100 km north-east of the capital city Harare and has been studied by the Blair Laboratory for a number of years (Chandiwana, 1987a,b; Chandiwana, Makaza and Taputaira, 1987; Chandiwana and Christensen, 1988). Information from previous research formed the basis of the initial plan for the study. There was good background information on this region, and only a limited amount of intervention had been carried out in the form of chemotherapy to all infected school children in 1991, four years prior to the start of this study. This current study was focused on pre-school children attending pre-school centres within 2 to 6 years old age range, who would not have participated in the previous intervention. However, with the help of the local Environmental Health Technician, some younger children, below pre-school age, were brought to the centres by their mothers and participated in the study. This was facilitated through community meetings between the research group and the parents of the children when consent was obtained. Due to previous work in the area, the community and especially the mothers, were already aware of the schistosomiasis problem. This resulted in a high level of participation.

4.2.2 PARASITOLOGY

Urine samples for parasitological examination for *S.haematobium* infections were collected from all the children on three consecutive days and were processed on the day of collection. Samples were collected between 10.00 hours and 14.00 hours to coincide

with peak egg excretion in urine (Warren, 1978). Urine samples were examined for urinary schistosomiasis by filtration of 10 ml samples after thorough mixing (Mott *et al.*, 1980, 1982) using polyamide 13 mm Nytrel™ filters and 5-10% Lugol's iodine stain, to help visualise the eggs. The filters were examined for eggs of *S.haematobium*, and expressed as eggs per 10 ml of urine (ep10ml). Intensity of infection was classified as negative - no eggs detected; light - <10 ep10ml; moderate - 10 to <50 ep10ml; or heavy - ≥50 ep10ml.

A stool sample was examined by the Kato-Katz technique (Katz *et al*, 1972 as modified by Peters *et al*, 1980). The prevalence of *S.mansoni* was known to be low, and those found to be infected with *S.mansoni* were excluded from the study.

4.2.3 COLLECTION OF VENOUS BLOOD FOR SERA

Prior to the first treatment, up to 2 ml of blood was collected by venepuncture using vacuum bleeding into vacutubes™. Blood was allowed to clot at room temperature, then stored overnight below 8^0C, the clot removed and the sera obtained by centrifugation at 300xg for ten minutes. The sera were stored at -20^0C in cryotubes until required for assays.

4.2.4 REPEATED TREATMENT WITH PRAZIQUANTEL

After collection of demographic data, three parasitology samples were collected over three consecutive days, after blood collection, the children were treated with 40 mg/kg body weight regardless of their infection status. Every eight weeks, after further collections of parasitology data and blood samples were made and the children were retreated on each occassion. During treatment, the nurse and research team observed

each child take the drug which was normally given with an orange juice drink and some bread. This was repeated during the follow-up period of 14 months for this aspect of the study. The study design was approved by the Medical Research Council of Zimbabwe and the Zimbabwe Ministry of Health ethical committee.

4.2.5 HUMORAL ASSAYS BY ELISAs

The optimum conditions for the assays used for antibody detection were initially set up using sera from another study area which had study populations ranging from 5 - 64 years old (Mutapi *et al*, 1997). For this current study, some confirmation and modification of the concentrations were carried out by titration of several dilutions of the sera from the study population. Some modifications were made of antigen concentrations, serum dilutions and incubation times. These are shown in the Appendix 6.41 and 6.42. The cohort for humoral assays was selected on the basis of those providing parasitological samples, who were free of *S.mansoni* infection and who were examined at each follow-up point.

4.2.5.1 ANTIGEN PREPARATION

Schistosoma haematobium egg and worm antigens were obtained from Schistosome Biological Supply Programme, Theodore Bilharz Research Institute, Giza (Egypt), with the egg antigens in soluble form. The worm antigen was obtained as intact freeze dried adult worms which were then homogenised by grinding in PBS buffer pH 7.4, centrifuged three times at 400xg for 25 minutes and the total protein concentration determined using Bicinchoninic Acid (BCA, Pierce) as outlined in the method supplied by the manufacturer (Smith, 1985). The SEA concentration was determined by the same method.

4.2.5.2 ANTIBODY DETECTION USING WWH

The whole worm homogenate was diluted in carbonate bicarbonate buffer (pH 9.6) to a concentration of 5 µg/ml and distributed as 100 µl of the antigen-buffer mixture per well in Immulon 4 plates (Dynatec Ltd) then left to react overnight at 4°C. The plates were then blocked with 200 µl per well of 5% skimmed milk for an hour at room temperature on a shaker. The plates were then washed three times with PBS, pH 7.4. Dilution of sera was carried out in PBS buffer at pH 7.4 and 100 µl aliquots placed in duplicate wells. The sera dilutions were 1:10 for IgE, IgG1, IgG2 and IgG3; 1:100 for total IgA, total IgG and IgG4; while for IgM it was 1:200. The plates were then incubated for two hours at room temperature on a shaker while for IgE assays the incubation was continued overnight at 4°C. Then the plates were washed three times and 100 µl of monoclonal conjugate specific for each antibody isotype was added in PBS. For all the isotypes, the monoclonal conjugates were horse radish peroxidase (HRP) conjugated and the final dilution was 1:2000. The monoclonal antibodies were obtained from Sigma, total IgG, total IgA and IgM; from DAKO, IgE and from Serotec all the IgG subclasses. The plates were incubated for one hour at room temperature except for IgE where incubation was extended overnight at 4°C. After washing four times, 100 µl of OPD substrate was added per well and allowed to react in the dark for total IgA, total IgG, IgM, IgG3 and IgG4 for 20 minutes, IgE for 30 minutes and IgG1 and IgG2 for 3 hours (Appendix 6.41). On each plate, standard pools were assayed together with the test samples. The standards were a pool of sera obtained from individuals from an area with high egg counts and a negative standard using sera from Norwegians. Duplicate wells were left with no sera as the blanks. The reaction was stopped with 25 µl of 10% H_2SO_4 and the absorbance read at 492 nm.

4.2.5.3 ANTIBODY DETECTION USING SEA

The ELISA detection of anti-egg antibodies was carried out using a protocol similar to that used in the WWH ELISA. The Immulon plates were coated with 100 µl of antigen at 10 µg/ml diluted in carbonate-bicarbonate buffer (pH 9.6) per well and left to react overnight at 4°C. Sera and conjugate dilutions and incubation times were similar to the assays for anti-worm antibodies (Appendix 6.26). Washing times and stages were also similar.

4.2.6 STATISTICAL METHODS

At pre-treatment parasitological examination, heavy infections were detected in some children while at the follow-up time points only very low levels of infection were recorded. Since the parasitology and antibody assay results were mostly not normally distributed, non parametric tests were applied and the Spearman rank correlation used. Kruskal-Wallis one-way ANOVA was used to compare the antibody isotype profiles obtained at each treatment point. The Mann-Whitney Rank sum test was used to compare the infected and uninfected categories before treatment. The Rank sum test was also used to compare the antibody isotypes levels between the two schistosome antigens, WWH and SEA at any of the follow-up points.

4.3 RESULTS

4.3.1 STUDY POPULATION AND PARASITOLOGY

Results from preliminary parasitological surveys of the children attending schools near the pre-school centres indicated that the area had high *S.haematobium* infection with some *S.mansoni* infection (Table 4.1). After examination of the preliminary sampling, the centres at the following schools were selected: Chimbira, Chihuri and Mupfure. For Mupfure primary school, two other centres in the school's catchment area were included, as the prevalence of infection in them was over 90%. At the start of the study, a total of 595 children between 2-6 years old were recruited. Blood samples could be obtained from only 32.8% (195), and at the 14 month follow-up about 42.1% (82) could be sampled of which 28.2% (55) provided blood samples.

NON HUMORAL ASSAY PARTICIPANTS

A total of 595 children below 6 years old were included in the study from the different pre-school centres identified by a primary school centre. The prevalence of infection was about 60% before treatment (at the start of the study). After the first treatment follow-up, the prevalence of infection was reduced to below 9% which later dropped to below 1% by 12 months follow-up (Figure 4.1). After the initial parasitological examination of the 595 examined children, the mean intensity of infection was about 110 eggs per 10 ml of urine. However, during the follow-up examination, the intensity of infection was low (Figure 4.2).

HUMORAL ASSAYS COHORT

The prevalence of infection patterns with follow-up time is shown in Figure 4.3. At the beginning of the study, before treatment, the prevalence of infection was about 51.8%

which was different from the follow-up prevalences after treatment, which were below 20% with the last examination showing no infected children.

The intensity of infection of the cohort was moderate before treatment giving a mean egg count of 18 ep10ml urine. After treatment the intensity of infection was less than 4 ep10ml of urine at 2 months and 9 ep10ml of urine by 4 months. Thereafter infection intensity was below 2 ep10ml of urine examined (Figure 4.3b). The children who were infected and belonging to the cohort for the humoral assays had moderate to heavy infection intensity (Figure 4.4). Before treatment of the infected children, the intensity of infection among them was 35 ep10ml of urine, the results of the infection intensity (among the infected only) are shown in Figure 4.4.

4.3.2 TREATMENT

Madziwa is an area endemic for schistosomiasis which is a major health problem since the area suffers from a lack of developed water for domestic use. Even after provision of treatment to the children some infection still occurred and the few reinfected children showed moderate to heavy infection intensities. Treatment with praziquantel was shown to be effective, however, at population level few individuals became reinfected at moderate intensity, in the eight week periods between re-treatments and re-examinations.

4.3.3 HUMORAL IMMUNITY - ANTIBODY PROFILES.

4.3.3.1 ANTI-EGG ANTIGEN ANTIBODY ISOTYPE LEVELS.

The cohort that provided sera and were treated at consecutive follow-ups had their sera assayed for these isotypes: IgA, IgE, IgG1, IgG2, IgG3, IgG4 and IgM. At each follow-up a comparison was made between the antibody responses of infected and the

uninfected children, even though the samples sizes varied, with only a few children becoming reinfected after treatment. On comparison of antibody responses of infected and uninfected children at any time point, no differences were observed for any isotype (Figure 4.5 - 4.11). Also, no differences were observed when comparing either infected children at different time points or uninfected children at different time points (Figure 4.5 - 4.11).

4.3.3.2 ANTI-WORM ANTIGEN ANTIBODY ISOTYPE LEVELS.

Only four isotypes (IgA, IgE, IgG4 and IgM) were assayed against the worm due to availability of the antigen and sera. No differences in antibody isotype levels were observed between the antibody responses of infected children at different time points or the uninfected children at different time points (Figure 4.12 - 4.15). Also, no differences were observed when comparing either infected children at different time points or uninfected children at different time points (Figure 4.12 - 4.15).

4.3.3.3 ANTIBODY RESPONSE PROFILES WITH TIME AND CORRELATION

Prior to praziquantel treatment the antibody responses of both infected and uninfected children to WWH and SEA antigens showed similar patterns (Figure 4.16a and 4.16b). The antibody profiles for each isotype against egg and worm for all the children examined with follow-up time showed similar patterns at each time point for the antigens used (Figure 4.18). The patterns showed higher levels of anti-egg IgE than anti-worm IgE at all the time points (Figure 4.18b). Of interest were the high correlations, using Spearman's rank correlation, of anti-egg IgE and anti-egg IgG4 to infection intensity measured as egg output at the start of the programme (Table 4.2, when all the children were considered $p=0.013$ and $p=001$ for IgE and IgG4, respectively). The infected

children showed a high correlation of anti-egg IgG4 to egg output and anti-worm IgM to egg output ($p<0.0001$ and $p=0.035$, respectively, Table 4.2).

Correlations were observed between infection intensity (egg output) and anti-egg IgA and IgG4 ($p=0.050$ and $p=0.027$, respectively) at four months after the treatment programme began. At 10 months, egg output and anti-egg IgE ($p=0.032$) were positively correlated yet infection intensity was low (Table 4.2), while at remaining examination points no correlations were found.

In general the patterns of the antibody isotypes profiles to WWH and SEA antigens were similar throughout the repeated treatment programme. Of interest was the difference between the anti-worm IgE and anti-egg IgE responses. Higher levels of anti-egg IgE antibodies were detected than anti-worm IgE antibodies throughout the study (Figures 4.16a, b, c; 4.17a-f). Anti-egg IgG3 and anti-egg IgG2 antibodies were detected at lower levels than any of the other isotypes at all of the examination points (Figures 4.16a-c; and 4.17a-f).

4.4 DISCUSSION

The results from the preliminary examinations indicated that the area has a major problem with schistosomiasis. Children living in this area were heavily exposed to infection. After selection of the study cohort, defined as those below school-attendance age, the results indicated that even among the young children the prevalence of infection was high and this was coupled with heavy infection intensities. For background information after obtaining these results, mothers who brought their children to the

centres were asked as to how their children became infected. One of their common answers was that these children accompanied their mothers to the rivers during their daily activities of tending vegetable gardens and some domestic chores like washing dishes and clothes and also bathing. They reported that during these activities the children had the opportunity to have contact with the water, thereby becoming exposed and ultimately infected.

Madziwa area was identified as one of the areas in Zimbabwe with high *S.haematobium* infection with some areas within the region having some *S.mansoni* infection (Taylor and Makura, 1985; Chandiwana, Makaza and Taputaira, 1987; Preliminary results, Table 4.1). The results obtained at the start of the study indicated that the children are at very high risk of the schistosome infections and the likely accompanying morbidity and pathology. Praziquantel is not available at the local clinics and treatment is instead with metrifonate, mothers rarely take the children back for the second and third doses. From the current study it became apparent that even after controlling schistosomiasis with retreatment in areas of intense transmission some children will became reinfected, most of those reinfected was at moderate to heavy intensity. The results confirmed similar findings in a *S.haematobium* study, in The Gambia by Wilkins and colleagues (1987) where children having frequent contact with water containing infected snails became reinfected at higher infection intensities than the adults.

When examining humoral responses, no major variations in the antibody isotypes were noted, possibly due to the age of the children. The development of demonstrable protective immunity has been reported to take place in older children above 10 years old (Butterwoth *et al.*, 1984, 1985, 1987, 1992; Wilkins *et al.*, 1987; Hagan *et al.*, 1987,

1991, 1994; Roberts *et al.*, 1993, Webster, *et al.*, 1997a,b). The correlations of IgE and IgG4 antibodies with infection probably indicates that they are a marker of infection intensity rather than an indicator of resistance to infection. Of the two isotypes, IgE was detected at higher levels compared with the IgG4 in response to egg antigen. But no major change in the antibody isotype profiles with time was seen, since the children in this study were below six years old. This may point to a possibility that development of protective immunity is not only duration of exposure and infection intensity dependent but age may also be necessary on which will depend the experience of schistosome parasite infection. This later point may support the report that residents in a new *S.mansoni* epidemic focus in Senegal (Gryseels *et al.*, 1994a, 1994b, 1995) lacked clear developed protective immunity after treatment irrespective of age suggesting that experience of infection acquired over a long period plays a major role in the development of protective immunity.

The isotype profiles showed small biological trend changes with the treatments. In other studies, greater changes (statistically significant) have been reported. Mutapi *et al.*, (1997 and submitted) reported that antibody isotype class switching of IgA to IgG1 take place in younger individuals after treatment. This change, which may be associated with resistance or susceptibility, has been documented as being protective when worm induced and pathological when egg induced. But age may be involved in development of such protection development since this was not demonstrable in the young children in this study. The close repeated treatment may not have assisted in any change of the antibody isotypes, perhaps challenge (repeated exposure) is more important in determining antibody responses. This can be achieved possibly by a wider treatment

intervals, although giving no opportunity for the worms to establish a stage of massive egg deposition.

Chemotherapy with praziquantel can be beneficial in the young children living in schistosomiasis endemic areas as found in this study showing reduction in prevalence and intensity of infection which become low throughout the follow-up. Even though the two monthly repeated treatment was very effective, with indications of a drastic reduction in prevalence and intensity of infection soon after the begining of the programme, it is possible that similar effects might be achieved when the treatment intervals are increased and this would warrant further investigation. The study has clearly demonstrated that, repeated treatment can be used to remove infections and minimise production of eggs. The egg are the main cause of pathology. But it is not clear whether removal of pathology also caused failure of development of protective immunity.The results can be interpreted as lack of development of humoral indices of protective immunity of IgE, IgG1, IgA may suggest a decline in immunity with repeated treatment due to unavailability of antigen in the immune system. Clear intepretation is difficult due to unavailability of a parallel group of children not receiving praziquantel. Follow-up of an untreated group of children was against the ethics of the Zimbabwe Medical Research Council and Ministry of Healthy ethical committees. However, there is a possibility of a follow-up of the treated children for a period of time after treatment is stopped to observe any imunological development pattern which is beyond the limits of this study.

Table 4.1: Results of a preliminary parasitology survey from six schools in the Madziwa area marking each central point in the catchment area for the study. The prevalence of *S.haematobium* determined by urine filtration of three samples collected on three consecutive days. *S.mansoni* infection prevalence determined by Kato-Katz method of three stool samples collected on three consecutive days.

School centre	No. of children examined	*S.haematobium* Prevalence% (n)	No. of children examined	*S.mansoni* Prevalence% (n)
Madziwa	97	42.71 (41)	86	4.65 (4)
Chihuri	95	71.58 (68)	100	18.0 (18)
Mupfure	90	90.0 (81)	74	28.38 (21)
Nyamaruro	97	66.0 (64)	96	14.58 (14)
Chimbira	99	61.62 (61)	97	12.37 (12)
Kaziro	99	58.59 (58)	97	7.22 (7)
Total	**577**	**64.64 (373)**	**550**	**13.82 (76)**

Table 4.2: The correlation between different antibody isotype profiles (measured in O.D at 492 nm) to egg output (infection) at each examination and treatment point.

Antibody isotype	before treatment			2 months after			4 months after		
	No.	Spearman coefficient	P-value	No.	Spearman coefficient	P-value	No.	Spearman coefficient	P-value
Anti-egg									
IgA	83	-0.0931	0.402	35	0.0910	0.603	66	-0.2421	**0.05**
IgE	83	0.2705	**0.013**	35	-0.3990	**0.018**	49	0.3070	**0.032**
IgG1	31	0.1120	0.549	13	0.1572	0.608	21	0.0000	1.000
IgG2	56	0.1587	0.243	23	-0.3574	0.094	43	0.0786	0.616
IgG3	23	-0.0067	0.976	7	0.1786	0.702	16	-0.3349	0.205
IgG4	83	0.3571	**0.001**	35	0.1322	**0.449**	62	0.2810	**0.027**
IgM	83	0.1163	0.295	35	-0.2690	0.118	66	0.2157	0.902
Anti-worm									
IgA	83	0.0762	0.494	35	0.0838	0.632	66	-0.0546	0.664
IgE	83	0.3695	**0.001**	35	-0.0698	0.690	55	0.3453	**0.010**
IgG4	83	0.0589	0.597	35	0.2238	0.196	66	0.0468	0.709
IgM	83	-0.0494	0.657	35	-0.0430	0.806	65	-0.0886	0.483

Table 4.3: Number of children examined for *S.haematobium* infection and number of children in the humoral assays cohort after parasitology examination and provision of blood samples. The numbers shown represents the total number of children assayed for the antibody isotype at each follow-up time point and in each infection status group.

Follow-up time (Months)	Total number examined	Number infected (%)	**Total number in cohort**	Number infected (%)
Start (Pre-treatment)	595	357 (60.0)	**83**	43 (51.8)
2	465	32 (6.7)	**35**	5 (14.3)
4	567	34 (6.0)	**66**	12 (18.2)
6	209	5 (2.4)	**55**	3 (5.5)
8	220	5 (2.3)	**47**	2 (4.3)
10	230	5 (2.2)	**49**	3 (6.1)
14	246	3 (1.2)	**25**	none

Figure 4.1: Prevalence of infection of the children examined including the children in the study cohort for humoral assays with the follow-up time. The prevalence determined by examining eggs in three 10 ml urine samples collected over three consecutive days. (The number of children examined at each time point is shown on the bar).

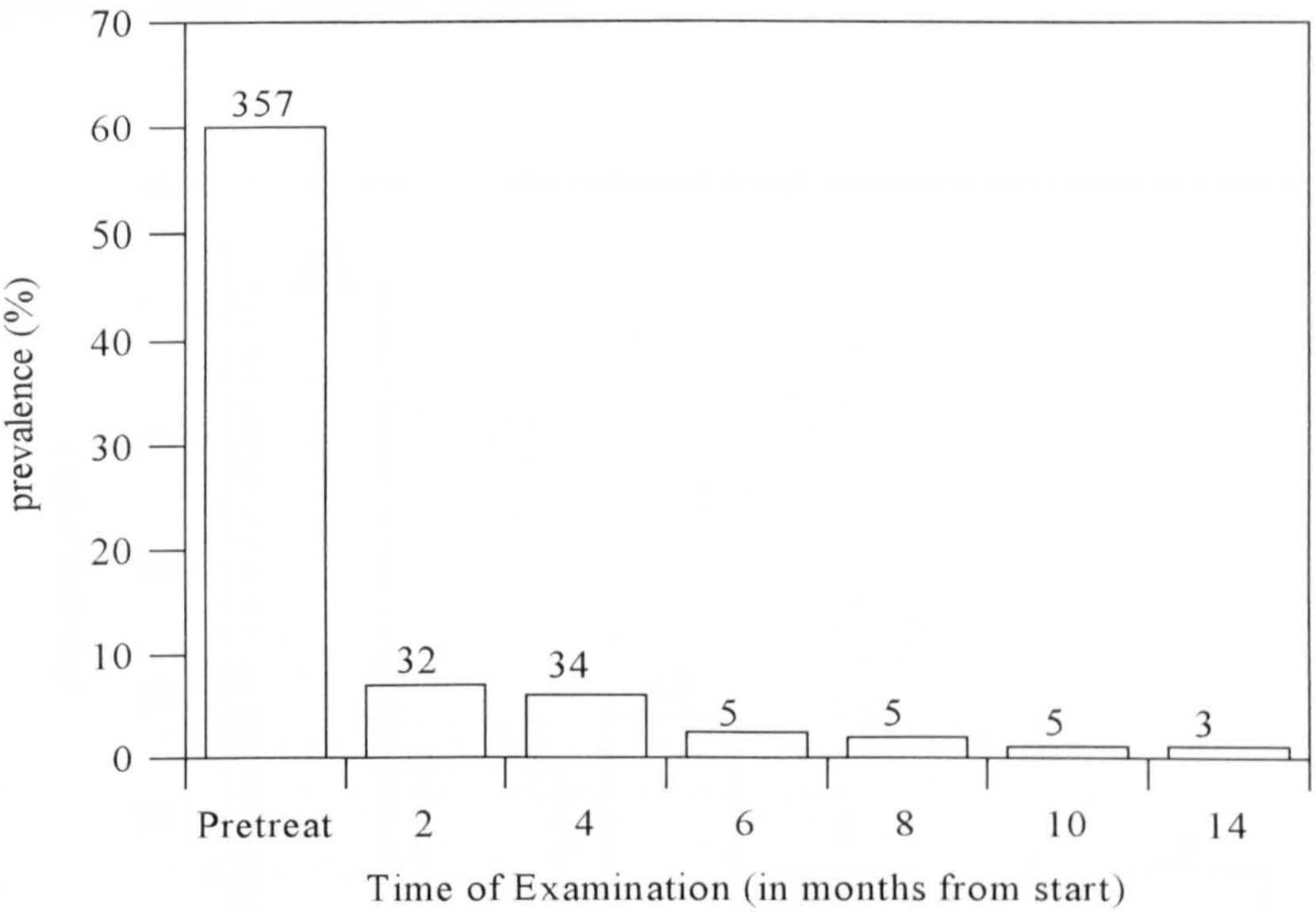


Figure 4.2: Intensity of infection (mean ep10ml, ±S.E.) of the children examined including the children in the study cohort for humoral assays with the follow-up time. The intensity of infection determined by examining eggs in three 10 ml urine samples collected over three consecutive days. (The number of children examined at each time point is shown in Table 4.3 and the graph above).

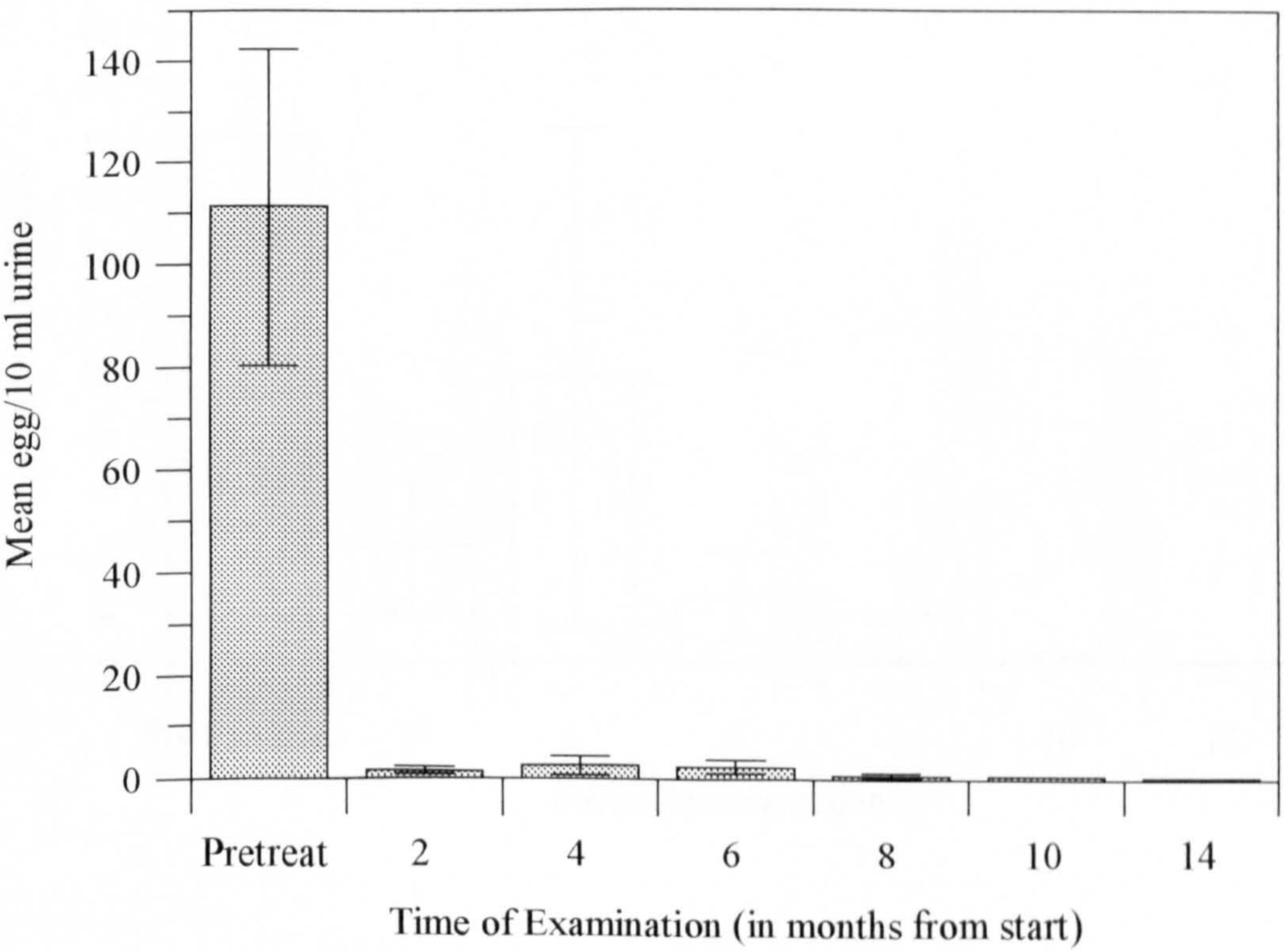


Figure 4.3: Prevalence and intensity (mean ±S.E) of infection in the children in the study cohort for humoral assays with the follow-up time. The prevalence and intensity of infection were determined by examining eggs in three 10 ml urine samples collected over three consecutive days. (The number of children in the cohort examined at each time point is shown as figures on bars)

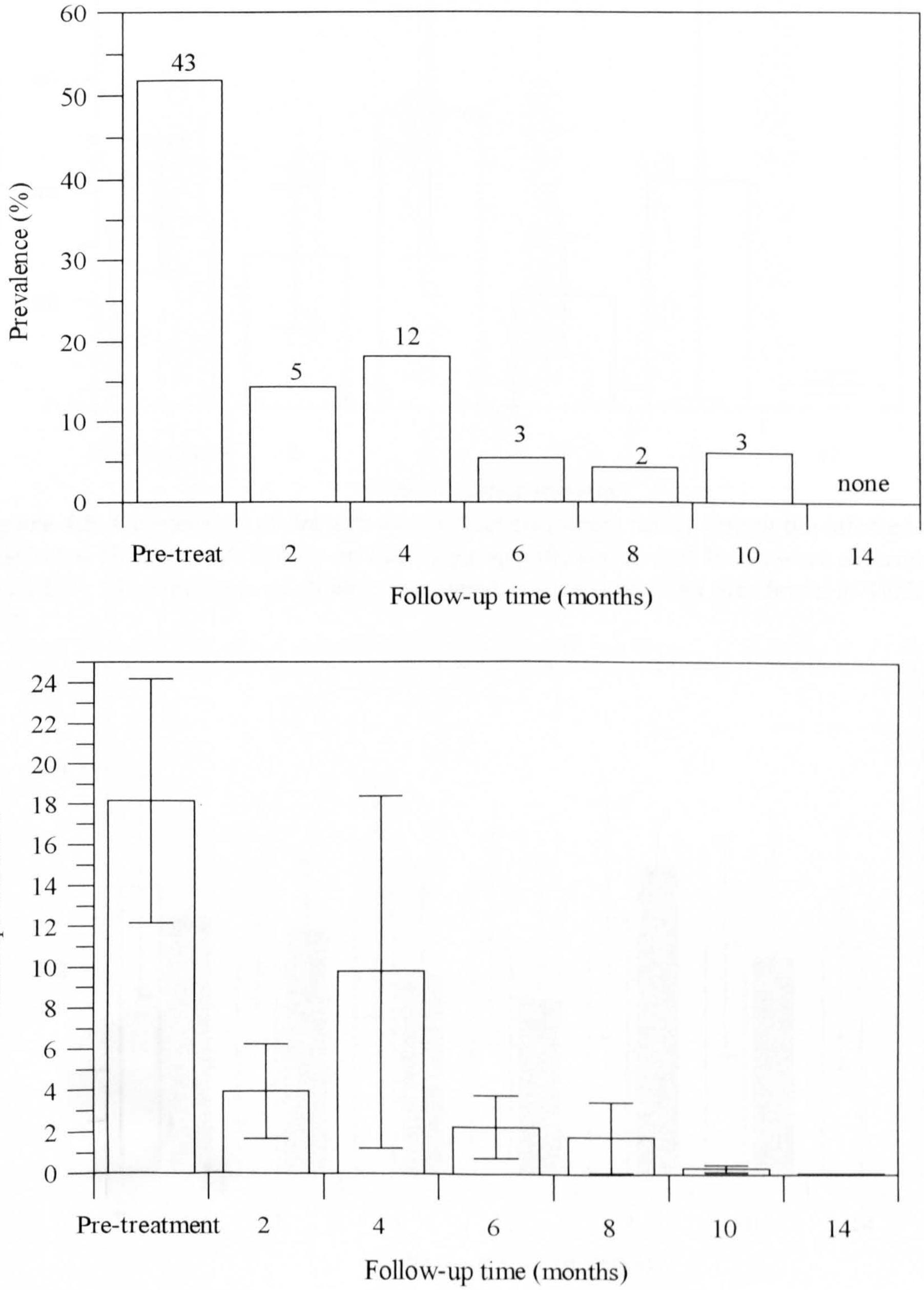


Figure 4.4: Intensity of infection (mean ep10ml ±S.E) among the infected children in the study cohort for humoral assays with the follow-up time. The intensity of infection were determined by examining eggs in three 10 ml urine samples collected over three consecutive days.(The number of infected individuals is shown on the bars).

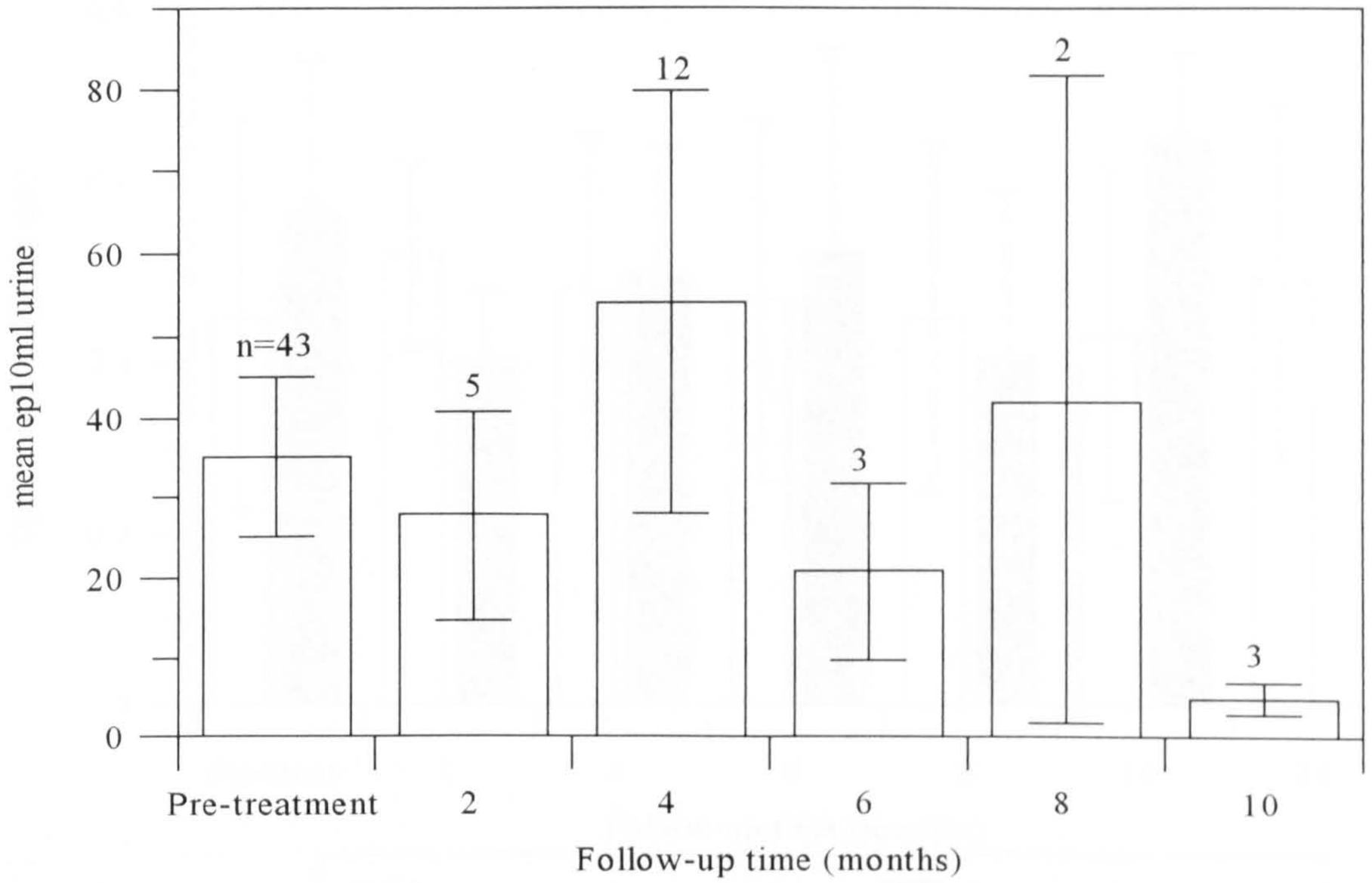


Figure 4.5: Comparison of the anti-egg IgA levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgA levels were determined by ELISA. (The numbers of children examined at each time point are shown in Table 4.3).

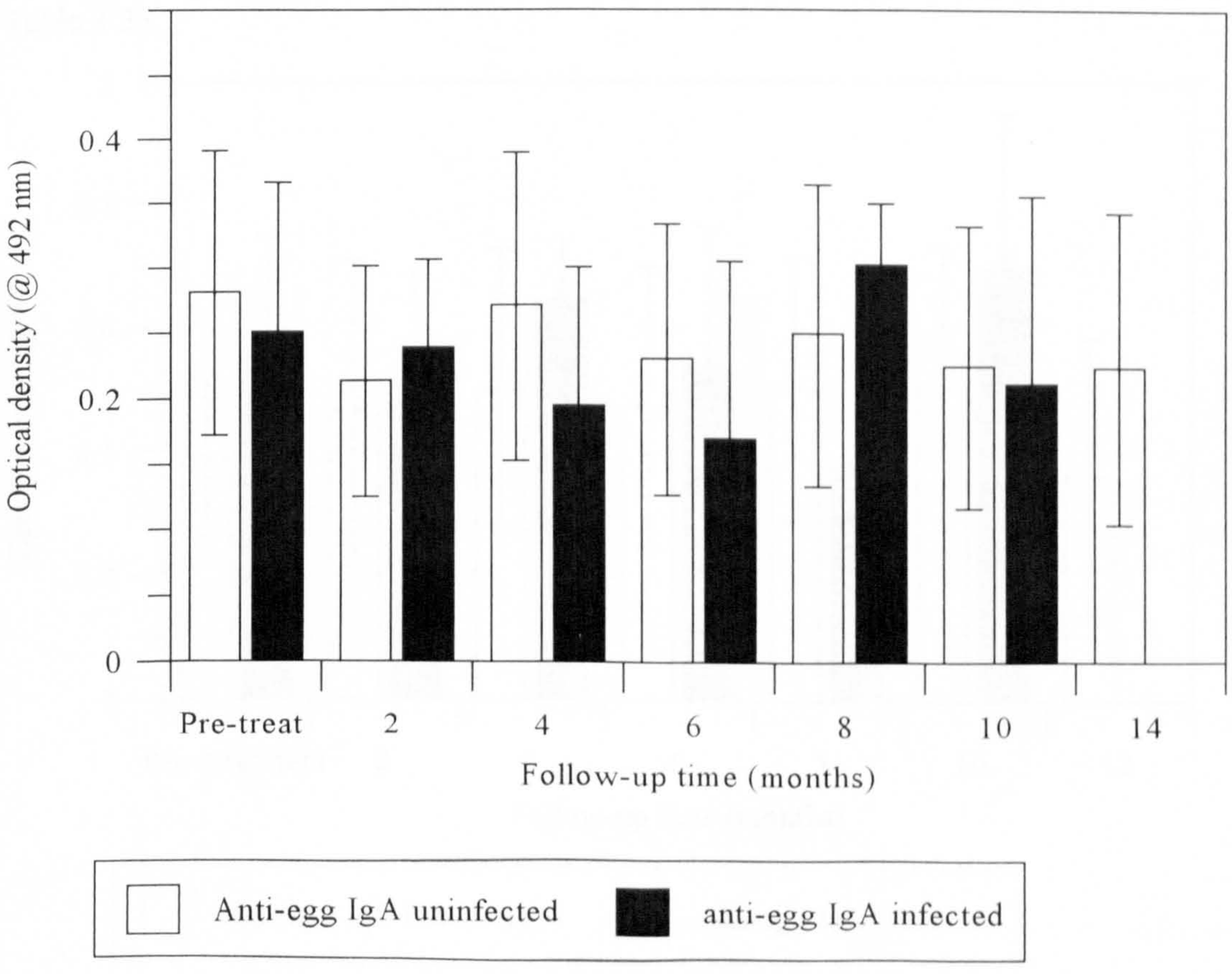


Figure 4.6: Comparison of the anti-egg IgE levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgE levels were determined by ELISA. (The number of children in each examined at each time point is shown in Table 4.3).

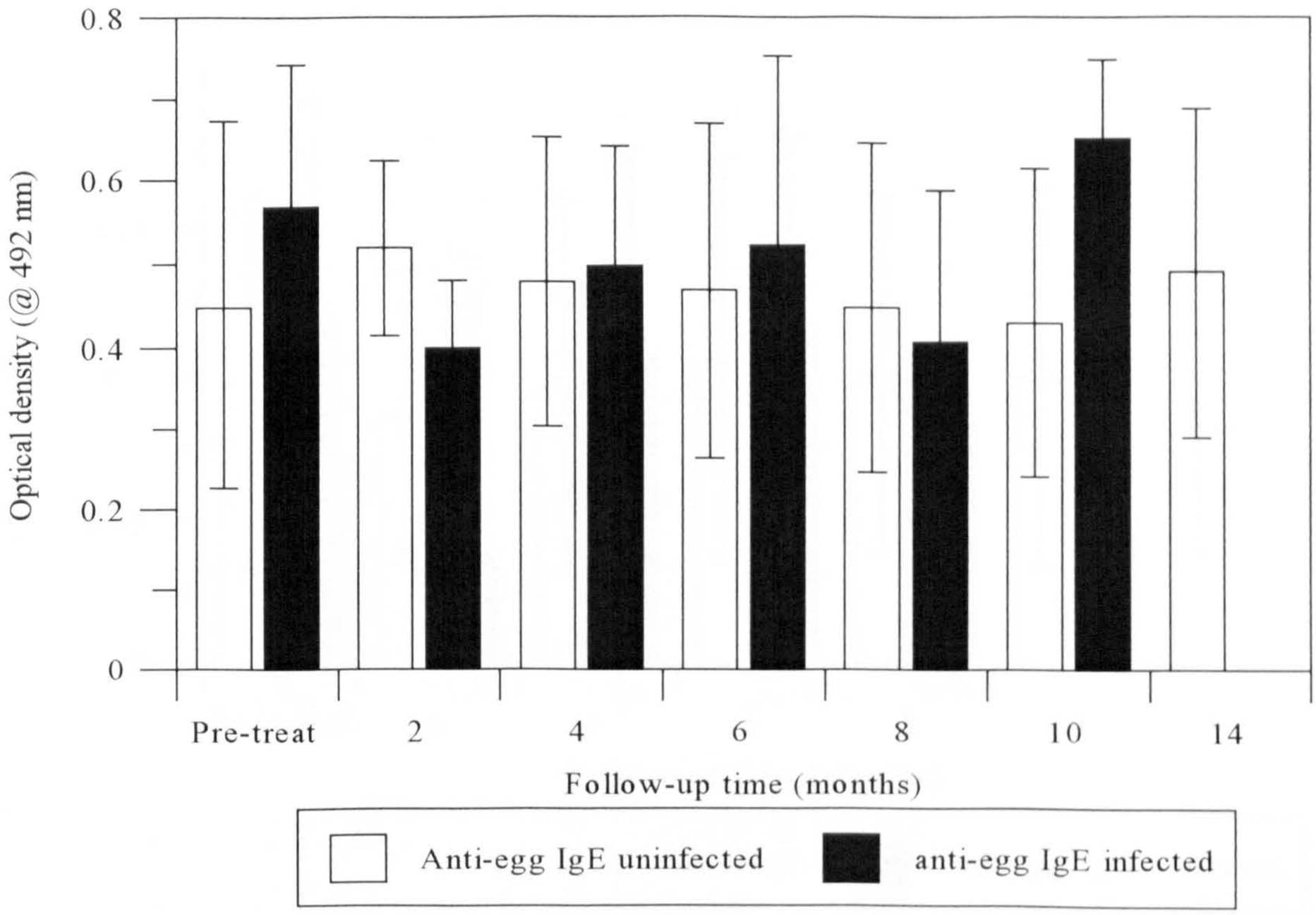


Figure 4.7: Comparison of the anti-egg IgG1 levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgG1 levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

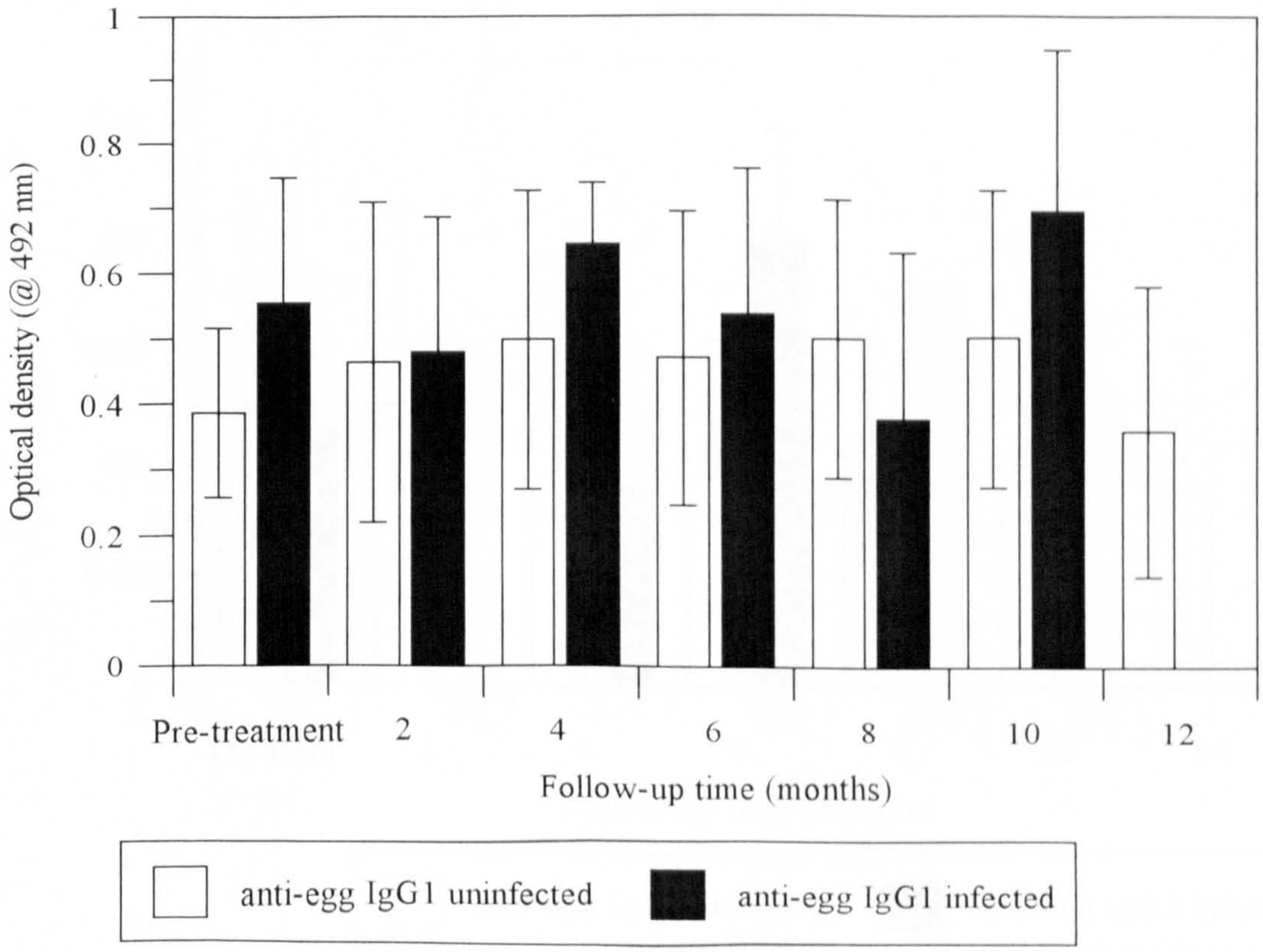


Figure 4.8: Comparison of the anti-egg IgG2 levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgG2 levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

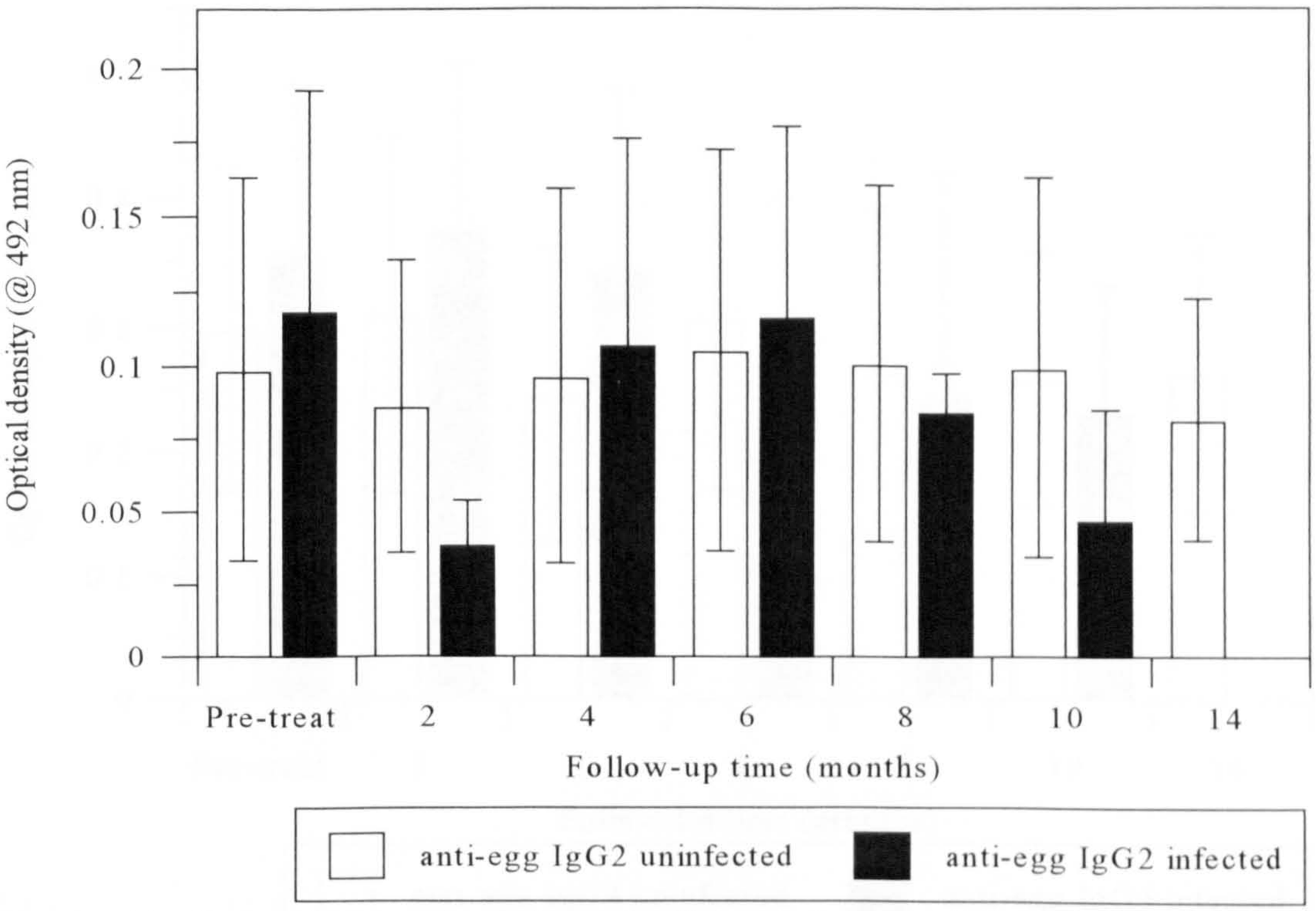


Figure 4.9: Comparison of the anti-egg IgG3 levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgG3 levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

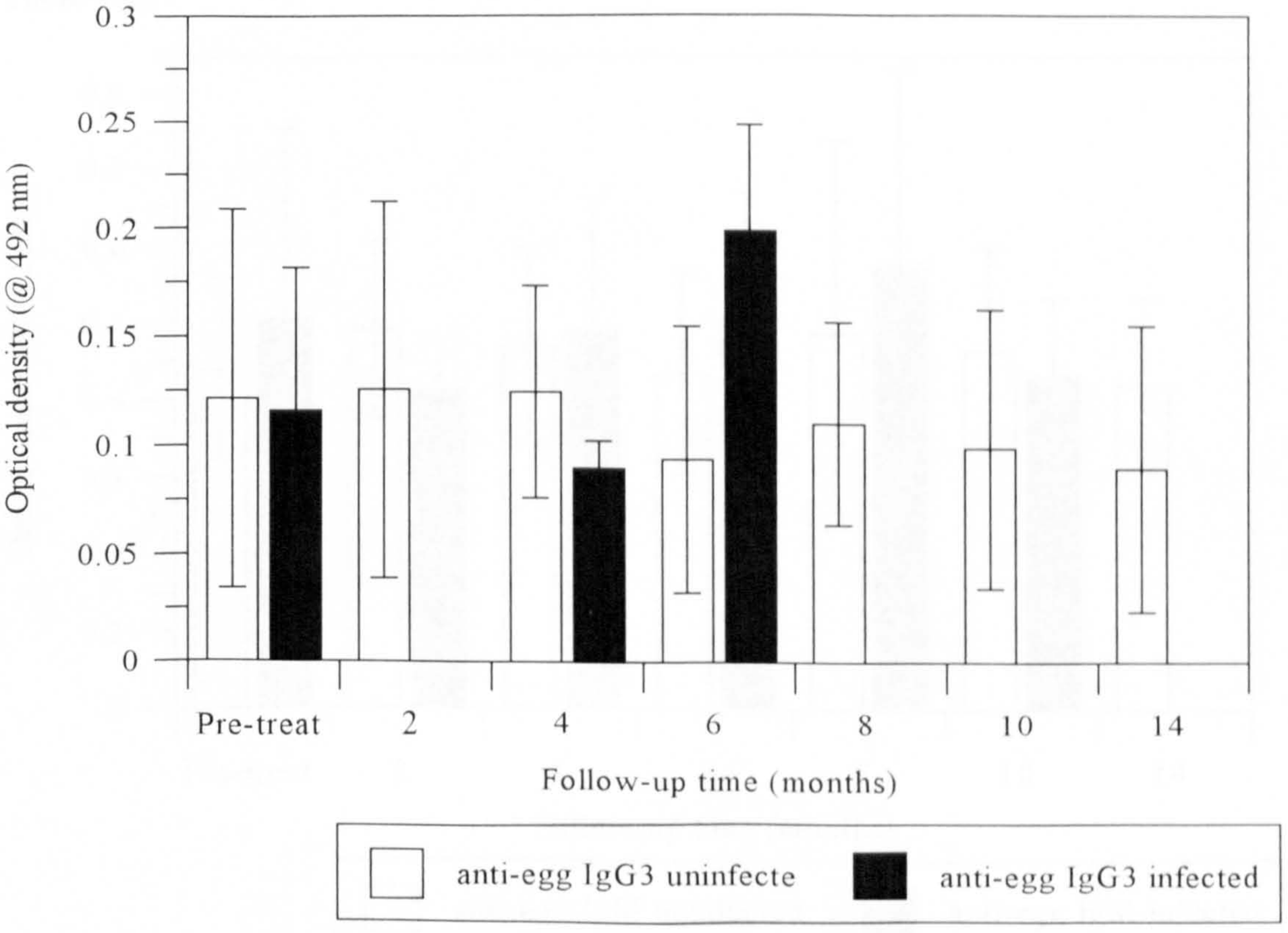


Figure 4.10: Comparison of the anti-egg IgG4 levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgG4 levels were determined by ELISA.(The number of children examined at each time point is shown in Table4.3).

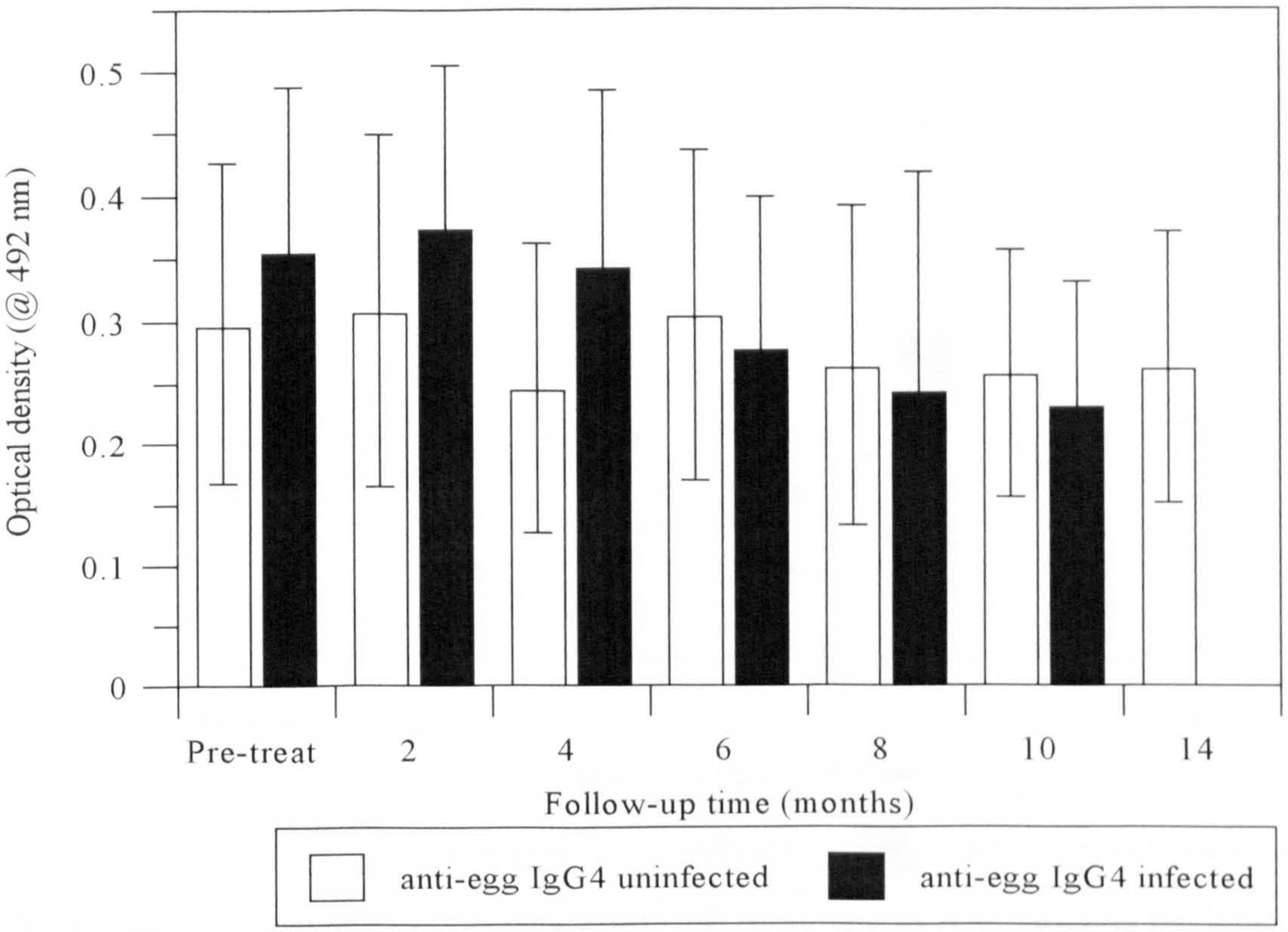


Figure 4.11: Comparison of the anti-egg IgM levels (mean, ±SD) among the infected and uninfected children with follow-up time. Egg-specific serum IgM levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

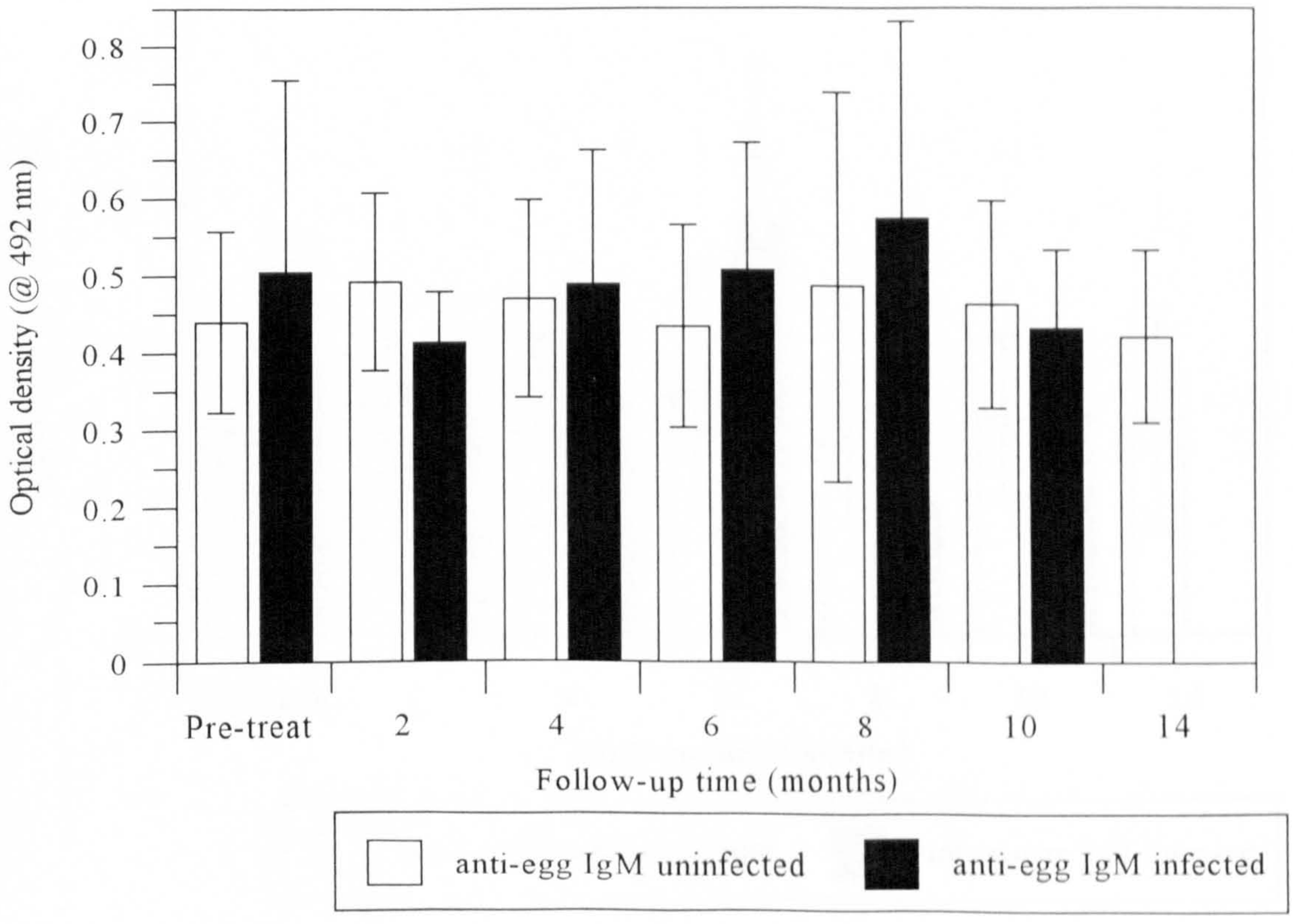


Figure 4.12: Comparison of the anti-worm IgA levels (mean, ±SD) among the infected and uninfected children with follow-up time. Worm-specific serum IgA levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

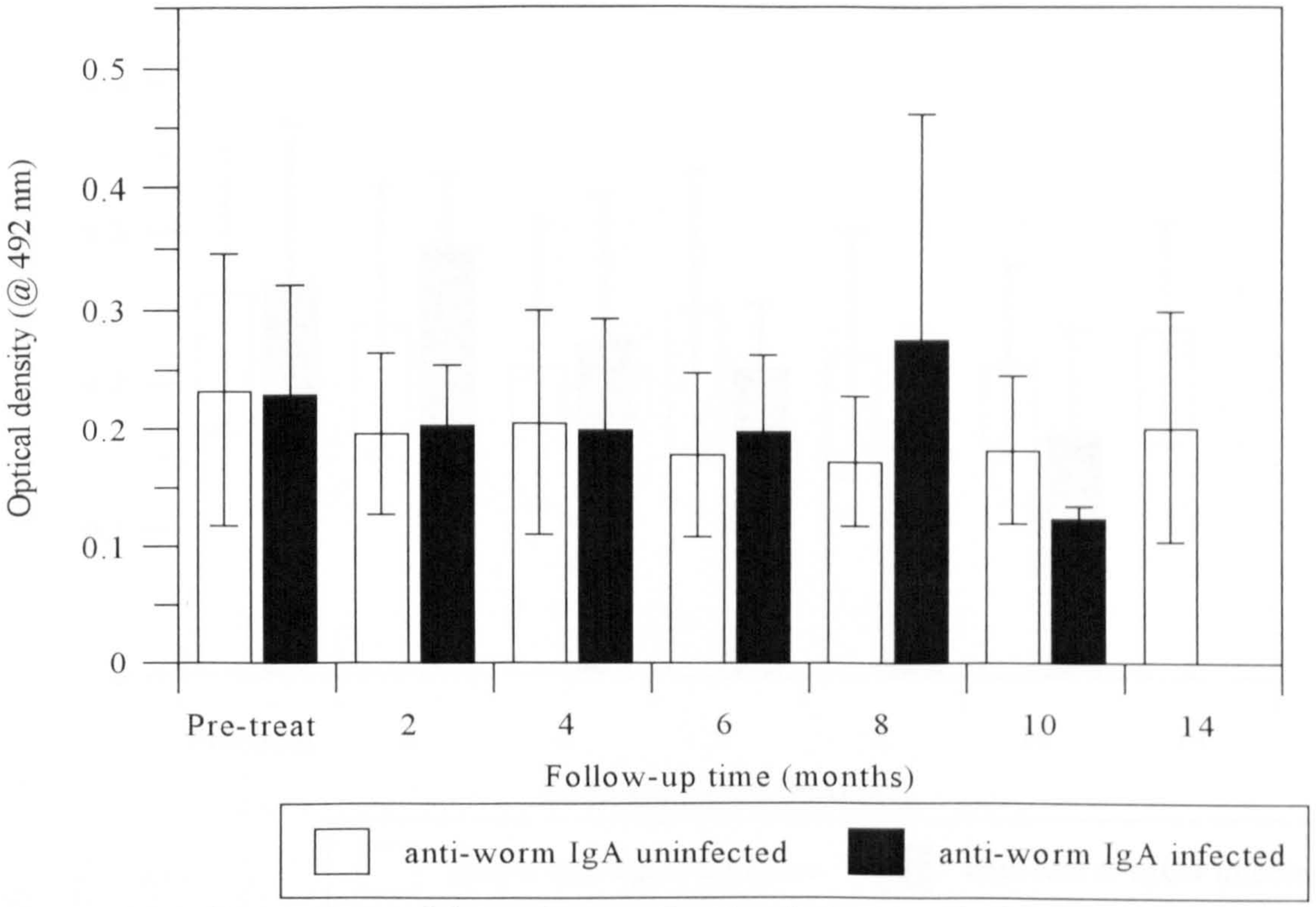


Figure 4.13: Comparison of the anti-worm IgE levels (mean, ±SD) among the infected and uninfected children with follow-up time. Worm-specific serum IgE levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

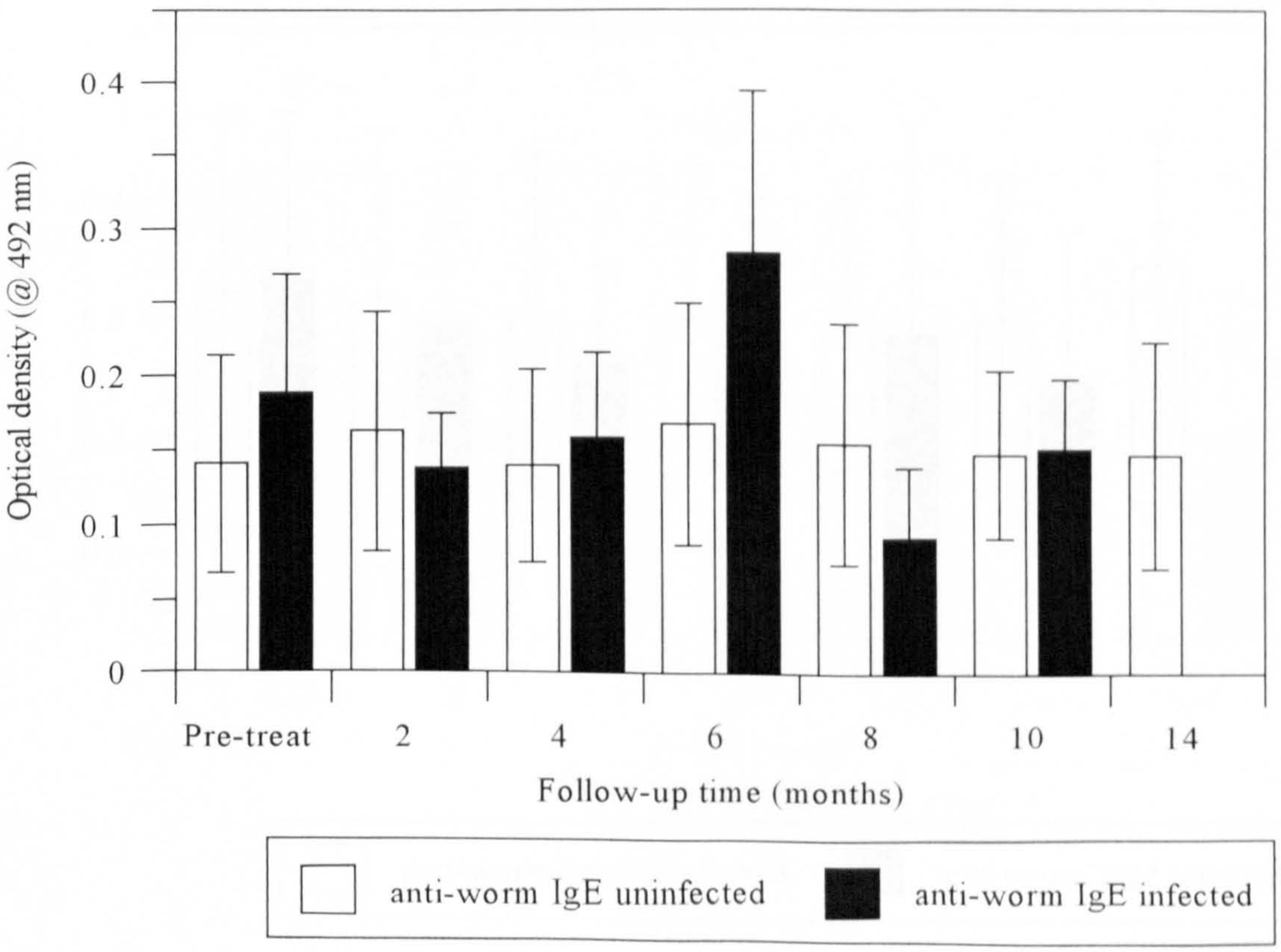


Figure 4.14: Comparison of the anti-worm IgG4 levels (mean, ±SD) among the infected and uninfected children with follow-up time. Worm-specific serum IgG4 levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

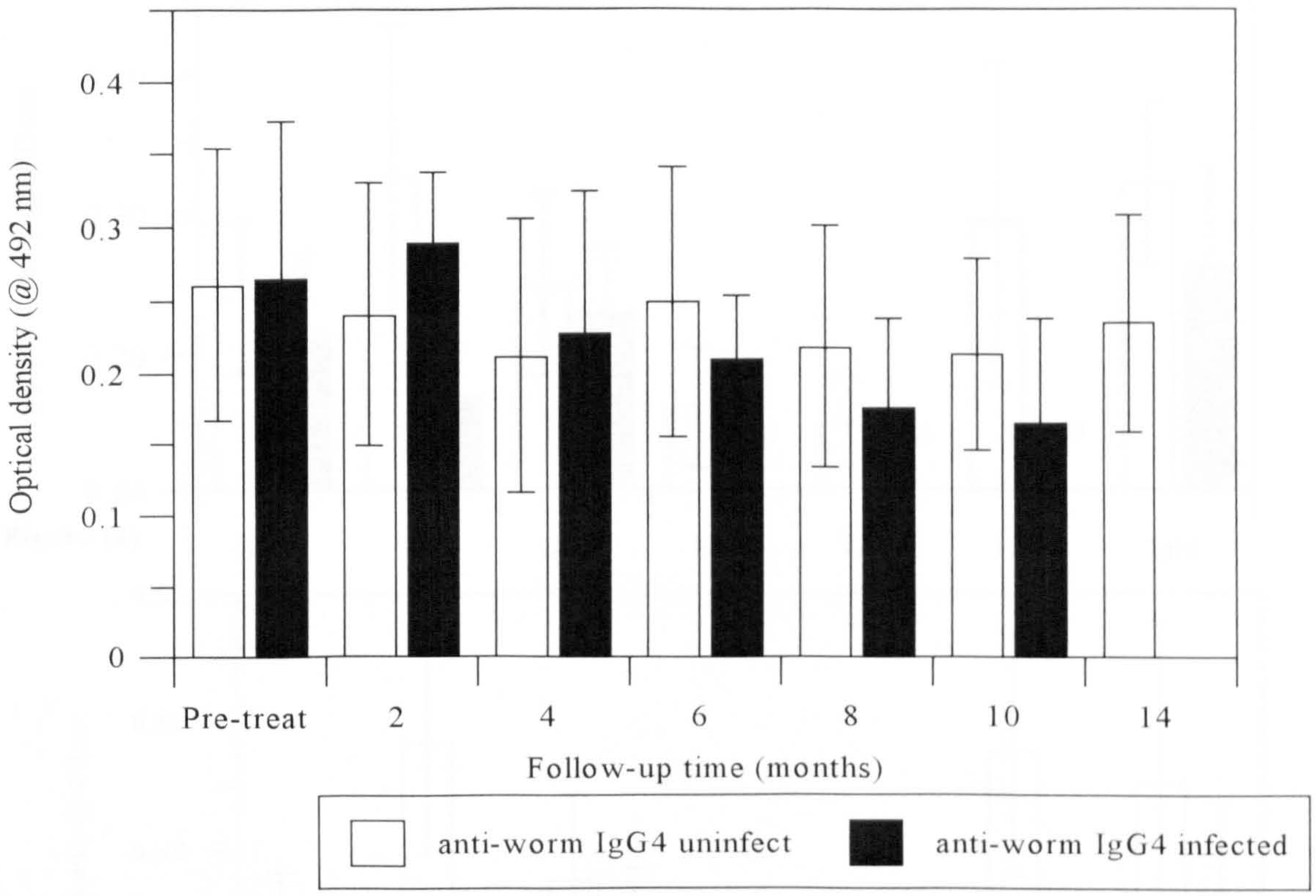


Figure 4.15: Comparison of the anti-worm IgM levels (mean, ±SD) among the infected and uninfected children with follow-up time. Worm-specific serum IgM levels were determined by ELISA. (The number of children examined at each time point is shown in Table 4.3).

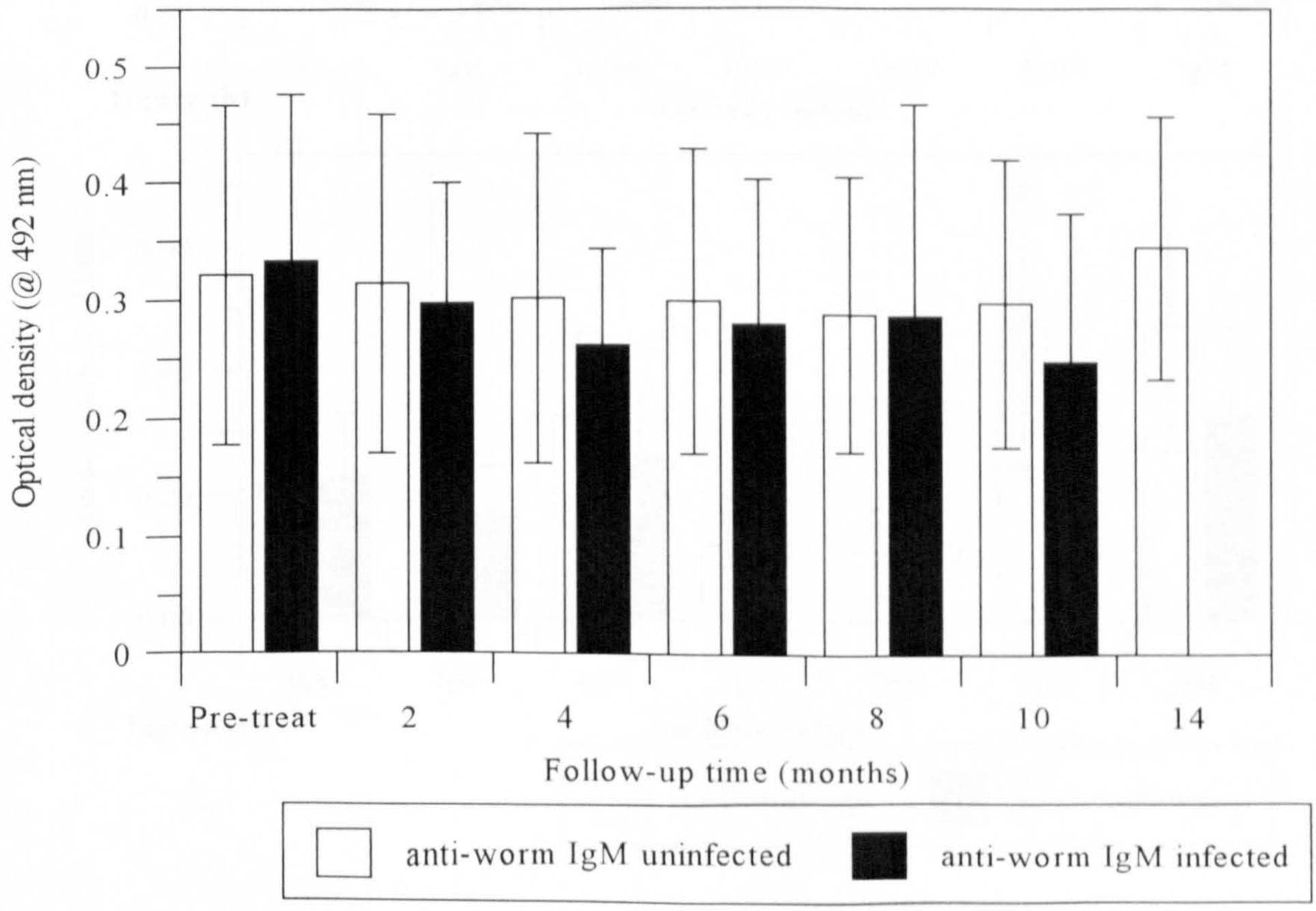


Figure 4.16: Showing antibody isotype profiles before giving chemotherapeutical cure with praziquantel; Figure (a) showing uninfected children; Figure (b) showing infected children and Figure (c) showing all the children. Egg- and worm-specific serum antibody levels were determined by ELISA. (ND=not determined).

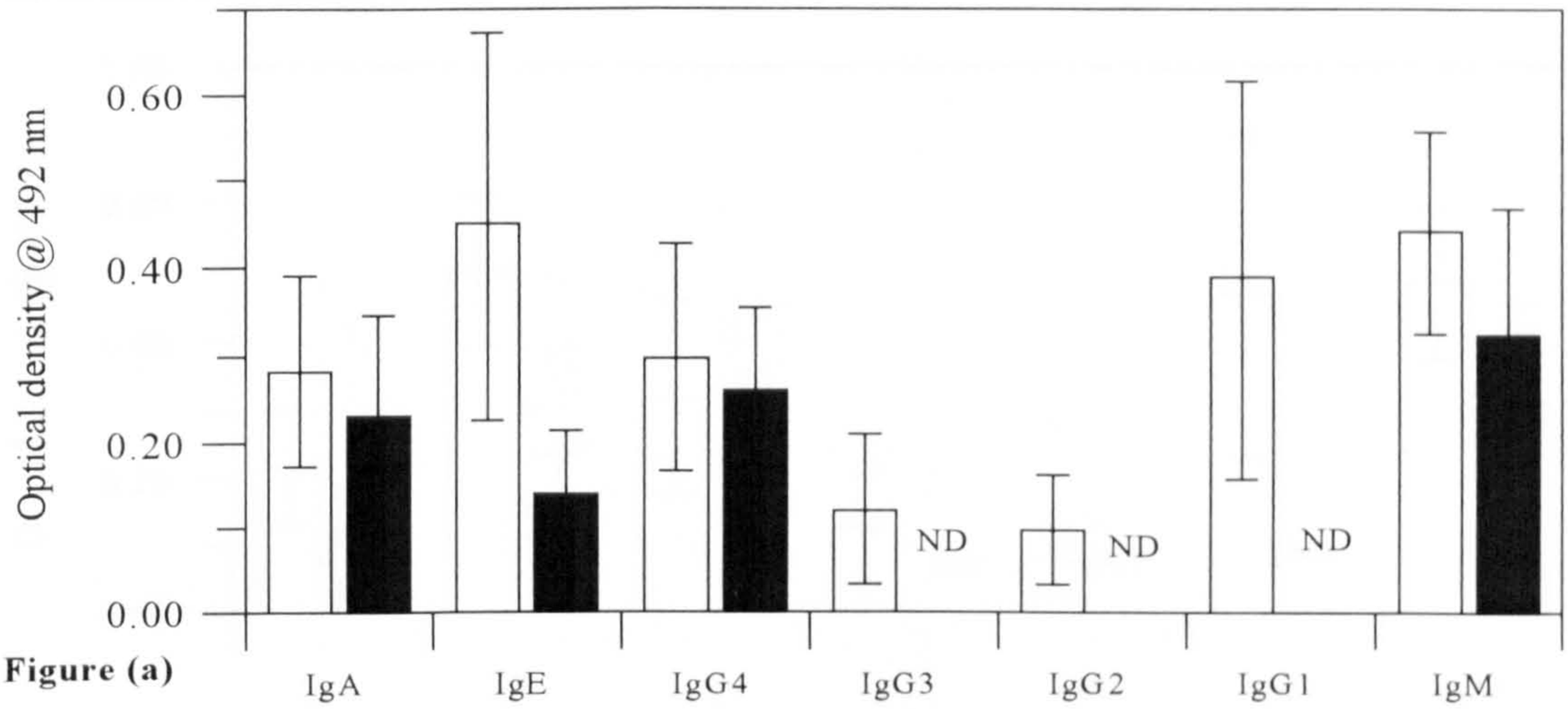


Figure (a)

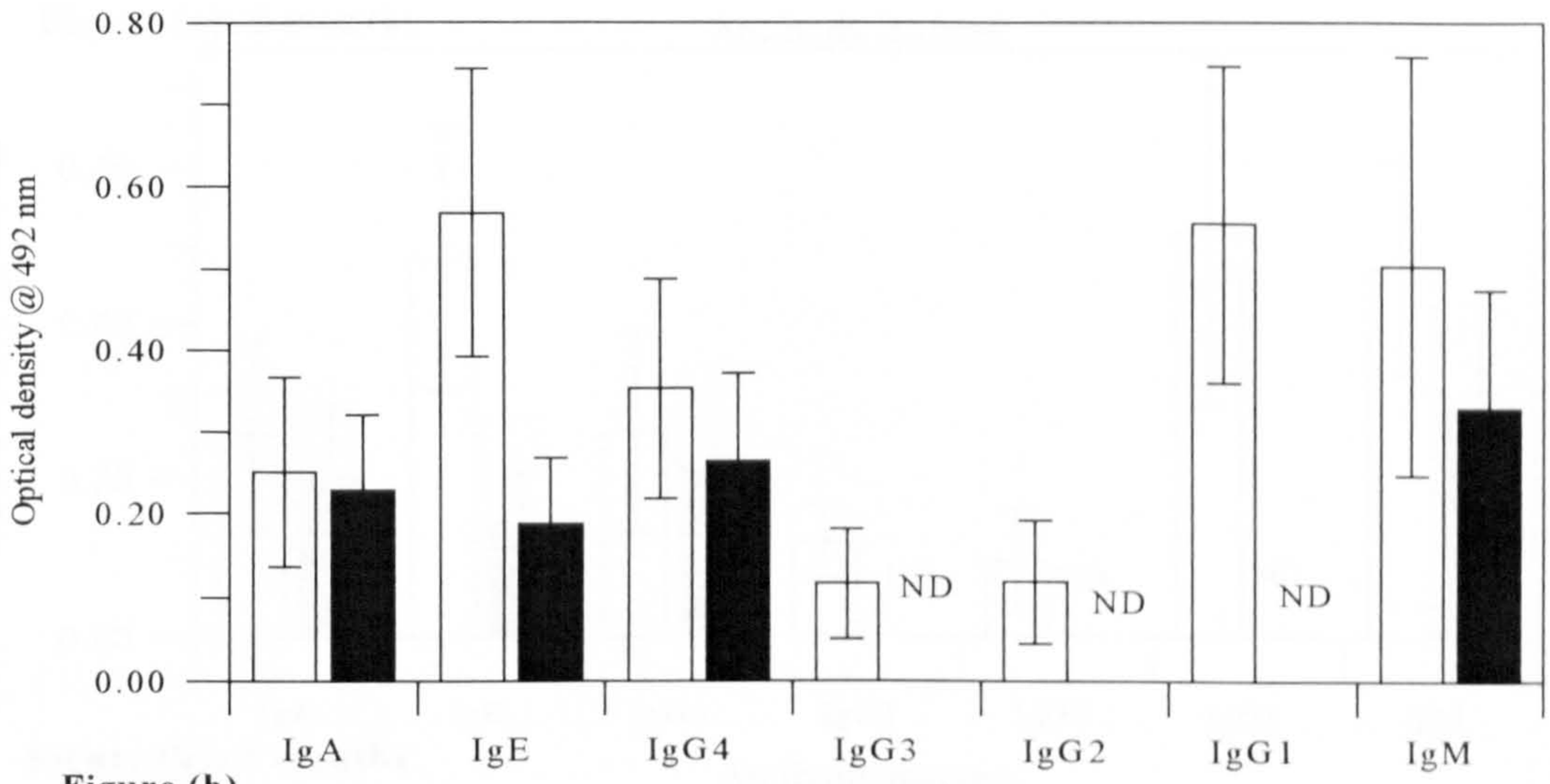


Figure (b)

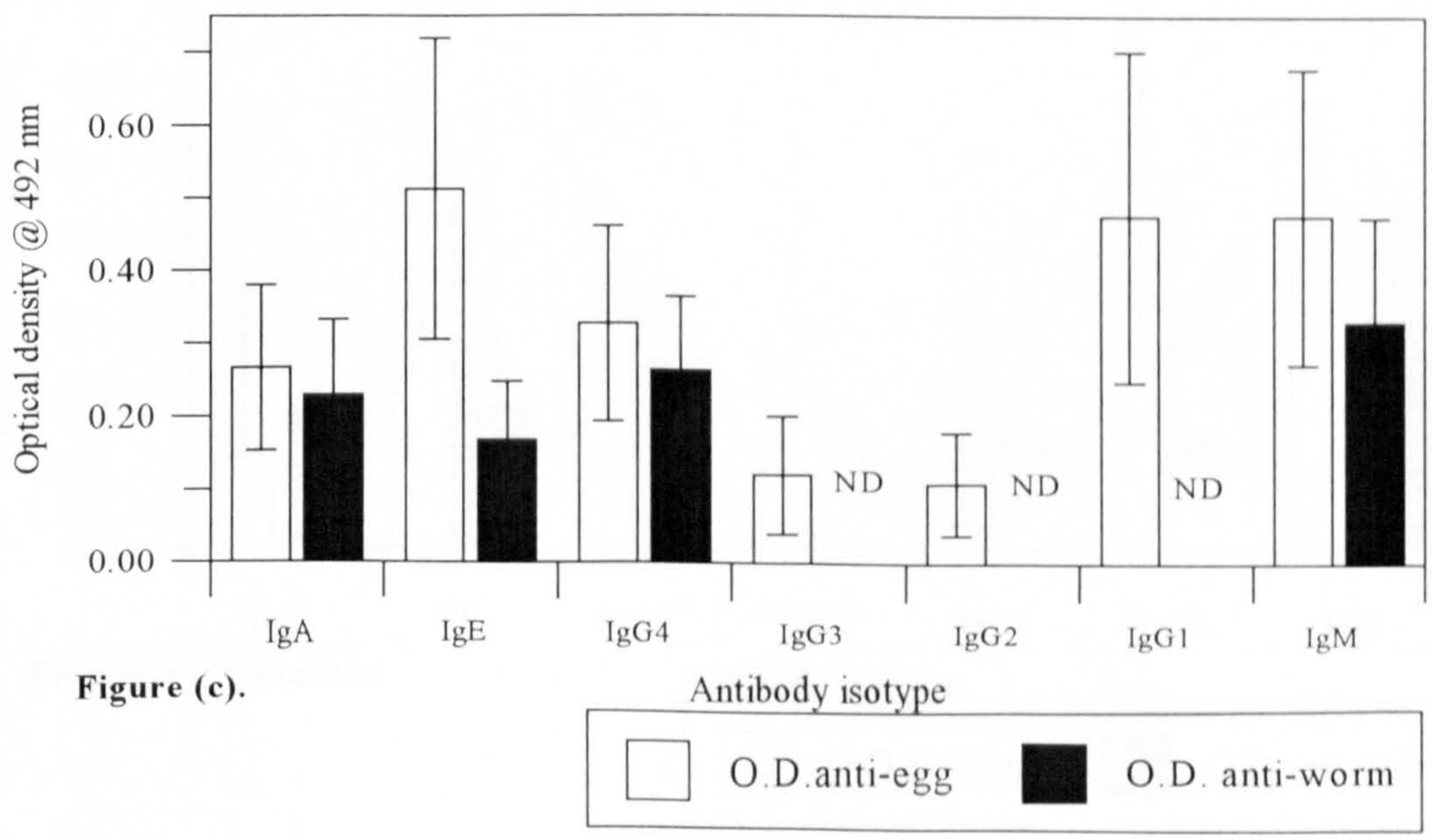


Figure (c).

Figure 4.17: Showing antibody isotype profiles after giving chemotherapeutical cure with praziquantel, at each examination point shown in months from start; Figure (a): 2 months; Figure (b): 4 months; Figure (c): 6 months; Figure (d): 8 months; Figure (e): 10 months; Figure (f): 14 months. Egg- and worm-specific serum antibody levels were determined by ELISA. (ND=not determined)

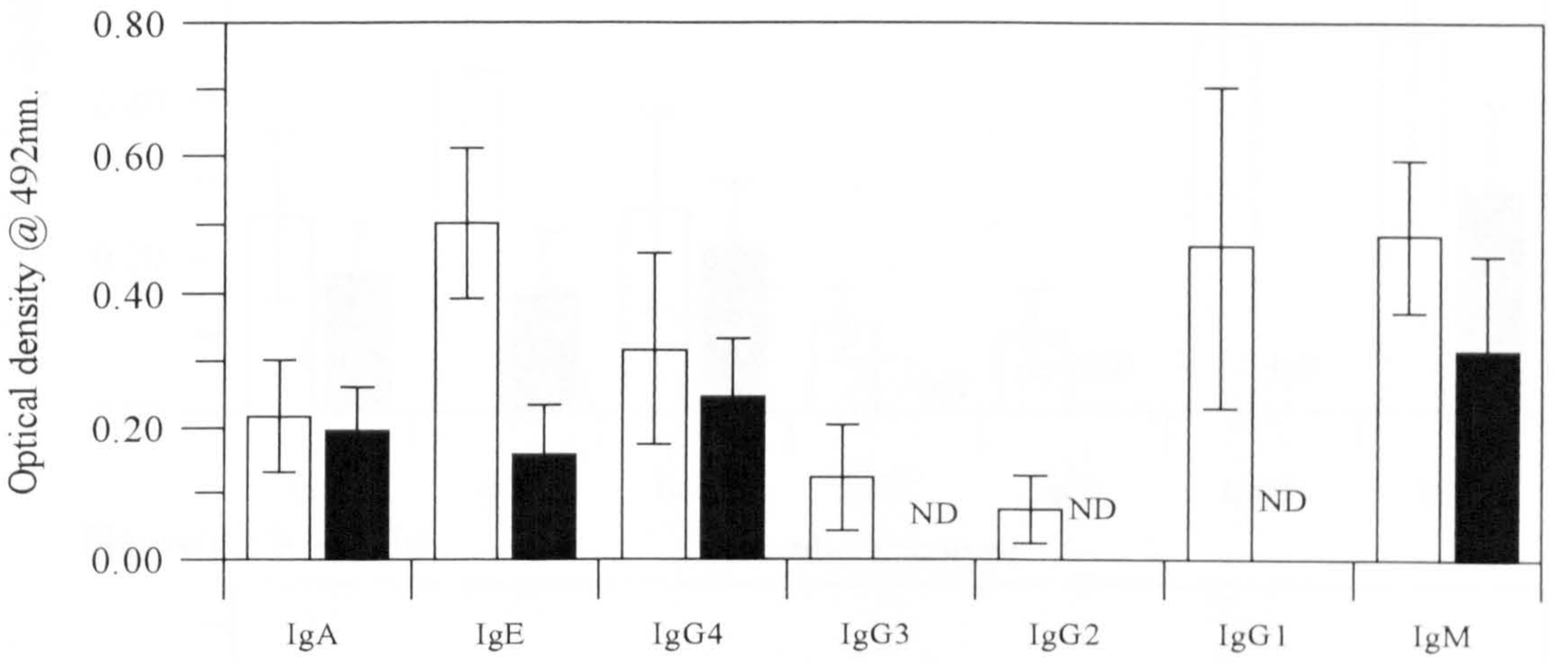


Figure (a): 2 months

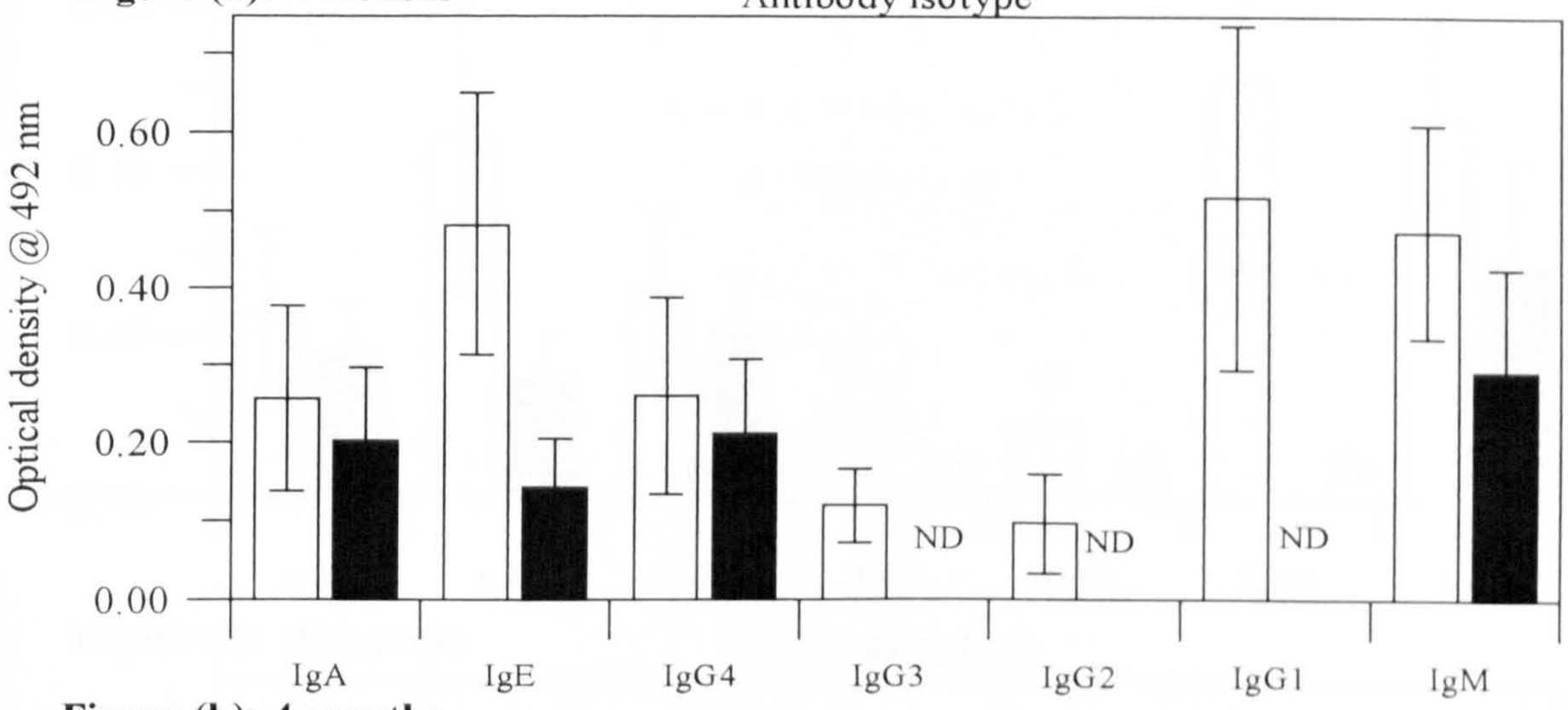


Figure (b): 4 months

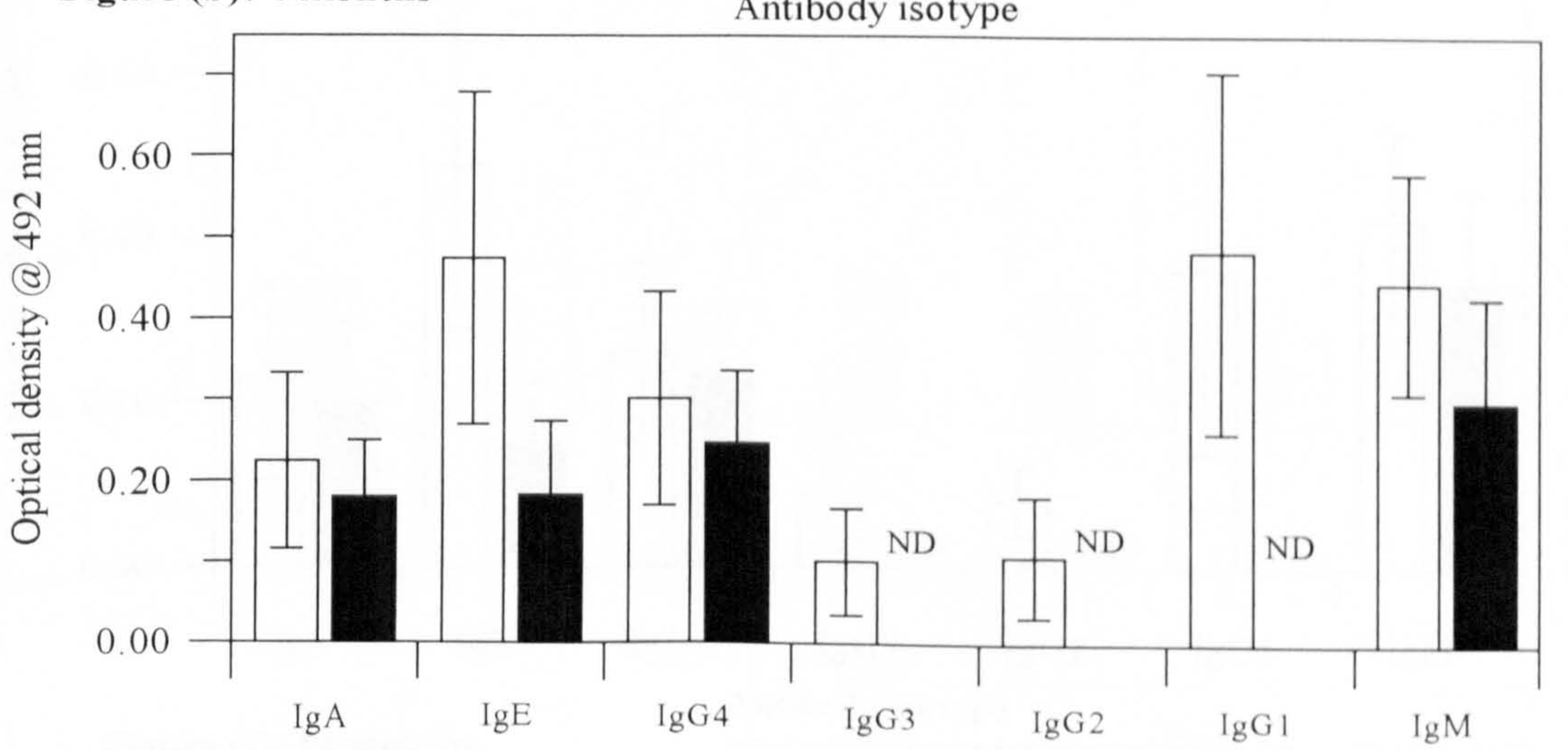
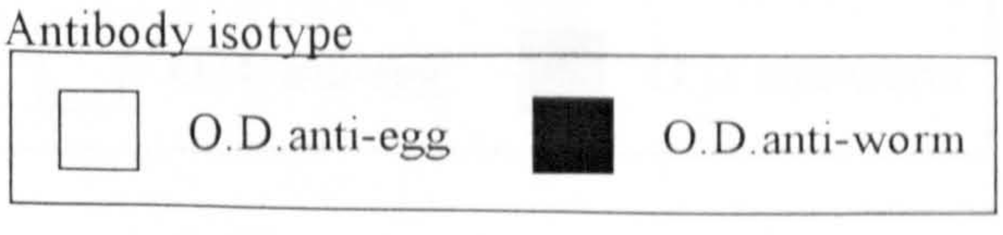
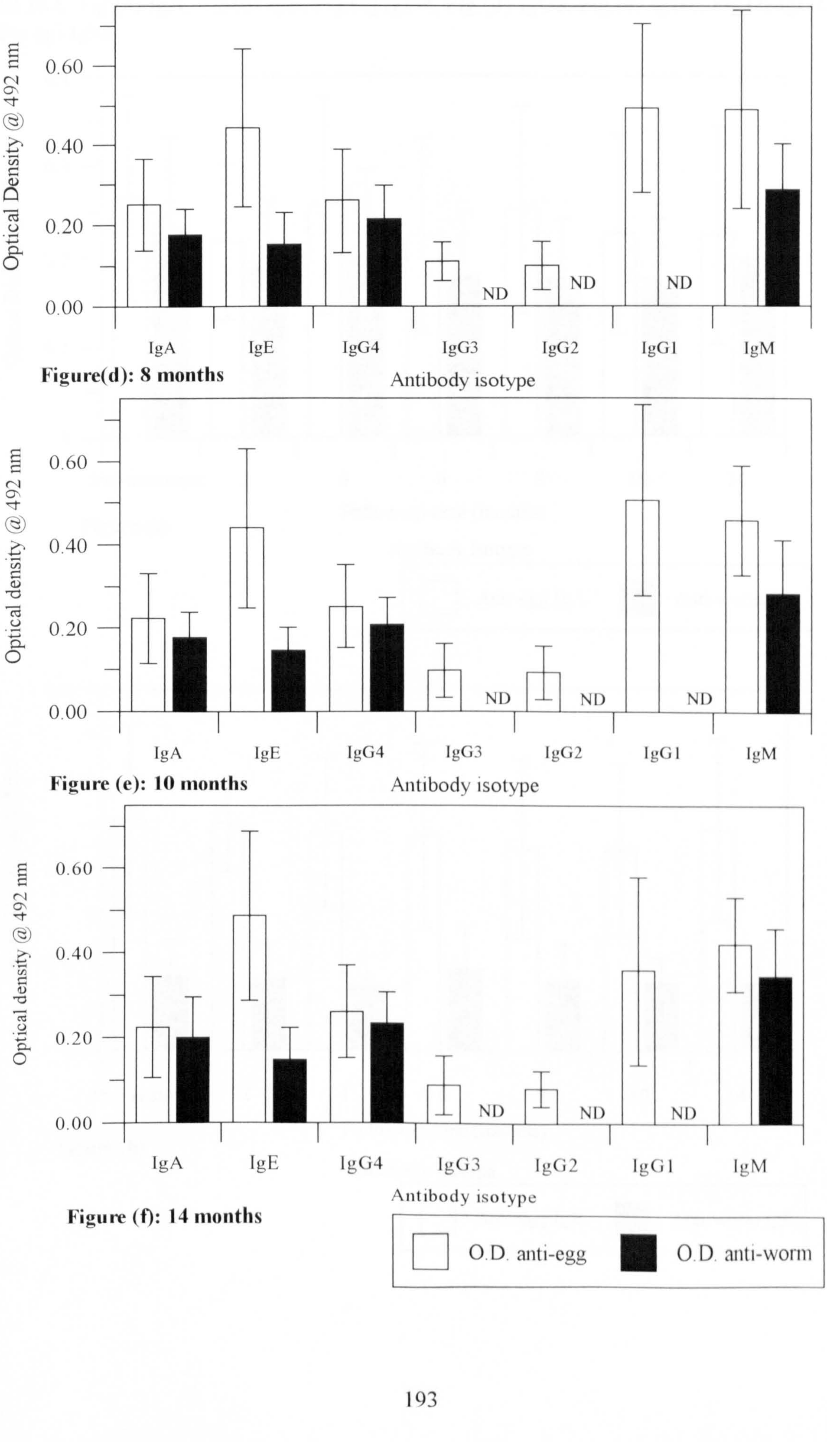


Figure (c): 6 months





Figure(d): 8 months

Figure (e): 10 months

Figure (f): 14 months

Figure 4.18: Showing antibody isotype profiles in all the children sampled according to time of sampling. Egg- and worm-specific serum antibody levels were determined by ELISA. Fig.(a) IgA, Fig.(b) IgE, Fig.(c) IgG4, Fig.(d) IgG3, Fig.(e) IgG2, Fig.(f) IgG1, Fig.(g) IgM.

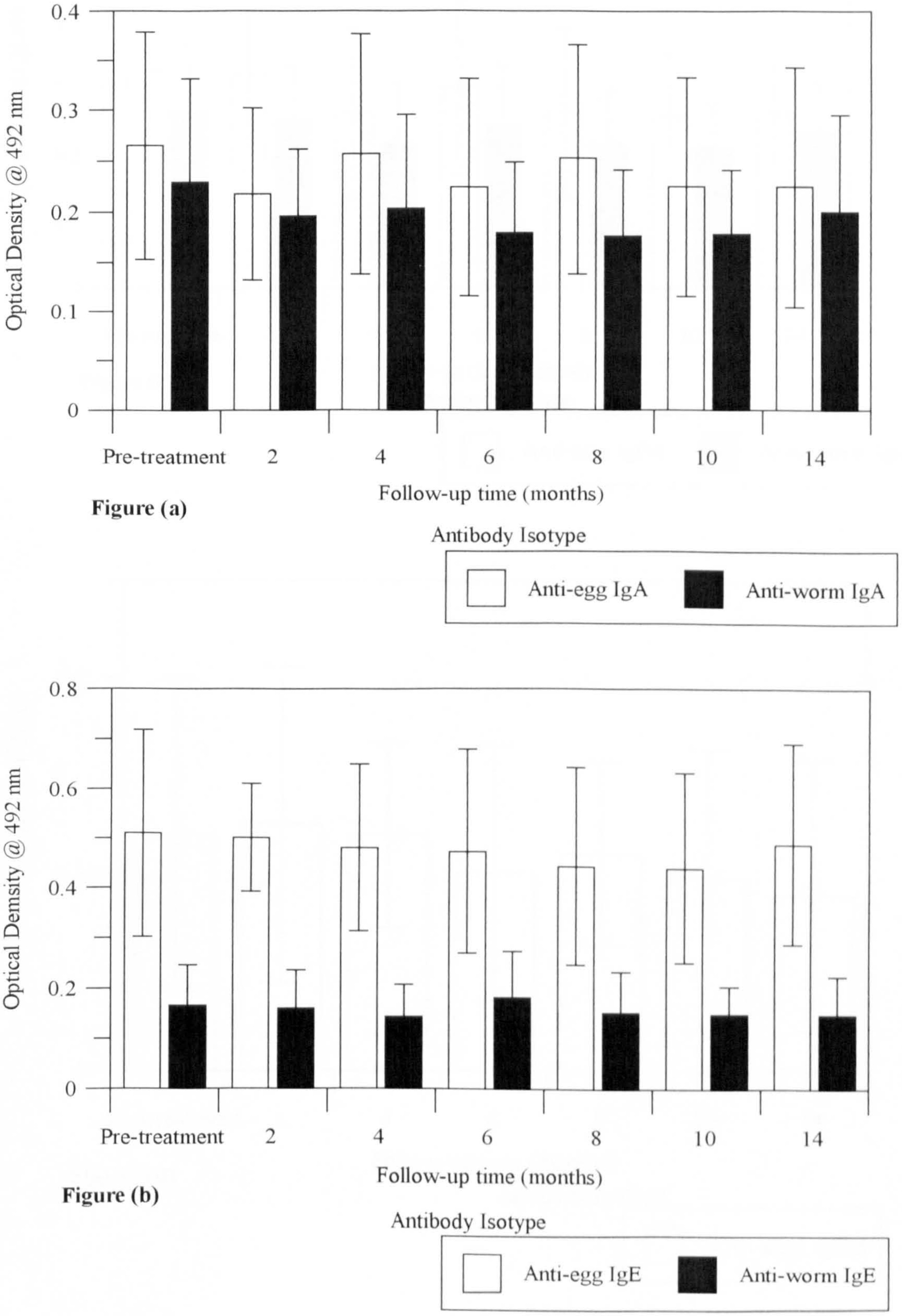


Figure (a)

Figure (b)

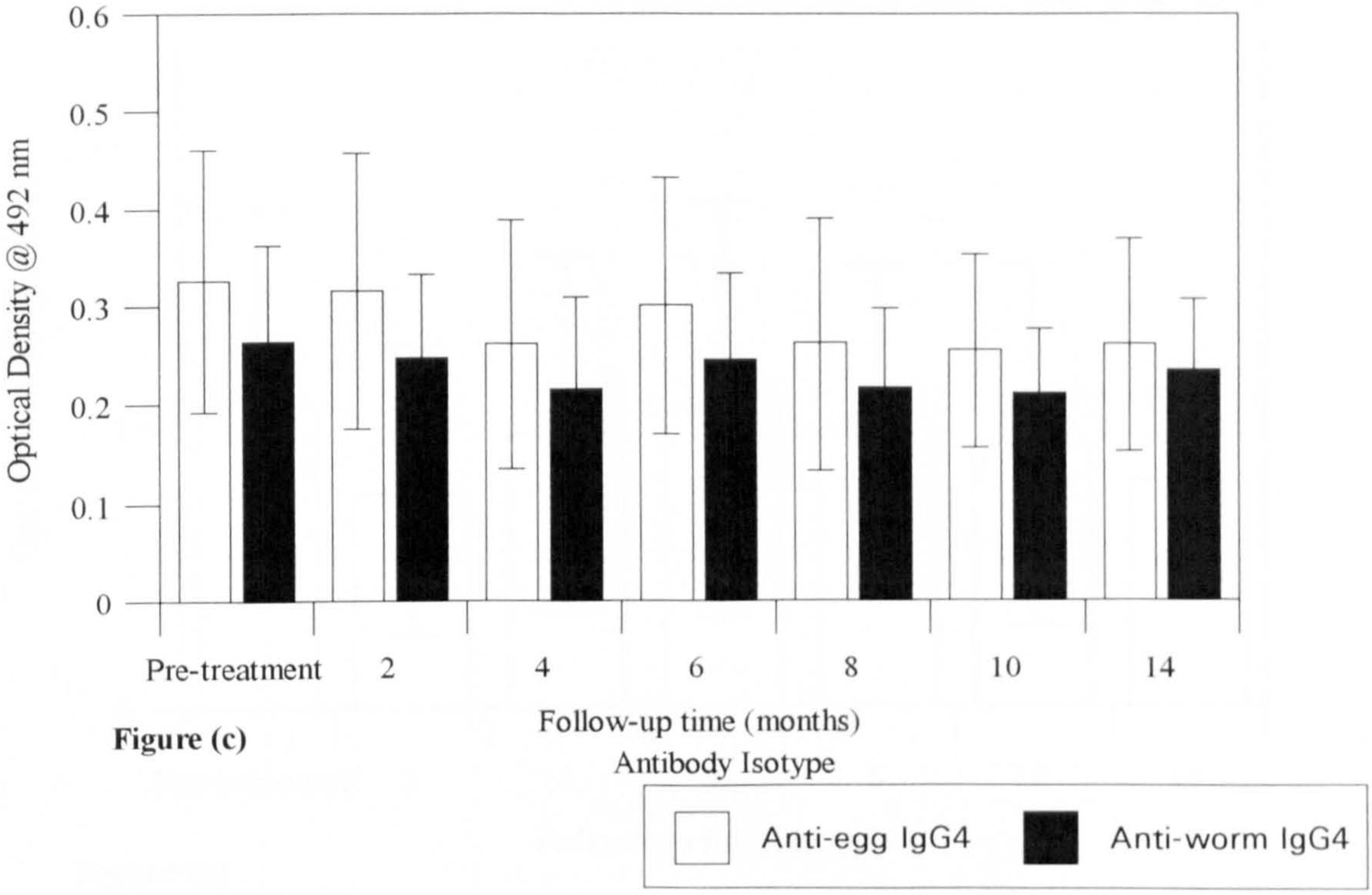


Figure (c)

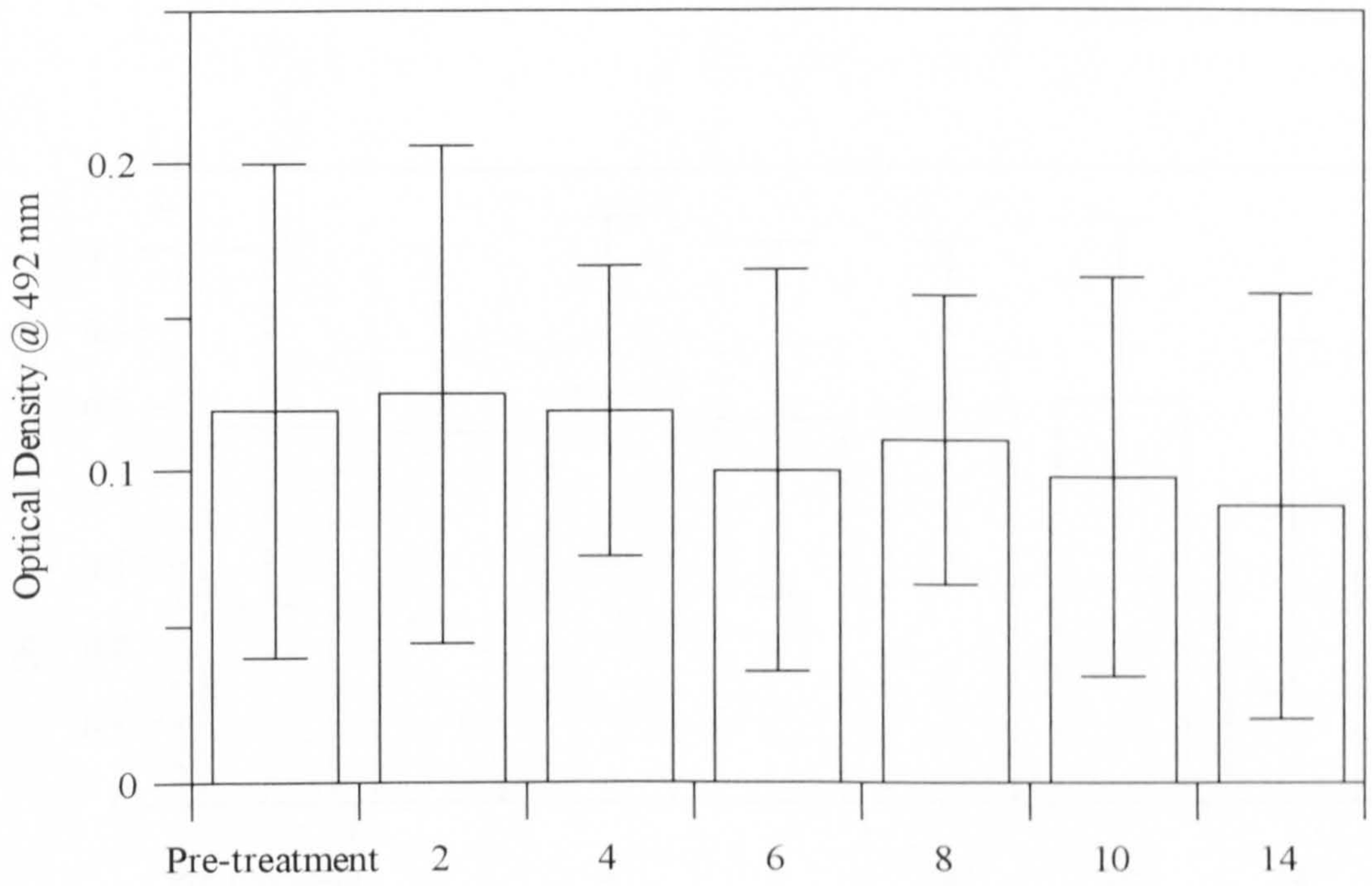


Figure (d)

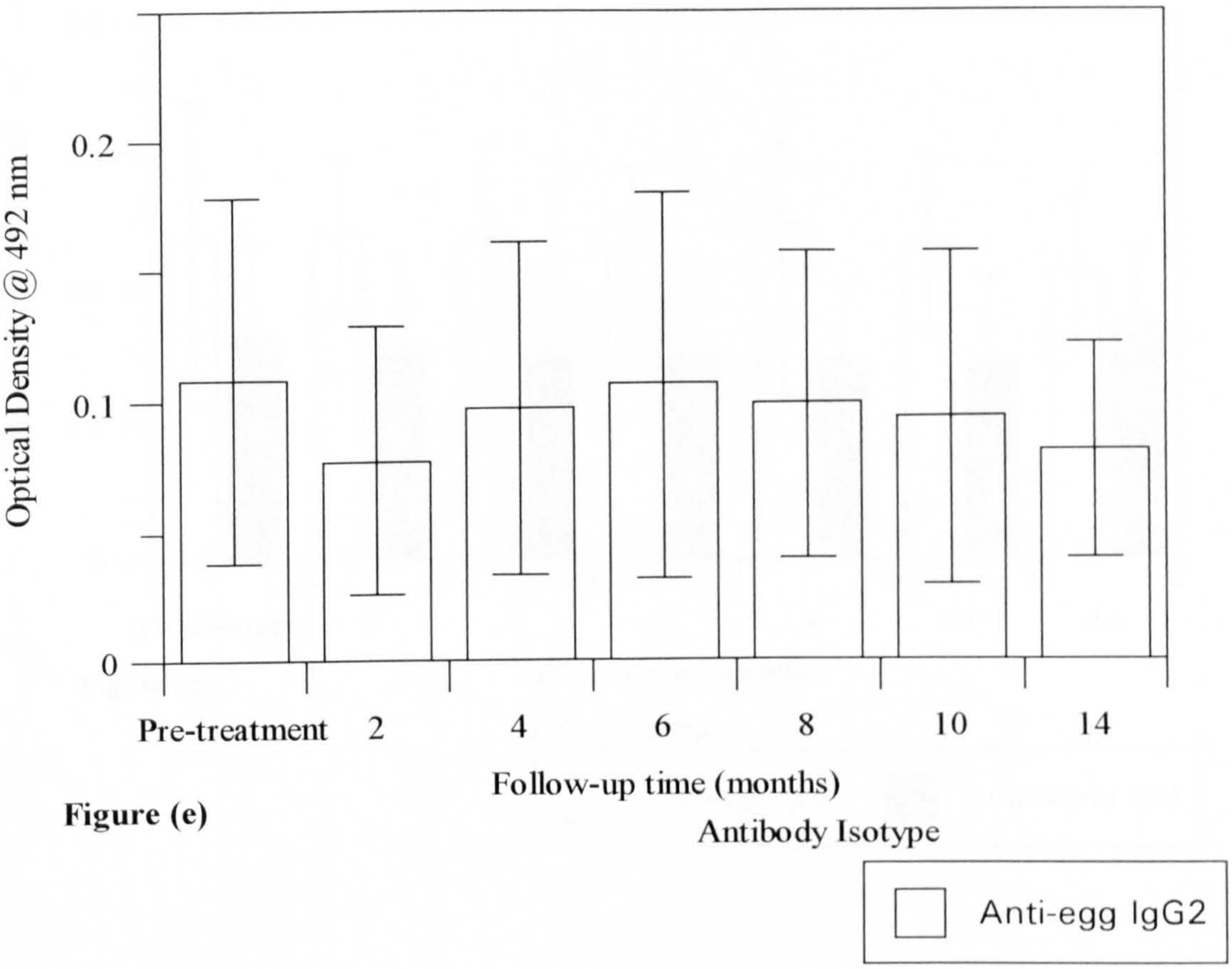


Figure (e)

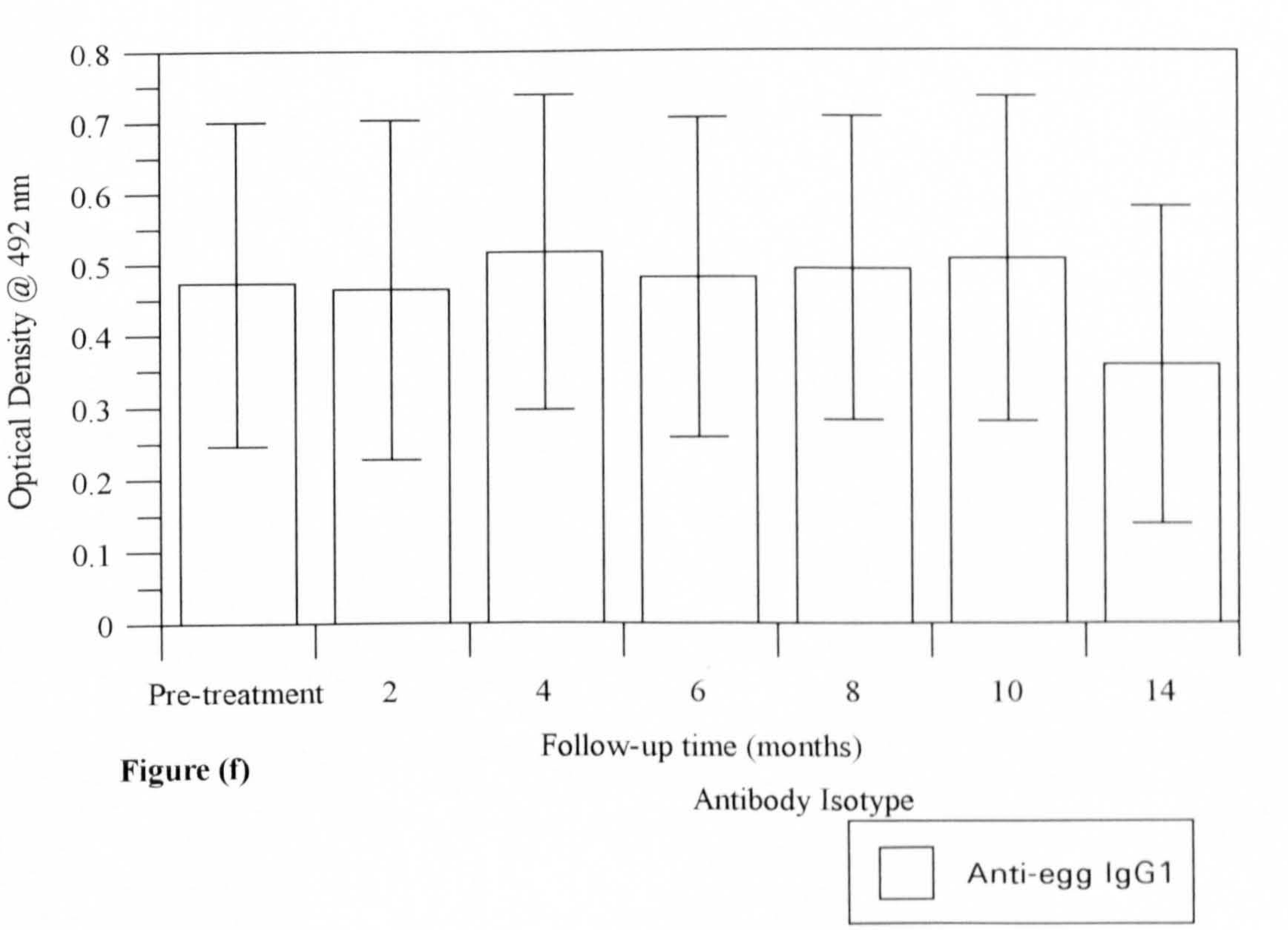


Figure (f)

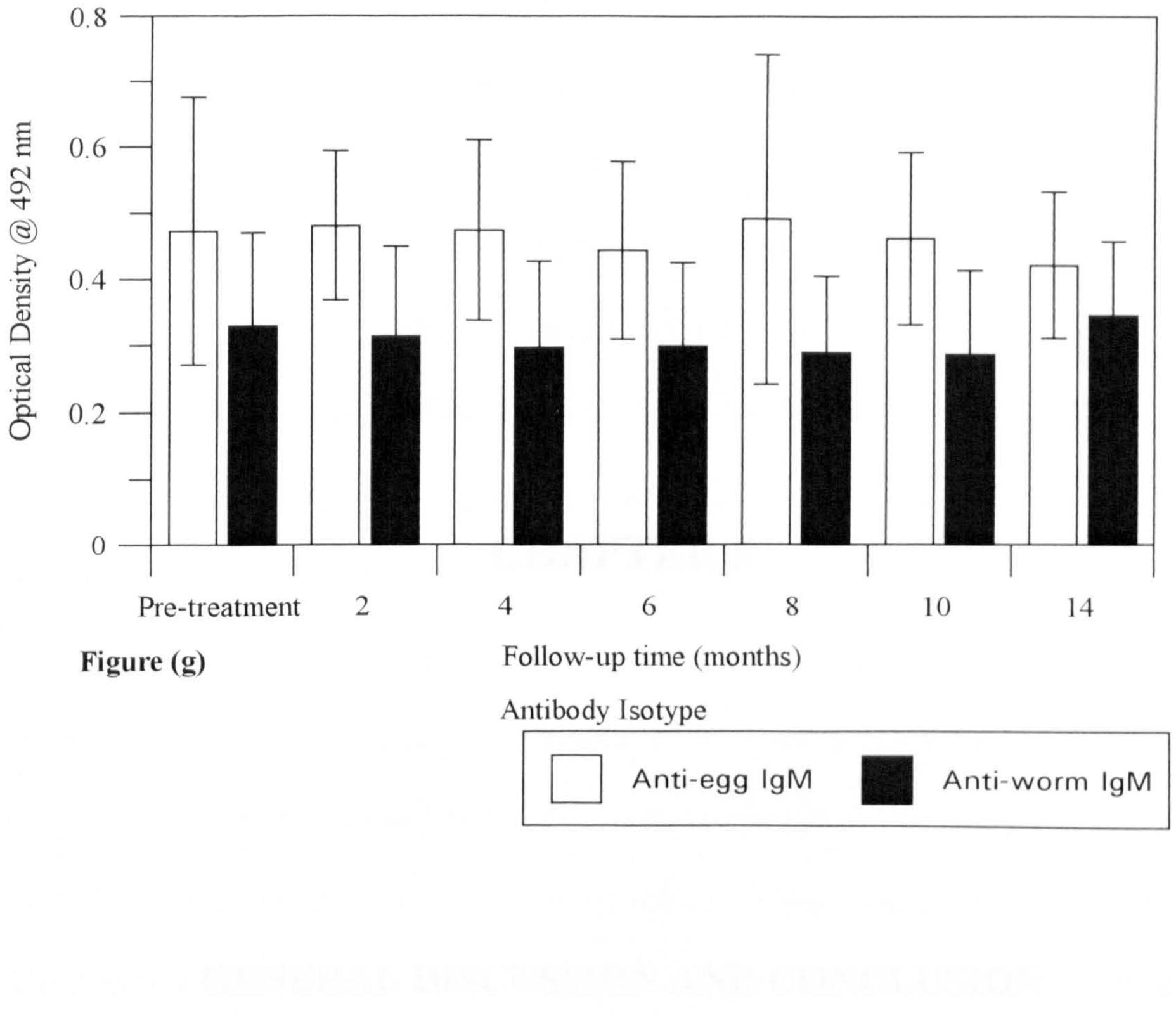

0.8
0.6
0.4
0.2
0
Optical Density @ 492 nm
Pre-treatment
2
4
6
8
10
14
Follow-up time (months)
Antibody Isotype
Anti-egg IgM
Anti-worm IgM

Figure (g)

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.0 GENERAL DISCUSSION

5.1 THE IMMUNO-EPIDEMIOLOGY OF *S.haematobium* INFECTIONS.

Human immune responses to schistosome infections have been extensively investigated with the main objective of understanding the mechanisms involved in determining susceptibility and resistance to infection or reinfection. Several researchers have reported on the potentially protective and 'blocking' roles of IgE, IgG4, IgA, IgG2 and IgM antibodies in determining resistance or susceptibility to reinfection after drug treatment (Butterworth *et al.*, 1985, 1987, 1991, 1992, Hagan *et al.*, 1987, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993 and Ndhlovu *et al.*, 1996). Others have studied the role of macrophages, eosinophils and other effector cells during ADCC, (Capron *et al.*, 1977; Capron and Capron, 1986; Hagan *et al.*, 1985a,b, 1987; Dunne *et al.*, 1987) but not much is known about the cellular immune responses of the vulnerable age groups in endemic regions, and how much these might reflect different transmission patterns and differences in infection prevalence and intensity, especially for *S.haematobium* infection.

Over the last decade, evidence has accumulated that following chemotherapeutic cure of *S.mansoni* and *S.haematobium* infections, older individuals display resistance to infection in comparison to younger children (Butterworth *et al.*, 1985, 1988; Wilkins *et al.*, 1987; Hagan *et al.*, 1987, 1991). The extent to which reinfection occurs is dependent in part upon age (Wilkins *et al.*, 1987; Butterworth *et al.*, 1988, 1991, 1992; Haggi *et al.*, 1990; Dunne, Hagan and Abath, 1995) and in conditions of continued transmission of infection, young children usually, and sometimes rapidly, become reinfected (Hagan *et al.*, 1987, 1991; Butterworth, 1991, 1992; Roberts *et al.*, 1993). In contrast, adults, if they become reinfected at all, normally become reinfected only at very low infection intensities.

The effect of age on the development of resistance has been observed in several studies of *S.mansoni* infection (Sturrock *et al.*, 1983; Dunne *et al.*, 1992; Dessein *et al.*, 1992) and of *S.haematobium* (Ndhlovu *et al.*, 1996). It has been a repeated finding in studies of human schistosomiasis, that resistance to infection develops slowly, often taking many years. This has been supported by the findings of the study described here, in which evidence of acquired immunity could not be detected in young children below six years old living in Madziwa an endemic area for *S.haematobium*. Two important factors in resistance to infection have been reported to be age and duration (history) of exposure to infections (Butterworth *et al.*, 1985, Wilkins *et al.*, 1987, Woolhouse *et al.*, 1991). The delay in the expression of resistance may be due to the slow maturation of certain protective immune mechanisms or to factors that inhibit the expression of immunity. The development of resistance has been said to take years in children but also in adults who move into endemic areas from non-endemic areas suggesting that the development of protection is dependent on repeated exposure to the parasite.

In an endemic area for schistosomiasis, the current understanding is of infection and reinfection in young children with gradual development of resistance. Since this resistance is apparently dependent on the history of exposure to infection, ('trickle' infections or 'the ghost of worms past'), then it is possible to speculate that in areas where transmission is at a high level, children would be exposed to higher quantities of infection and at a higher rate and therefore that they might develop resistance to infection more rapidly than in areas where children had low levels of exposure to infection (Woolhouse *et al.*, 1991; Hagan *et al.*, 1994). Woolhouse *et al.*, (1991) reported this early development of acquired immunity as a 'peak shift' in *S.haematobium* infections,

while Fulford *et al.*, (1992) reported similar changes taking place in *S.mansoni* infection. Peak shifts have now been reported for other helminth parasites. The early peak in intensity of infection found in this study, in individuals from the High Transmission area post-treatment, may represent a 'peak-shift' effect and could be due to the earlier development of at least an acquired partial resistance. Resistance to re-infection was also displayed by the adults from the High Transmission area after treatment. Butterworth *et al.*, (1985) and Wilkins *et al.*, (1987), in studies in Kenya and The Gambia respectively, attributed the lack of reinfection in treated adults previously infected to depend on previous experience of infection which had induced an acquired immunity. Other workers consider that some age dependent physiological changes including hormonal changes cannot be excluded as important factors in the development of resistance to infection (Fulford *et al.*, 1992). Whether physiological or immunological, the development of resistance is age-dependent and a long period of exposure to repeated challenges is necessary for its full development (Wilkins *et al.*, 1987; Butterworth and Hagan 1987; Woolhouse *et al.*, 1991; Hagan *et al.*, 1991; Dessein *et al.*, 1992; Dunne *et al.*, 1992 and Ndhlovu *et al.*, 1996).

5.1.1 CELLULAR IMMUNITY OF *S.haematobium* INFECTION.

Although much work has been done on the role of antibodies in resistance to infection with schistosomiasis, less work has been done on the role of cellular factors in resistance. Levels of proliferation of PBMC in response to specific and non specific stimulants have been exploited as a measure of cellular immunity in individuals with schistosomiasis. It has been a repeated finding that only low levels of proliferation of PBMC could be detected from heavily or chronically infected patients following schistosome antigen stimulation (Domingo and Warren, 1968; Ottesen *et al.*, 1977; Colley *et al.*, 1977a,b,

1986). However, similar studies by Gazzinelli *et al.*, (1985), Roberts *et al.*, (1993), Ribeiro de Jesus *et al.*, (1993) on *S.mansoni* infected individuals and Grogan *et al.*, (1996) on *S.haematobium* infected individuals reported that low levels of proliferation to schistosome antigens take place before treatment. The same PBMC responded well to unrelated non-schistosome stimulants before treatment. The PBMC were observed to show elevated levels of proliferation after treatment. This unresponsiveness of PBMC, before treatment, to schistosome-specific antigen stimulation appears to be related to an immunomodulation that takes place during schistosome infection. The immunoregulation was said to involve several other poorly defined agents in sera, reportedly causing anergy in T cell responses during heavy and chronic infections (Colley *et al.*, 1977b, 1983; Fieldmeier, Gastl and Poggensee, 1985a,b; Ellner *et al.*, 1985).

Immunoregulation was also thought to be involved in the responses shown by PBMC from individuals living in the High Transmission area studied here. Before treatment, PBMC from individuals with a heavy infection intensity showed lower levels of proliferation than PBMC from uninfected individuals from the same area. However, after treatment proliferation levels were reversed, and were observed to be higher among the re-infected than the uninfected individuals. One explanation for this is that praziquantel treatment had an effect on the immune response. The antigens released by dead and damaged schistosome worms as a result of the treatment boosted cellular responsiveness. As a consequence, reinfection is marked by a higher level of cellular reactivity indicated by increased levels of proliferation to schistosome-specific antigen stimulation.

In contrast, individuals of low infection intensity and prevalence from the Low Transmission area, do not experience massive schistosome invasion by the parasite. PBMC from the infected individuals from the Low Transmission area showed higher levels of proliferation to schistosome antigen stimulation than PBMC from uninfected individuals. The same pattern was observed at the follow-up points after treatment.

5.2: CYTOKINE PROFILES AND T HELPER CELL SUBSETS IN HUMAN *S.haematobium* INFECTION.

T lymphocytes are now well understood as the central regulatory cells of the immune system, with their function being mediated by cytokines whose production is induced as a result of antigen stimulated cellular activation. The T lymphocytes involved in this cross regulation have been designated as T helper type 1 (Th1) and Th2 subsets, and it is recognized that the pattern of cytokines produced may directly determine whether responses are protective in character or may induce susceptibility. The role played by the T cell subsets and cytokines in directing immune responses in schistosomiasis is still unclear. Animal studies have not helped clarify the situation since results of protection studies using mice seem to conflict with those obtained from the human studies (see below). Observing cellular and cytokine responses to *S.haematobium* in humans from areas of different endemicity have added to our understanding of how individuals respond to infection and in this study have helped in the identification of the roles played by particular cytokines in determining susceptibility or resistance to further *S.haematobium* infection. Although members of each of the cytokine classes play important regulatory roles in the immune responses, attention has been focused in this and in other studies on IFN-γ as a cytokine which is a dominant feature of the Th1 response and IL-4, IL-5 and IL-10 which are dominant features of the Th2 response. The

possible role of these 'signature' cytokines in the control of *S.haematobium* immunity has been examined in this study, principally in the context of regulation of immune responsiveness by T cells. The Th1-like responses have been found to dominate early during infection while Th2-like responses begin to be elevated during infection and dominate responses post-treatment in uninfected individuals.

Cytokines normally function in concert with one another and may be involved in generating a network or cascade of other cytokines that interact with other cell regulators such as hormones and nueropeptides that are in turn capable of stimulating the generation of many others (as reviewed by Romagnani, 1991, 1997; Abbas, Murphy and Sher, 1996). In all infected individuals examined before treatment, IFN-γ, IL-4, IL-5 and IL-10 were found at high levels. This presumably reflects the 'normal' situation in which the outcome of particular responses is determined by the interaction of a variety of cytokines which may have cross-regulatory activities, a condition which may be essential to ensure fine modulation of the immune responses as the host immune response deals with newly establishing infection.

Studies on murine schistosomiasis have shown that Th1 responses indicated by IFN-γ production are associated with protection whereas Th2 responses appear to cause immunopathology (Sher and Coffman, 1992). In contrast, studies on reinfection after chemotherapy in humans have shown that resistance is associated with eosinophilia and with production of specific IgE (Hagan *et al.*, 1985a,b, 1991; Rihet *et al.*, 1991). Production of eosinophil is dependent on IL-5 while the production of IgE is dependent on IL-4 both Th2 type responses (Lopez *et al.*, 1988; Sher *et al.*, 1990; Mahanty *et al.*, 1997). However, it may be considered that both Th1 and Th2 may be involved in

immunity. Roberts and colleagues, (1993) in a Kenyan study reported high levels of IL-5 and IFN-γ production in response to *S.mansoni* antigens in resistant individuals.

Another approach to such work is to examine T cell lines or clones derived from the PBMC of infected or exposed individuals. This has the advantage that the cells producing the cytokines can be reasonably accurately defined but the disadvantage that cytokines derived from other cell types are no longer represented in the system. Nevertheless despite the efforts involved in generating them, T cell lines and clones are proving to be a valuable source of information on human responses in general and to infections in particular. Th1 clones or cell lines through their production of IFN-γ and TNF-β have been reported to induce enhanced activity in macrophages (enhanced cellular immunity), while the Th2 clones or cell lines make products which are well adapted to promoting B cells to antibody producing cells (Del Prete, Maggi and Romagnani, 1994). The products of Th2 cells IL-4, IL-5, IL-6, IL-10 and IL-13 may each potentially enhance some aspects of the B cell response and thus of antibody production (Coffman *et al.*, 1988). A Th2-like response, by opposing the effects of IFN-γ on macrophages through the action of IL-10 and IL-4, and possibly suppressing production of IFN-γ and other cytokines of Th1 cells, could exert influence on the control of antibody production. Th2 induction may depend on the route of immunisation or infection or on the nature and concentration of the antigen (Bradley *et al.*, 1993). IFN-γ secreted by T cells causes induction of MHC class II on macrophages. More APCs expressing MHC class II results in more antigen presentation to T cells and more IFN-γ availability in the environment (Marrack and Kappler, 1994; Sprent, 1994). This may explain the high levels of IFN-γ found in the infected individuals in the current study as IFN-γ is suspected to be one of the first cytokines to be produced in abundance during

schistosome infection perhaps indicating cellular mobilisation to combat newly acquired infection.

In the study reported here, the dominance of Th2-like cytokines after treatment allows the speculation that the Th2-like cytokine environment favoured immune reactivities, possibly including ADCC mechanisms, which could have contributed to resistance to reinfection. This interpretation would be entirely consistent with the evidence from other studies which indicates that Th2-associated responses appear to be associated with resistance to reinfection (Hagan *et al.*, 1985a,b, 1991; Rihet *et al.*, 1991, 1992, Dunne *et al.*, 1992, Dessein *et al.*, 1992).

The Th1- and Th2-like cytokine patterns have been implicated in several other disease states including helminths infections, where the Th2 subset has been linked to pathogenesis. But from the critical analysis of these studies it appears the T helper cell-dependent effector and regulatory mechanisms as well as the critical role played by the cytokines in controlling immune responses during schistosomiasis need further elucidation.

In contrast to intracellular microbes, extracellular pathogens including helminths, trigger Th2-dominated responses (Urban *et al.*, 1994). In some studies these Th2 responses which dominate after infection have been related to immunological unresponsiveness. This may be suspected to be the situation in the individuals from the High Transmission area before treatment and in those with heavy infections. The Th2-like responses are involved in driving eosinophil production and activation and in antibody production by B cells. The dominance of the Th2 response results in downregulating the effects of IFN-γ that is involved in DTH. As a results of long-term dominance of this response it is suspected that T cell anergy develops and

the T cells lose responsiveness. The PBMC from the High Transmission area gave low levels of proliferative responses to stimulation with schistosome-specific antigens and this could have been due to the simultaneously elevated levels of IL-4, IL-5, IL-10 and IFN-γ before treatment.

Cytokine studies carried out over the last decade have demonstrated that T cells produce cytokines that serve as autocrine growth factors for their own lineage (Coffman *et al.*, 1991). This may be the reason that the uninfected people in the study cohort maintained an elevated Th2 type cytokine environment at the 12 and 18 months post-treatment examination points. Prior to treatment, it seems that the T cell responses maintained a balanced state of both Th1- and Th2- like cytokine environment which was observed in both infected and uninfected children having low IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ ratios. After treatment it appears that the state of T cell anergy is removed resulting in increased proliferation to specific antigen stimulation, and consequently setting up of a Th2-like cytokine environment (Coffman and von der Weid, 1997).

After early cloning *in vitro* the $CD4^+$ T cell pool does not always fit easily into the Th1/Th2 classification leading to the proposition that there are Th0 T cells which only differentiate into Th1 and Th2 upon prolonged *in vitro* culture (Romagnani, 1991; Couissinier and Dessein, 1995). This interpretation challenges the importance of the Th1 and Th2 dichotomy and has raised the possibility that it might be an artifact of *in vitro* differentiation. Another view is that, depending on the signals received, the duration of the stimulation and maturity of the immune effector responses, T cells can produce different pattern of cytokines. Various groups have attempted to determine if human T cells fit into the Th1/Th2 dichotomy. To date there is little hard and fast evidence to

support this concept, with many groups reporting PBMC cultures producing IL-2, IL-4, IL-5 and INF-γ. The lack of confirmation of the Th1/Th2 concept in human schistosomiasis may suggest that the observed differences in the cytokine production in the mouse are more likely to be a characteristic unique to the murine models. What is clear and demonstrated once again in this study is that in human schistosomiasis, no distinct polarised cytokine types for Th1/Th2 subsets exist, due to production by single T cell clones of both 'signature' cytokines which are normally used to identify each T cell subset.

5.3 CONCLUSIONS

5.3.1 INDIVIDUALS FROM AREAS OF DIFFERENT TRANSMISSION PATTERNS HAVE DIFFERENT INFECTION AND RE-INFECTION PATTERNS.

This study has shown that individuals living in *Schistosoma haematobium* endemic areas show different patterns of infection prevalence and intensity depending on the transmission pattern in the area. This study added to the findings reported by other workers that infection prevalence and intensity peaks in young children. No differences were detected in infection levels for all age groups below 16 years old, who were found to be heavily infected. However, the infection prevalence and intensity was found to be low in the adult age groups for both study cohorts living in Low and High Transmission areas. After treatment the patterns were seen to differ between the transmission areas. The individuals from High Transmission area showed a sharp rise in infection prevalence and intensity relative to age, with the infection prevalence peaking earlier than in the age group in the Low Transmission area. Considering infection intensity, it was found that the lower age groups were re-infected earlier and at higher intensities than the older age

groups with no reinfection being recorded in the adult group. For the individuals living in Low Transmission area the peak reinfection prevalence occurred in an older age group than in High Transmission area and in this case the age group was found to be most heavily infected prior to treatment and was most heavily re-infected.

5.3.2 INDIVIDUALS RESIDENT IN AN AREA WHERE INFECTION PREVALENCES AND INTENSITIES ARE HIGH, DEVELOP RESISTANCE TO INFECTION EARLIER DUE TO GREATER 'EXPERIENCE' OF PARASITES.

In this study, cellular immune response levels appear to reflect the different infection patterns and exposure histories of the people in the High and Low Transmission areas.

The individuals from the Low Transmission area are exposed to low levels of infection and, as a result, only experience a low antigen load. Consequently their infection status does not progress beyond an 'acute' phase and this is reflected in their continuing susceptibility to infection and immunological reactivity to stimulation with schistosome antigens *in vitro*. Those living in this area who are not infected are probably not infected due to the relatively restricted opportunities for exposure. Therefore they have only limited, and possibly no, experience of the parasite. Reflecting this 'naive' status, PBMC obtained from them fail to respond to schistosome antigen.

The failure of cells derived from individuals living in High Transmission area to respond to schistosome antigen stimulation probably reflects a state of T cell anergy. This is a characteristic feature of schistosomiasis mansoni and is well documented in the literature. Occurring as a result of intense infection this relatively simple host adjustment to the

persistent antigenic stimulation can be regarded as beneficial for the host and thus essential for the long-term maintenance of the adult parasite and of its life-cycle.

The individuals from the High Transmission area who were not infected showed a high response to schistosome antigen stimulation. This may be indicative of some degree of resistance to infection which is constantly re-stimulated through repeated exposures. Immunological reactivity is maintained while invading parasites are eliminated. In contrast to these infected individuals from High Transmission area, infected individual from the Low Transmission area gave even higher levels of responses presumably reflecting the absence of development of any immunomodulation, whereas the uninfected individuals in Low Transmission area who gave very low levels of response to stimulation.

Treatment with praziquantel caused some form of immune boosting which was shown in this study to result in increased reactivity to stimulation with all schistosome antigens relative to pre-treatment reactivity levels. Although the overall response to stimulation increased, the infected individuals from the Low Transmission area remained high responders while the non-infected still showed low responses to schistosome antigen stimulation. The individuals in the High Transmission area showed a reverse of their responses possibly indicating the development of protective immunity post-treatment perhaps resulting from the enhanced exposure to parasite antigens induced by treatment. This may also have been reflected in reinfection after treatment with the adults remaining uninfected. In contrast, adults from the Low Transmission area were re-infected after treatment. This would be consistent with the view that as yet they had failed to develop an effective protective immune response and that the low levels of infection (antigenic

load) they had were insufficient to induce an enhanced reactivity following treatment. Taken together these findings suggest that repeated exposure to the parasite is essential for the development of protective immune responses. Although the increased reactivity after treatment of individuals from the High Transmission area may in part be explained by the lifting of the observed immunoregulation it was noted that following treatment this status was not readily regained as re-infected individuals in the High Transmission area showed high responses to specific schistosome antigens even at 18 months post-treatment compared with the pre-treatment infected groups.

5.3.3: THE CYTOKINE PROFILES VARY ACCORDING TO INFECTION PATTERNS OF EACH AREA AND THE ANTIGENS STIMULATING THEIR PRODUCTION.

Individuals living in different areas of schistosome transmission show different patterns of cytokine production after schistosome-specific antigen stimulation. The individuals from the High Transmission area where infection was high produce, overall, high levels of all the cytokines as measured in this study (IL-4, IL-5, IL-10 and IFN-γ), compared to the individuals from Low Transmission area of schistosomiasis. Even though the PBMC from individuals living in the High Transmission area were found to give low levels of proliferation to stimulation, they were capable of producing higher levels of cytokines when compared with the responding PBMC obtained from individuals living in the Low Transmission area. Within each transmission area, the levels of each cytokine have been found to differ according to infection status. Before treatment, the infected individuals from both areas produced high levels of IFN-γ (a Th1-type cytokine) which may be involved in early stages of the development of immune responses aimed at curbing infection. However, it was clearly shown when examining the PBMC from the High

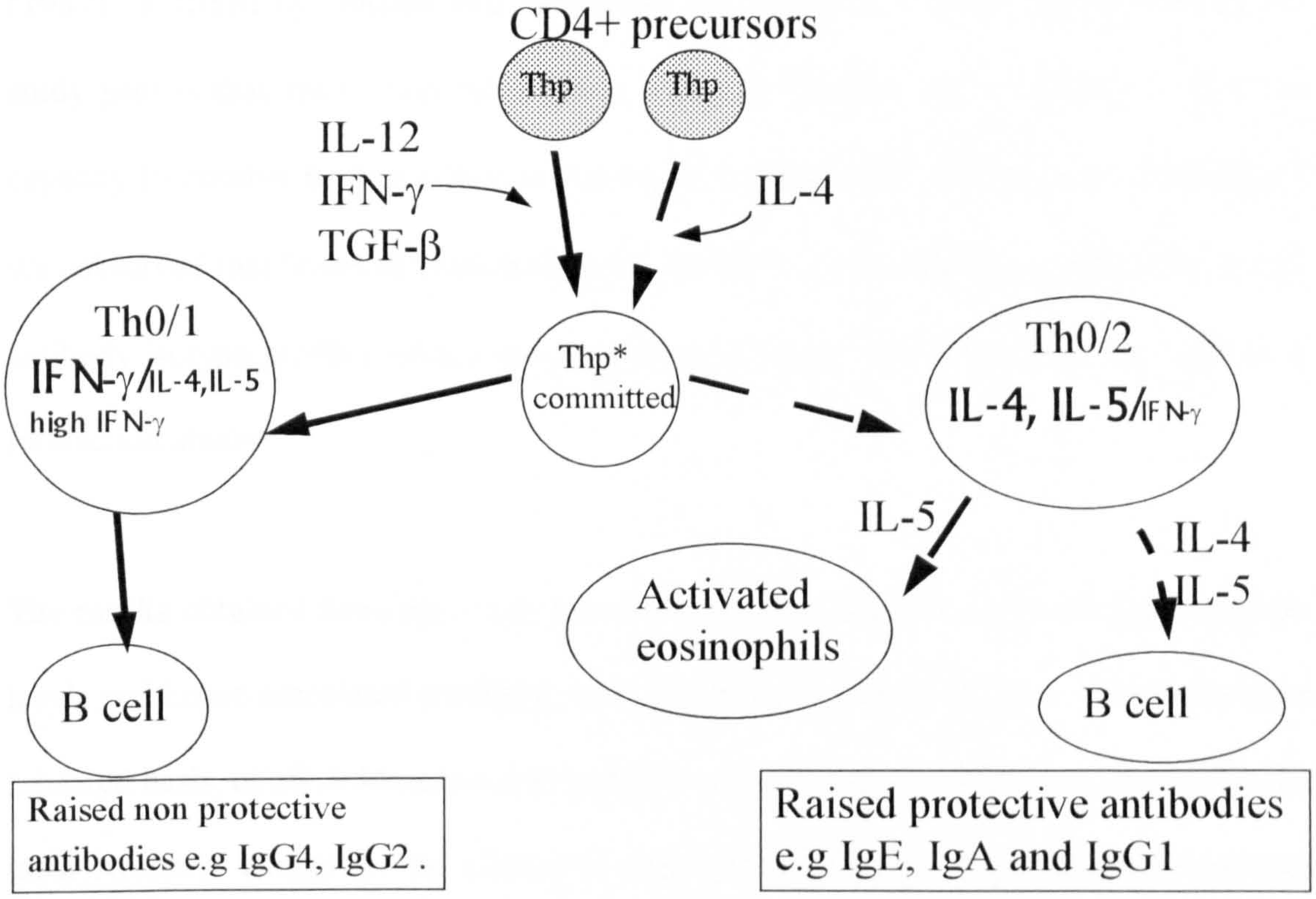
Transmission area, that the Th2 cytokines (IL-4, IL-5 and IL-10) were also produced at high levels during infection and before treatment. This raises the possibility of having an immune response geared both to mobilise cell-mediated immunity and drive humoral responses, by involvement of both Th1 and Th2 cytokines. As Th1 and Th2 cytokines are normally considered to be cross-regulatory, further work is needed to confirm if this combined cytokine production is reflected in both cellular and antibody responses to schistosome infection. This study has revealed that following treatment the individuals who remain uninfected in the High Transmission area had a dominating Th2-like response with high levels of IL-4, IL-5 and IL-10 relative to IFN-γ. However, individuals who become re-infected had high levels of IFN-γ compared with those who were uninfected but the ratios of the Th1/Th2 cytokines showed that the cytokines for these classes (IL-4:IFN-γ, IL-5:IFN-γ) were found at a similar level both before and after treatment.

5.3.4 THE EXISTENCE OF TH0/1- AND TH0/2-LIKE CYTOKINE SUBSETS EVIDENCED FROM T CELL CLONING.

The work in this study on T cell cloning for the characterisation of T cell responses involved in schistosomiasis immune responses produced clearly unpolarised cytokine profiles of the T helper cell subsets. This is in contrast with the findings in other studies cited above, many of them from studies of murine models of *S.mansoni* infections. However, the findings on the T cell cloning mirrored the results reported in human *S.mansoni* infection reported by Couissinier and Dessein, (1995). In their experiments Couissinier and Dessein found that both 'signature' cytokines were co-expressed, with the T cell clones having a dominating Th2-like cytokine profiles being from resistant individuals. In this current study the PBMC were obtained from individuals from the

High Transmission area prior to treatment. Age and duration of infection, (history of exposure to infection) may play a role in influencing the development of unpolarised T helper cells with the combined 'signature' cytokine profiles which have been recorded. In terms of IL-4:IFN-γ and IL-5:IFN-γ the 'signature' cytokines for defining each T helper cell subset, most of the clones derived from all the children showed co-expression of the cytokines, resulting in the subsets being classified as Th0/1 and Th0/2, depending on the ratio. Although the extremes of each subset class, i.e. Th1 and Th2 were recorded, these were relatively rare. Although the sample size is limited, from this work it is concluded that the balance of the cytokines relative to each subset marks the immune state of an individual. Those able to resist parasite invasion and establishment mounted protective immune responses reflected in elevated levels of IL-4, IL-5 and IL-10, cytokines marking Th0/2-like responses. In contrast, the dominance of IFN-γ marks a state of susceptibility to infection of a given individual. However, the balance between these Th1- and Th2-like responses appears to be finely tuned and depending on the timing, quantity and duration of exposure to infection, the cytokine balance may shift, possibly resulting in a change in the immune response from putative protective to susceptibility or *vice versa* (Figure 5.1). Additional work is required to confirm this hypothesis.

Figure 5.1: T helper subsets according to cytokine phenotypes in human schistosomiasis susceptibility and protection.



5.3.5 PRAZIQUANTEL IS USEFUL AS A CHEMOPROPHYLAXIS IN PREVENTING FURTHER HEAVY INFECTION IN YOUNG CHILDREN, ALTHOUGH PROTECTIVE IMMUNITY SEEMS NOT TO DEVELOP.

The study has shown that praziquantel can be useful as a chemoprophylaxis without any adverse effects on young children. Reinfection at low intensity was observed taking place in the study children during the early stages of the repeated treatment programme. The period during which reinfection took place has been reported to be the high transmission season in Zimbabwe, during which the hot weather is conducive to snail population propagation and the weather also contributing to high water contact activities, even in small children accompanying their mothers to the rivers (Chandiwana, Makaza and

Taputaira, 1987). Similar to studies undertaken in Kenya by Butterworth and colleagues on *S.mansoni* (1988), in The Gambia by Wilkins and colleagues on *S.haematobium* (1987), in Brazil by Dessein and colleagues on *S.mansoni* (1992); the findings of this study proves that reinfection takes place in young children who are likely to lack the capacity to combat further infection. However with repeated praziquantel treatments it was observed that levels of reinfection were markedly reduced in the study children. The antibody isotype profiles gave a similar picture to those often reported from children in reinfection studies.

The results obtained from this study provide a possible effective way to reduce infection levels and hence associated morbidity and pathology of *S.haematobium.* Treatment on a repeated basis, of all children living in endemic areas at staggered intervals remains as the most attractive alternative for alleviating suffering in children whose immune responses are not yet fully developed. However due to the cost of such programmes, a targeted approach may be helpful, in which only infected children are treated. Of course this has an inherent cost in the need for relatively skilled manpower for diagnosis before treatment but with schistosomiasis, simpler diagnostic methods (dipsticks) are being investigated. Mass treatment of children without diagnosis is also possible but costly in terms of drug use. The need for skilled manpower for drug administration can be met ideally by using existing health systems in the endemic areas. Although children in endemic areas start to acquire schistosome infections from the age of 2 to 4 years, they do not become readily immune to infection. In this study reinfection continued to take place and there was a lack of development of protective antibody isotypes.

5.4 RECOMMENDATIONS

Human immuno-epidemiological studies are always desirable for an improved understanding of the disease conditions in the actual host and ultimately for the final understanding and application of vaccine candidates when they become available. However, such studies have to contend with several hurdles as far as the use of, and working with, the human host is concerned, including the participation and willingness to provide the required biological samples, especially blood. The use of communities in many endemic areas may require some application of visible immediate benefits during implementation and progress of the study, e.g. provision of treatment for the disease under study. In an ideal situation, studies of this type would be fully integrated into the existing health care system of the areas where they are carried out.

The findings on *S.haematobium* reported in this thesis seem to be in line with other work reported from other endemic areas on *S.mansoni* and *S.haematobium*. The use of relatively unpurified antigenic extracts of different life cycle stages can be justified on the basis that the organism is so complex, that immunity and immune responsiveness is unlikely to be determined by responses to a single antigen. Instead the net level of immunity may depend on a combination of different responses to different antigens. However it is still valid to select to study responses to individual purified or recombinant antigens which can provide useful information in the development of vaccines and in eliciting particular immune responses.

The findings presented in this work indicate that Th2-like immune effects may be important in mediating human protective immunity to schistosome infection. This does not exclude the possibility that the mechanisms of resistance reported from studies of the mouse model of immunity to schistosomes, with a reported Th1-like bias involving a predominance of IFN-γ

are not also important in human schistosomiasis. IFN-γ was produced early and in abundance during infection indicating a useful role during this infection stage.

T helper cell subset determination is still complex and requires more work. Considering the number of patients' samples that can be handled at any one time in the determination of the subsets using single cell assays, the methodology needs to be modified and simplified to make it more practical for routine field use. Although over-modification can sometimes result in a weakening of the specificity of the methods, the use of two or more methods for a determination may help strengthen the significance of the findings, e.g. T cell cloning by cell cultures and the use of mRNA detection of cytokines genes by reverse transcriptase-polymerase chain reaction (RT-PCR) at the same time. The use of RT-PCR and/or intra-cellular cytokine staining may help the study of the T helper subsets in a wider age range of a population living in endemic areas. This may be important as cytokine polarisation may be present in a particular age group or group with a particular history of exposure to infection. Another method currently available is the use of ELISPOT, the method is useful in determining the specific number of cells in a clone involved in the cytokine production.

This study, has looked at an age group within the range where peak infection prevalence and intensity is found and coincidentally this may be the age group where cytokine polarisation is lacking due to the effects of infection intensity. Therefore, a cautious interpretation of the data demands that a distinct cytokine polarisation of Th1/Th2 subsets should not as yet be excluded, possibly being found in adults living in the High Transmission area, who had more experience of infections.

The use of praziquantel as a chemoprophylaxis should now be regarded as the only useful alternative to protect young children living in schistosome endemic areas from infection and subsequent pathology. Since the cost of the drug has now been reduced and significant drug resistance has not been reported, the duration of retreatment can be extended six to 12 month intervals. Several studies have reported reinfection prevalence reaching half the pre-treatment levels from six months post-treatment. Thus a six monthly treatment schedule may make the programmes supportable in terms of the cost of drug purchase. This approach would benefit greatly the young children in these endemic areas by protecting them from further re-infection and the heavy infection levels that result in morbidity and pathology, since it is well documented that the severity of clinical disease is dependent on intensity of infection rather than simply presence or absence of infection.

The findings summarised in this concluding chapter, like those presented elsewhere in this thesis, give some additional information for the development of a vaccine for use against schistosome infections in human. Immunity can be demonstrated in naturally infected individuals after subsequent chemotherapy, and the indications are that it is the nature of the response that is important, and not just the exact identity of a particular antigen which provides protective immunity. The most important and crucial issue appears to be the achievement of the right balance of protective as opposed to inappropriate responses. Considerable work is required on ways to elicit the correct responses in humans already primed to infection, which seem to be different where infections exist naturally and the period post-treatment. This leaves a huge gap that requires filling especially for the possibly developed vaccines, the stages at which they may have to be administered, with or before treatment, and the age groups that may require the vaccines.

6.0 APPENDIX

APPENDIX:

Table 6.1: Low Transmission area - Infection intensity (ep10ml) according to response categories at pre- and post-treatment determined using dye reduction method.

Antigen/ mitogen	PHA		WORM		EGG		CAP	
	low	mod/high	low	mod/high	low	mod/high	low	mod/high
Pre-treatment								
n	47	41	60	28	45	43	60	28
mean	14.0	3.8	11.0	6.1	10.0	9.0	10.4	6.6
± SD	40.0	11.0	34.0	21.0	35.0	25.0	33.0	21.0
median	1.0	0	0	0.5	0	1.0	0	0.5
range	0-210	0 - 58.0	0 - 210	0 - 110	0-210	0 - 114	0-210	0 - 110
Post-treatment								
n	26	22	43	5	40	8	44	4
mean	1.0	1.0	1.0	0	0.7	0	0.6	0
± SD	1.7	2.1	2.0	0	2.1	0	2.0	0
median	0	0	0	0	0	0	0	0
range	0 - 7	0 - 10	0 - 10	0	0 - 10	0	0 - 10	0

Table 6.2: High Transmission area - Infection intensity (ep10ml) according to response categories at pre- and post-treatment, determined using dye reduction method.

Antigen/ mitogen	PHA		WORM		EGG		CAP	
	low	mod/high	low	mod/high	low	mod/high	low	mod/high
Pre-treatment								
n	63	46	92	17	68	41	91	18
mean	52.0	41.0	47.0	45.0	54.0	35.0	54.0	12.0
± SD	113.0	86.0	101.0	109.6	112.0	84.6	110.0	26.0
median	0	5.0	6.0	2.0	9.0	2.0	8.0	2.0
range	0-567	0 - 448	0- 567	0 - 448	0- 567	0 - 448	0- 567	0 - 93
U							1035.5	602.5
p-value								0.078
Post-treatment								
n	29	32	57	4	55	6	58	3
mean	2.0	22.0	10.0	40.0	8.0	49.0	13.0	0
± SD	5.3	50.0	33.6	79.0	29.4	76.2	38.5	0
median	0	0	0	0	0	0	0	0
range	0 - 26	0 - 192	0- 192	0 - 158	0- 192	0 - 158	0- 192	0
U	356.0	572.0	110.5	117.3	142.0	188.0		
p-value	0.121		0.93		0.586			

Table 6.3: Low Transmission Area, Stimulation Index, determined by using tritiated thymidine.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	3	5	9	3	9	4
mean	81.2	73.8	44.8	125.3	115.5	160.1
± SD	69.1	56.8	55.8	102.3	123.0	78.2
median	96.9	62.2	36.6	124.0	93.9	194.3
range	5.7 - 141.1	21 - 163.2	3.5 - 181.1	23 - 227.6	8.2 - 380	44 - 208.1
WORM n	3	5	9	3	9	4
mean	10.3	135.1	29.4	137.8	18.1	108.8
± SD	5.1	96.5	26.6	49.0	16.1	74.0
median	11.1	83.5	25.8	136.0	11.7	108.8
range	4.7 - 14.9	68 - 298	2.0 - 90.1	89 - 187	1.4 - 43	19.6 - 196
EGG n	3	5	9	3	9	4
mean	12.2	224.2	45.1	229.1	30.2	120.6
± SD	10.6	214.3	50.6	0.9	31.7	65.9
median	12.3	125.0	35.0	229.6	8.7	145.3
range	1.5 - 22.6	46 - 581	2.0 - 170	229 - 230	2.7 - 87	24 - 167.5

Table 6.4 High Transmission Area Stimulation Index, determined using tritiated thymidine.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	4	11	5	4	5	8
mean	77.5	23.4	59.0	34.6	35.4	91.1
± SD	35.8	21.6	24.5	23.6	29.5	96.4
median	67	18.7	57.4	40.7	37.0	41.0
range	49 - 125	2.6 - 76	25 - 94	2.8 - 54	3.0 - 73	3.5 - 235
WORM n	4	11	5	4	5	8
mean	28.3	6.3	24.9	21.6	4.8	17.0
± SD	6.0	8.4	4.7	20.7	1.8	22.2
median	31.0	2.8	24.9	21.5	4.9	5.7
range	20 - 32	1.1 - 30	18.2 - 32	3.3 - 40	2.1 - 7	1.7 - 66
EGG n	4	11	5	4	5	8
mean	48.0	7.3	19.8	38.7	18.1	31.5
± SD	13.1	8.4	12.8	14.8	21.2	41.3
median	45.7	3.6	15	37.5	6.2	17.9
range	35 - 66	1.1 - 23	10 - 41	22.5 - 57	4.6 - 55	2.3 - 129

Table 6.5 Low transmission area Infection intensity (ep10ml) according to response categories at pre- and post-treatment, determined using tritiated thymidine.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
Pre-treatment						
n	3	5	3	5	3	6
mean	0	6.2	0	6.0	0	5.0
± SD	0	8.9	0	8.7	0	5.0
median	0	1.0	0	1.0	0	1.0
range	0	0 - 21	0	1 - 21	0	1 - 21
U	1.0	7.0	0.00	15.0	1.0	11.0
p-value	0.3830		0.0369		0.1336	
Post-treatment						
n	6	19	10	15	8	17
mean	0	1	0.2	2.0	0	2.0
± SD	0	2.0	0.6	2.2	0	2.1
median	0	0	0	0	0	0
range	0	0 - 7	0 - 2	0 - 7	0	0 - 7
U	13.5	40.5	21.5	88.5	17.5	80.5
p-value	0.1914		0.0201		0.0207	

Table 6.6: High Transmission area - Infection intensity (ep10ml) according to response categories at pre- and post-treatment determined using tritiated thymindine.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
Pre-treatment						
n	6	9	11	4	11	4
mean	34.0	28.0	39.0	6.0	39.0	6.0
± SD	40.0	61.9	59.3	11.5	59.3	11.5
median	16.0	0	6.0	0	6.0	0
range	1 - 103	0 - 185	0 - 185	0 - 23	0 - 185	0 - 23
U	41.5	12.5	35.5	8.5	35.5	8.5
p-value	0.0990		0.0896		0.0896	
Post-treatment						
n	5	17	13	9	11	11
mean	2.4	11.2	6.2	13.4	4.0	15.0
± SD	3.2	26.3	13.0	33.7	7.5	31.7
median	2.0	1.0	1.0	2.0	0	2.0
range	0 - 8	0 - 103	0 - 43	0 - 103	0 - 25	0 - 103
U	109.0	70.0	80.0	67.0	93.0	78.0
p-value	0.375		0.750		0.731	

Table 6.7:Values of IFN-γ determined from the individuals from the Low Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months post-treatment		18 months post-treatment	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	3	7	12	3	9	4
mean	289.1	945.5	865.1	980.2	471.7	2146.9
± SD	229.0	742.7	1879.0	666.9	242.2	2169.3
median	186.7	728.5	251.0	1318.7	436.0	1203.5
range	129-552	280-2444	159-6800.0	212 - 1410	171 - 935.0	803 - 5377
WORM n	3	7	12	3	9	4
mean	86.3	155.6	62.0	149.7	91.5	132.2
± SD	35.1	65.0	43.5	37.0	68.2	77.4
median	103.0	140.0	42.5	140.0	71.0	159.4
range	46 - 110	100 - 284	10 - 161	119 - 190	27 - 214	20 - 190.0
EGG n	3	7	12	3	9	4
mean	69.3	176.2	55.2	179.0	64.4	85.0
± SD	42.0	86.0	51.1	79	57.1	47.1
median	70.0	138.0	26.5	140	51.0	106.3
range	27 - 111	110 - 359.3	12 - 181.0	126 - 270	9 - 186	14.5 - 113

Table 6.8: Values of IL-4 determined from the individuals from the Low Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months post-treatment		18 months post-treatment	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	3	5	5	2	4	4
mean	64.9	83.0	173.7	91.0	130.2	112.0
± SD	7.8	44.0	98.0	48.0	74.3	80.7
median	68.0	78.0	169.0	91.0	144.9	108.1
range	56 - 70.7	19.2 - 128	55 - 310	57 - 125	27 - 204	42 - 189.7
WORM n	3	5	5	2	4	4
mean	54.0	45.0	99.4	15.0	91.8	68.7
± SD	9.5	19.0	52.6	7.0	44.9	52.7
median	54.0	40.0	105.0	15.0	105.0	57.5
range	43 - 62.0	20 - 69	16 - 161	10 - 20	29 - 128.0	23.7 - 136
EGG n	3	5	5	2	4	4
mean	25.0	47.0	131.2	34.0	91.8	41.6
± SD	8.1	28.0	90.7	21.0	54.6	35.5
median	27.0	36.0	125.5	34.0	117.5	30.0
range	16 - 32.0	14 - 80	15 - 262	19 - 48	10 - 122	14 - 92.0

Table 6.9: Values of IL-5 determined from the individuals from the Low Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	3	6	12	3	10	4
mean	1470.6	1042.0	755.8	821.2	526.6	1547.0
± SD	1276.0	1017.0	623.6	947.5	265.7	1029.7
median	1568.3	709.6	565.8	890.0	546.5	1703.0
range	149 - 2694	204 - 2923	210 - 2481	108 - 1896	115 - 896.7	272 - 2511
WORM n	3	6	12	3	10	4
mean	115.7	243.5	191.6	388.4	277.5	283.0
± SD	32.7	122.0	145.3	291.0	258.6	250.1
median	134.2	279.0	145.0	370.0	168.0	192.0
range	78 - 135	40 - 371.8	60 - 569	106 - 689	103 - 884	108 - 640.3
EGG n	3	6	12	3	10	4
mean	151.1	323.0	252.7	283	200.5	265.4
± SD	36.0	211.0	329.3	230.0	126.1	176.0
median	169.0	339.0	170.4	270.0	172.0	208.0
range	110 - 174.2	90 - 600	70 - 1275.2	60 - 519	107 - 509.0	121.5 - 522

Table 6.10: Values of IL-10 determined from the individuals from the Low Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	3	5	9	2	9	4
mean	124.2	47.0	127.2	74.5	115.4	129.3
± SD	19.4	25.0	56.0	6.4	39.8	39.4
median	118.0	41.0	127.0	74.5	113.0	118.0
range	109 - 146.0	16 - 84	40 - 209	70 - 79	51 - 182.0	99 - 183.0
WORM n	3	5	9	2	9	4
mean	68.0	17.6	80.4	9.0	59.6	50.0
± SD	32.7	17.0	63.1	1.4	27.8	32.0
median	80.0	10.0	101.0	9.0	60.0	49.0
range	31 - 93	9 - 48	13 - 200	8.0 - 10.0	23 - 90	18 - 84.0
EGG n	3	5	9	2	9	4
mean	154.0	18.0	121.8	7.5	76.7	42.5
± SD	44.7	9.4	71.3	0.7	42.8	27.9
median	149.0	18.0	116.0	7.5	60.0	41.0
range	112 - 201	9 - 32	23 - 245.0	7.0 - 8.0	40 - 170	17 - 70

Table 6.11: Values of IFN-γ determined from the individuals from the High Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months post-treatment		18 months post-treatment	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	5	8	5	7	6	7
mean	1565.6	4019.0	1111.0	4419	534.1	1935.7
± SD	673.0	2124.0	554.0	1779.0	379.5	1581.0
median	1613.0	4068.0	889.0	4864.0	335.5	2300.7
range	917 - 2563	500 - 7169	630 - 2033	1907 -7240	227 - 1127	265 - 4163
WORM n	5	8	5	7	6	7
mean	641.2	3779.9	452.0	3190.0	165.7	1119.4
± SD	137.0	1749.0	356.0	2173.0	127.6	828.3
median	611.0	3836.0	279.0	2010.0	124.0	1070.0
range	470 - 819	1276- 6992	118 - 942	1229 -7000	40 - 407	200 - 2157
EGG n	5	8	5	7	6	7
mean	403.0	3662.0	354.0	2877.0	173.1	1418.4
± SD	69.0	2312.0	267.0	2386.0	61.7	1133.9
median	421.0	3201.0	319.0	1640.0	160.4	1102.0
range	290 - 470	1347-8648	98 - 770	1240 -7780	112 - 249	125 - 2860

Table 6.12: Values of IL-4 determined from the individuals from the High Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months post-treatment		18 months post-treatment	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	4	8	3	4	2	5
mean	460.0	1871.0	1499.0	418.0	7711.0	1521.2
± SD	362.0	1688.0	560.0	324.0	2179.0	2034.3
median	361.0	1109.0	1254.0	365.0	7711.0	848.0
range	141 - 979	936 - 5936	1104- 2140	89 - 853	6170- 9252	31 - 5070
WORM n	4	8	3	4	2	5
mean	269.0	1414.0	1536.0	258.0	2803.0	1116.0
± SD	216.0	1054.0	558.0	120.0	1862.9	1291.2
median	229.0	992.0	1380.0	261.0	2803.0	772.0
range	79 - 541	490 - 3527	1073-2155	110 - 399	1486 -4120	12 - 3294.0
EGG n	4	8	3	4	2	5
mean	359.0	1226.0	2156.0	188.0	2326.3	1452.7
± SD	292.0	829.0	892.0	107.0	1108.4	1990.2
median	317.0	931.0	1895.0	178.0	2326.0	760.0
range	63 - 738	820 - 3267	1423-3150	69 - 328	1543- 3110	10 - 4923.5

Table 6.13: Values of IL-5 determined from the individuals from the High Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	4	9	6	5	5	8
mean	1378.0	5262.0	4539.0	6641.0	3516.1	3391.5
±SD	411.0	4031.0	2496.0	2783.0	2340.0	3454.0
median	1424.0	4827.0	4235.0	6658.0	3265.1	2344.8
range	908 - 1758	1316-13370	1654-8700	2115-9134	598-7797.0	208-9645.0
WORM n	4	9	6	5	5	8
mean	987.0	5194.0	3612.0	2874.0	2614.4	1978.2
± SD	283.0	2684.0	3255.0	2505.0	2264.1	2393.5
median	926.0	4613.0	2111.0	1205.0	2104.0	869.3
range	732-1366	1134 -10060	1152-9820	780 - 5840	120 - 6066	128 - 6241
EGG n	4	9	6	5	5	8
mean	1096.0	4494.0	3757.0	1540.0	2416.6	1470.9
±SD	291.0	2787.0	2075.0	861.0	1816.6	1531.8
median	1093.0	4329.0	3319.0	1158.0	2460.0	853.6
range	800 - 1399	1299-10160	1626-6882	670 - 2572	370 - 5217	270 - 4539

Table 6.14:Values of IL-10 determined from the individuals from the High Transmission Area.

Antigen/ mitogen	Pre-treatment		12 months	post-treatment	18 months	post-treatment
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
PHA n	4	9	6	5	5	8
mean	1030.0	2387.0	1495.0	1503.0	1607.8	544.3
±SD	110.0	1740.0	1027.0	1183.0	1040.0	537.6
median	996.0	1724.0	919.0	1106.0	990.0	290.0
range	941 - 1189	841 - 5583	755 - 3109	330 - 3409	801 - 3012	74 - 1515
WORM n	4	9	6	5	5	8
mean	861.0	1558.0	1099.0	698.0	744.2	484.8
± SD	119.0	1348.0	511.0	476.0	294.9	535.7
median	906.0	870.0	875.0	661.0	690.0	189.0
range	689 - 944	211 - 3933	740 - 2110	201 - 1340	416 - 1210	30 - 1395.0
EGG n	4	9	6	5	5	8
mean	727.6	2466.0	878.0	564.0	618.6	483.3
± SD	211.0	2218.0	361.0	208.0	245.6	583.1
median	828.0	1110.0	745.0	678.0	740.0	219.5
range	411 - 844	420 - 6098	560 - 1498	310 - 741	244 - 840.0	56 - 1705.0

Table 6.15: Value of cytokine levels according to response categories determined from the individuals from the Low Transmission area before treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IFN-γ n	1	7	3	5	2	6
mean	552.0	780.0	86.3	169.8	91.0	169.0
± SD	-	801.0	35.0	72.0	28.0	109.0
median	552.0	687.0	103.0	170.0	90.0	151.0
range	-	129 - 2444.0	46 - 110.0	105 - 284.0	70 - 111.0	27 - 359.0
IL-4 n	1	6	3	4	2	5
mean	71.0	69.9	53.0	46.5	24.0	46.0
± SD	-	35.0	9.5	21.0	11.0	28.0
median	71.0	69.0	54.0	48.0	24.0	36.0
range	-	19 - 128.0	43 - 62.0	20 - 69.0	16 - 32.0	14 - 80.0
IL-5 n	1	7	3	5	2	6
mean	156.8	1098.0	116.0	228.0	140.0	252.0
± SD	-	1193.0	32.7	129.0	41.0	166.0
median	156.8	640.0	134.0	240.0	139.0	206.0
range	-	149 - 2923.0	78 - 135.0	40 - 372.0	110 - 169.0	90 - 470.0
IL-10 n	1	6	3	4	2	5
mean	108.7	76.3	68.0	20.0	131.0	57.0
± SD	-	49.0	32.7	19.0	26.0	81.0
median	108.7	71.0	80.0	11.0	130.0	21.0
range	-	16 - 146.0	31 - 93.0	9 - 48.0	112 - 149.0	10 - 201.0

Table 6.16: Value of cytokine levels according to response categories determined from the individuals from the Low Transmission area after treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IFN-γ n	6	19	10	15	8	17
mean	393.3	876.0	101.0	87.0	60.0	82.0
± SD	213.0	1168.0	60.0	70.0	38.0	72.0
median	380.0	436.0	87.0	41.0	53.0	78.0
range	163 - 613.0	170 - 5377.0	27 - 190.0	10 - 214.0	9 - 110.0	14 - 270.0
IL-4 n	4	10	5	9	4	10
mean	111.0	127.0	84.0	71.0	102.0	69.0
± SD	87.0	61.0	62.0	49.0	118.0	45.0
median	98.0	137.0	85.0	90.0	68.0	70.0
range	27 - 220.0	42 - 204.0	16 - 161.0	10 - 136.0	10 - 262.0	14 - 126.0
IL-5 n	5	19	10	14	8	16
mean	1051.0	793.0	315.0	203.0	201.0	215.0
± SD	830.0	686.0	285.0	164.0	144.0	130.0
median	808.0	530.0	165.0	118.0	133.0	190.0
range	310 -2481.0	107 - 2511.0	79 - 884.0	60 - 689.0	100 - 509.0	60 - 522.0
IL-10 n	4	16	10	10	8	12
mean	136.0	112.0	68.0	46.0	109.0	47.0
± SD	35.0	41.0	33.0	35.0	65.0	32.0
median	128.0	113.0	83.0	35.0	107.0	50.0
range	102 - 186.0	51 - 183.0	17 - 110.0	8 - 116.0	42 - 221.0	7 - 110.0

Table 6.17: Value of cytokine ratios according to response categories determined from the individuals from the Low Transmission area before treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IL-4:IFN-γ						
n	1	6	3	4	2	5
mean	0.13	0.23	0.68	0.32	0.24	0.44
± SD	-	0.16	0.23	0.22	0.04	0.37
median	0.13	0.23	0.60	0.22	0.26	0.48
range	-	0.07 - 0.43	0.40 - 0.93	0.10 - 0.66	0.20 - 0.29	0.07 - 1.00
IL-5:IFN-γ						
n	1	7	3	5	2	6
mean	2.84	3.37	1.65	1.36	1.55	2.40
± SD	-	5.10	1.14	0.76	0.03	2.31
median	2.84	1.16	1.31	1.31	1.55	1.81
range	-	0.08-14.43	0.7-2.92	0.30-2.29	1.54-1.57	0.45-6.45
IL-10:IFNγ						
n	1	6	3	4	2	5
mean	0.20	0.32	1.00	0.10	1.57	1.57
± SD	-	0.41	0.70	0.05	0.79	3.28
median	0.20	0.09	0.90	0.10	1.57	0.11
range	-	0.02-0.91	0.20-1.74	0.05-0.17	1.01-2.13	0.07-7.44

Table 6.18: Value of cytokine ratios according to response categories determined from the individuals from the Low Transmission area after treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IL-4:IFN-γ						
n	4	10	5	9	4	10
mean	0.43	0.21	1.11	1.88	3.42	1.90
± SD	0.61	0.17	1.21	2.50	5.25	2.50
median	0.17	0.20	0.45	0.56	1.20	0.75
range	0.04-1.34	0.07-0.48	0.16-2.84	0.07-6.80	0.10-11.15	0.07-6.97
IL-5:IFN-γ						
n	5	19	10	14	8	16
mean	2.78	1.33	4.61	4.65	4.95	5.02
± SD	2.01	0.77	5.60	6.35	4.03	4.17
median	1.35	1.28	2.50	3.22	3.02	4.28
range	1.29-5.27	0.007-2.95	0.55-19.64	0.54-25.7	0.90-12.20	0.20-14.50
IL-10:IFNγ						
n	4	16	10	10	8	12
mean	0.44	0.21	0.81	1.42	2.49	1.40
± SD	0.47	0.15	0.45	1.98	1.85	1.54
median	0.23	0.16	0.67	0.27	1.69	0.74
range	0.16-1.14	0.02-0.57	0.19-1.67	0.04-5.80	0.70-5.64	0.02-4.34

Table 6.19: Value of cytokine levels according to response categories determined from the individuals from the High Transmission area before treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IFN-γ n	4	8	8	4	8	4
mean	4036.0	2859.0	3449.0	1321.0	3325.0	1067.0
± SD	2411.0	1926.0	2106.0	1202.0	2576.0	1396.0
median	4979.0	2491.0	3836.0	776.0	2678.0	408.0
range	500 - 5684.0	917 - 7169.0	470 - 6992.0	611 - 3120.0	470 - 8648	290 - 3160
IL-4 n	4	7	8	3	7	4
mean	1446.0	1518.0	1144.0	964.0	1269.0	486.0
± SD	524.0	1988.0	1088.0	1124.0	885.0	414.0
median	1474.0	1030.0	802.0	541.0	934.0	475.0
range	936 - 1900.0	141 - 5936.0	79 - 3527.0	112 - 2239.0	820 - 3267	63 - 929.0
IL-5 n	4	8	9	3	8	4
mean	6539.0	3129.0	4940.0	1623.0	4792.0	1302.0
± SD	1983.0	4197.0	2996.0	1289.0	2821.0	598.0
median	6334.0	1618.0	4613.0	1037.0	4412.0	1150.0
range	4827.-.8659	907 - 13370	815 - 10060	732 - 3101	1299-10160	800 - 2110
IL-10 n	4	8	9	3	8	4
mean	2557.0	1805.0	1565.0	897.0	2690.0	685.0
± SD	1722.0	1595.0	1343.0	75.0	2259.0	197.0
median	2573.0	1102.0	876.0	937.0	2368.0	743.0
range	1008 - 4073	841 - 5583.0	211 - 3933	810 - 944.0	420 - 6098	4511 - 844

Table 6.20: Value of cytokine levels according to response categories determined from the individuals from the High Transmission area after treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IFN-γ n	4	15	12	12	12	12
mean	1034.0	2007.0	922.0	1555.0	482.0	2004.0
± SD	1142.0	1611.0	1198.0	1638.0	728.0	2259.0
median	590.0	1902.0	315.0	1070.0	212.0	1241.0
range	265 - 2690.0	227 - 5416.0	109 - 4020.0	118 - 4860.0	98 - 2240.0	112 - 7780
IL-4 n	3	9	6	5	4	7
mean	5242.0	1624.0	1251.0	1078.0	1556.0	1098.0
± SD	5670.0	2313.0	1506.0	1308.0	1147.0	1758.0
median	5242.0	444.0	751.0	772.0	1371.0	328.0
range	1232 - 9252	31 - 6170	110 - 4120	12 - 3294	370 - 3110	10 - 4924
IL-5 n	5	14	12	7	9	10
mean	3881.0	3810.0	2059.0	2580.0	2070.0	1817.0
± SD	1992.0	3320.0	1963.0	2251.0	1492.0	1534.0
median	3375.0	3267.0	1632.0	1894.0	1626.0	1152.0
range	2115-7136.0	208 - 9645.0	120 - 6066.0	214 - 6241.0	280 - 5217	270 - 4539
IL-10 n	4	15	13	6	9	10
mean	1414.0	874.0	615.0	848.0	509.0	652.0
± SD	1207.0	612.0	412.0	452.0	335.0	449.0
median	1253.0	868.0	661.0	933.0	499.0	645.0
range	138 - 3012.0	74 - 2430.0	30 - 1340.0	40 - 1395.0	56 - 916.0	70 - 1705.0

Table 6.21: Value of cytokine ratios according to response categories determined from the individuals from the High Transmission area before treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IL-4:IFN-γ						
n	3	6	6	3	5	4
mean	0.87	0.55	0.27	0.55	0.55	0.70
± SD	1.07	0.56	0.14	0.31	0.59	0.71
median	0.35	0.43	0.23	0.72	0.28	0.42
range	0.10-2.1	0.07-1.63	0.10-0.50	0.10-0.74	0.10-1.55	0.20-1.75
IL-5:IFN-γ						
n	3	7	7	3	6	4
mean	3.98	0.59	1.33	1.23	1.11	2.30
± SD	4.90	0.20	0.54	0.40	0.61	1.29
median	1.43	0.56	1.19	1.00	1.02	2.50
range	0.80-9.65	0.20-0.91	0.50-1.94	0.90-1.70	0.20-2.04	0.60-3.54
IL-10:IFNγ						
n	3	7	7	3	6	4
mean	1.05	0.48	0.55	1.03	0.44	1.43
± SD	1.07	0.21	0.61	0.67	0.26	0.86
median	0.71	0.47	0.22	1.29	0.42	1.68
range	0.10-2.25	0.10-0.78	0.10-1.86	0.20-1.53	0.10-0.77	0.20-2.14

Table 6.22: Value of cytokine ratios according to response categories determined from the individuals from the High transmission area after treatment.

Antigen/ mitogen	PHA		WORM		EGG	
	low	mod/high	low	mod/high	low	mod/high
IL-4:IFN-γ						
n	2	9	6	5	4	7
mean	18.51	4.14	6.93	3.75	7.89	2.44
± SD	19.60	7.5	12.4	7.14	5.17	4.76
median	18.51	0.37	2.16	0.72	9.07	0.09
range	4.6-32.4	0.01-18.57	0.07-31.94	0.006-16.47	0.60-12.80	0.003-12.89
IL-5:IFN-γ						
n	4	14	11	7	8	10
mean	10.08	6.19	9.31	8.0	13.0	4.42
± SD	11.6	10.6	13.66	11.6	9.73	6.14
median	6.34	2.12	7.31	3.0	13.23	0.81
range	0.70-26.9	0.05-35.33	.05-47.02	0.10-31.21	0.10-23.96	0.09-17.17
IL-10:IFNγ						
n	3	15	12	6	8	10
mean	5.78	1.3	2.87	2.77	3.72	1.78
± SD	4.72	1.83	2.97	3.45	3.27	2.35
median	5.7	0.47	2.07	1.0	2.73	0.57
range	1.1-10.54	0.02-6.39	0.06-9.38	0.02-7.42	0.05-8.28	0.02-6.61

Table 6.23: Cytokine levels of all the antigen derived clones according to the antigen and subject.

Subject/antigen		IFN-γ	IL-4	IL-5	IL-4:IFN-γ	IL-5:IFN-γ
309worm	n	19	19	19	19	19
	mean	229.4	670.7	3394.0	4.3	45.9
	± SD	(373.7)	2176	5925	12.6	122.5
	median	61.0	18.0	495.6	0.4	12.2
	range	0.4 - 1404	1.7 - 9483	69 - 17920	0.03 - 55	0.7 - 527
309egg	n	14	14	14	14	14
	mean	640.6	789.4	4650.3	3.1	36.1
	± SD	897.3	1824.0	6077.8	9.4	64.5
	median	183.3	160.3	2415.0	0.4	12.5
	range	13 - 2984	0.2 - 6669	8.6 - 18180	0.004 - 36	0.05 - 244
309PPD	n	15	15	15	15	15
	mean	2098.9	608.9	3153.2	0.1	3.5
	± SD	3540.0	1998.5	5872.0	0.2	2.7
	median	286.9	24.7	447.0	0.1	3.6
	range	57 - 11600	0.5 - 7777	277 - 17630	0.001 - 0.7	0.07 - 8.4
350worm	n	68	68	49	67	48
	mean	1391.1	662.3	2590.1	2.5	11.9
	± SD	1430.7	978.0	3154.2	6.8	23.8
	median	812.3	438.5	1289.0	0.4	1.3
	range	24 - 5406	13 - 7118	8 - 12630	0.02 - 40.8	0.004 - 112.5
350egg	n	12	12	12	12	12
	mean	1137.3	1537.0	230.0	17.9	3.6
	± SD	1562.3	1336.7	158.1	45.6	11.0
	median	433.8	1387.0	190.0	1.5	0.3
	range	13 - 5244	54 - 4363	39 - 501	0.02 - 161	0.03 - 112.5
350PPD	n	9	9	9	9	9
	mean	2061.8	1673.3	184.0	1.0	0.2
	± SD	1146.6	975.9	120.9	0.7	0.2
	median	1996.0	1791.0	155.0	0.9	0.1
	range	409 - 3735	359 - 3647	6 - 455	0.2 - 2.7	0.003 - 0.6

Table 6.24: Cytokine levels of all the antigen derived clones according to the antigen and subject.

Subject/antigen		IFN-γ	IL-4	IL-5	IL-4:IFN-γ	IL-5:IFN-γ
329worm	n	43	43	39	43	39
	mean	3664.9	1193.0	2423.4	1.2	1.6
	± SD	4852.4	1829.5	3920.4	3.0	2.4
	median	2702.5	754.0	996.0	0.3	0.6
	range	98 - 30560	10 - 9434	51 - 15680	0.002 - 16.6	0.007 - 7.8
329egg	n	21	21	18	21	18
	mean	515.3	750.2	3348.3	3.5	18.1
	± SD	346.2	843.7	4566.2	6.3	40.0
	median	542.0	326.0	2108.0	0.7	4.8
	range	34 - 1017	31 - 2627	2 - 19040	0.1 - 27.1	0.003 - 173
329PPD	n	13	13	20	13	13
	mean	1917.8	1772.5	4667.9	1.2	3.0
	± SD	2190.7	2543.5	15670.0	1.3	5.7
	median	1255.0	694.0	159.0	1.0	0.4
	range	331 - 8572	30 - 9139	20 - 70440	0.02 - 4.2	0.003 - 18.5
311worm	n	16	17	22	16	16
	mean	89.7	1244.2	31970.0	8.4	1347.4
	± SD	193.4	2503.5	98820	19.2	3503.9
	median	25.7	36.4	788.9	2.2	60.4
	range	0.2 - 774	0.3 - 8561	26 - 42290	0.02 - 78	0.4 - 11850
311egg	n	36	37	41	36	36
	mean	667.9	536.4	6183.5	3.6	82.3
	± SD	1922.0	1125.0	12350.0	4.8	241.9
	median	68.9	65.0	644.8	1.2	19.7
	range	1.3 - 11130	12 - 4868.9	13.4 - 43990	0.4 - 19.3	0.2 - 1454.7
311PPD	n	8	8	8	8	8
	mean	281.1	2666.3	13010.0	13.1	60.5
	± SD	337.5	4552.3	18180.0	24.6	93.3
	median	220.0	28.0	1052.5	0.4	6.8
	range	27 - 1065	9.4 - 10490	100 - 40300	0.009 - 68	0.7 - 260

Table 6.25: Cytokine levels (mean, ± SD and median) of all the antigen specific clones according to the antigen used initially to derive them.

Subject/antigen		IFN-γ	IL-4	IL-5	IL-4:IFN-γ	IL-5:IFN-γ
309worm	n	2	2	2	2	2
	mean	721.8	4747	9140.3	2.5	8.6
	± SD	768.0	264.3	10110.0	3.7	4.3
	median	109.4	38.9	451.6	0.4	9.0
	range	40 - 1404	11.5 - 94824	360 - 17920	0.3 - 6.8	9 - 12.8
309egg	n	3	3	3	3	3
	mean	714.7	946.8	7077	0.2	3.0
	± SD	695.5	146.8	2215.7	0.3	4.1
	median	1035.2	109.8	1636.1	15.0	4327.3
	range	73 - 1527	6 - 2620.8	69 - 17960	0.004 - 37	0.05 - 244
350worm	n	18	18	18	18	18
	mean	1043.6	821.6	2450.9	1.7	5.9
	± SD	939.1	1603.5	3384.7	2.9	8.2
	median	672.5	509.0	1025.0	0.6	1.2
	range	145 - 2960	37 - 7118	351 - 12630	0.1 - 10.7	0.3 - 22
350egg	n	1	1	1	1	1
	mean	219.5	205.0	39.0	0.9	0.2
	± SD					
	median	219.5	205.0	39.0	0.9	0.2
	range					
329worm	n	11	11	11	11	11
	mean	6343.2	374.9	2257.0	0.4	1.3
	± SD	8400.0	338.5	3739.0	0.9	2.3
	median	3509.0	276.0	1163.0	0.1	0.5
	range	98 - 30560	10 - 994	218 - 13330	0.002 - 3.1	0.007 - 7.8
329egg	n	2	2	2	2	2
	mean	877.8	218.5	2729.0	0.3	2.7
	± SD	197.0	152.0	3856.6	0.2	3.8
	median	877.8	218.5	2729.0	0.3	2.7
	range	739 - 1017	111 - 326	2 - 5456	0.1 - 0.4	0.003 - 5.4
311worm	n	4	4	4	4	4
	mean	88.0	1981.0	61950.0	28.6	5142.3
	± SD	123.5	2542.5	10480.0	33.4	5975.3
	median	40.7	1178.9	13090.0	15.1	4327.3
	range	0.2 - 270.1	2.2 - 5563.9	2725 -218900	6.4 - 77.9	68.5 - 11850
311egg	n	7	7	7	7	7
	mean	170.2	1104.4	15770.0	9.3	288.8
	± SD	249.3	1255.4	16830.0	6.3	521.0
	median	62.7	809.3	6972.8	7.7	111.2
	range	10 - 707	36 - 3415	53 - 38580	3.1 - 19.3	3.2 - 1455

Table 6.26: Concentration of reagents used for the determination of specific immunoglobulin against *S.haematobium* soluble egg antigen (SEA)

Antibody	SEA concentration	Serum dilution	Conjugate dilution	Substrate incubation
Total IgA	10 ug/ml	1 : 100	1 : 2000	20 minutes
IgE	10 ug/ml	1 : 10	1 : 2000	30 minutes
IgM	10 ug/ml	1 : 200	1 : 2000	20 minutes
IgG1	10 ug/ml	1 : 10	1 : 2000	3 hours
IgG2	10 ug/ml	1 : 10	1 : 2000	3 hours
IgG3	10 ug/ml	1 : 10	1 : 2000	20 minutes
IgG4	10 ug/ml	1 : 100	1 : 2000	20 minutes

Table 6.27: Concentration of reagents used for the determination of specific immunoglobulin against *S.haematobium* whole worm antigen (WWH)

Antibody	WWH concentration	Serum dilution	Conjugate dilution	Substrate incubation
Total IgA	5 ug/ml	1 : 100	1 : 2000	20 minutes
IgE	5 ug/ml	1 : 10	1 : 2000	30 minutes
IgM	5 ug/ml	1 : 200	1 : 2000	20 minutes
IgG1	5 ug/ml	1 : 10	1 : 2000	3 hours
IgG2	5 ug/ml	1 : 10	1 : 2000	3 hours
IgG3	5 ug/ml	1 : 10	1 : 2000	20 minutes
IgG4	5 ug/ml	1 : 100	1 : 2000	20 minutes

REAGENTS PREPARATION FOR ELISAs

1. Carbonate/Bicarbonate coating buffer pH 9.6:

Solution A: (Carbonate):

K_2CO_3 3.45 g dissolve in 1 litre

or

Na_2CO_3 1.55 g dissolve in 500 ml

Solution B: (Bicarbonate):

$KHCO_3$ 2.5 g dissolve in 1 litre

or

$NaHCO_3$ 1.05 g dissolve in 500 ml

Adjust to correct pH by adding solution A to solution B

2. Phosphate Buffered Saline (PBS) x10: pH 7.4

NaCL	80 g
KH_2PO_4	2 g
$Na_2HPO_4,2H_2O$	29 g
KCL	2 g
H_20	1 LITRE

For cytokine ELISAs PBS 1x + Tween 20 (0.5 ml/l)

3. Phosphate/citrate buffer pH 5.0

Na_2HPO_4	7.19 g
Citric acid	5.19 g
H_2O	1 litre

4. Substrate stock:

Ortho-phenyl diamine (OPD)	100 mg
Methanol	10 ml

This is kept at 4°C

5. HRP substrate:

Phosphate/citrate buffer	100 ml
Stock OPD	1.0 ml
H_2O_2	10 ul

The H_2O_2 should be added last immediately before use

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