# Cellular Immuno-Epidemiology of *Schistosoma haematobium* Infection in Humans.

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*To Grandad and Grandma,*
*Mr. James Trapp and Mrs. Janet Trapp*
*who gave me an understanding of the past*
*and optimism for the future.*

# Declaration.

**This thesis is submitted in accordance with the regulations for the degree of Doctor of Philosophy in the University of Glasgow. No part of it has been previously submitted by the author for a degree at any university.**

**Data regarding diagnosis of *Schistosoma haematobium* infection reported in chapter 4 was made available as a result of a collaboration between the Universities of Glasgow and Oxford and The Blair Research Institute.**

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## Abbreviations.

| | |
|---|---|
| °C | (degrees centigrade). |
| ADCC | (antibody-dependent cell-mediated cytotoxicity). |
| CERC | (cercarial antigen). |
| CSF | (colony stimulating factor). |
| DMSO | (dimethyl sulphoxide) |
| ELISA | (enzyme linked immuno-sorbant assay). |
| FCS | (foetal calf serum) |
| g | (gram). |
| GM-CSF | (granulocyte-macrophage colony stimulating factor). |
| GST | (glutathione-S-transferase). |
| hAB | (human AB) |
| IFN | (interferon). |
| Ig | (immunoglobulin). |
| IS | (international standard). |
| IL | (interleukin). |
| iu | (international units). |
| kDa | (kilo Dalton). |
| L | (litre). |
| LPS | (Lippo-polysaccharide). |
| mAb | (monoclonal antibody). |
| µg | (micro-gram). |
| mg | (milli gram) |
| ml | (milli-litre). |
| mM | (milli molar) |
| mRNA | (messenger ribo-nucleic acid) |
| MWU | (Mann-Whitney U comparison of independent groups) |
| n | (sample size). |
| NIBSC | (National Institute for Biological Standards and Control). |
| PBMC | (peripheral blood mononuclear cells). |
| PBS | (phosphate buffered saline). |
| PHA | (phytohaemagglutinin). |

| | |
|---|---|
| PPD | (purified protein derivative). |
| RPMI | (Roswell Park Memorial Institute). |
| RT-PCR | (reverse transcriptase-polymerase chain reaction). |
| SDS/DMF | (sodium dodecyl suphate/N,N-dimethyl formamide). |
| SEA | (schistosome egg antigen). |
| sh | (*Schistosoma haematobium*) |
| sj | (*Schistosoma japonicum*) |
| sm | (*Schistosoma mansoni*) |
| spp. | (species). |
| TGF | (transforming growth factor). |
| TNF | (tumour necrosis factor). |
| u | (units). |
| WWH | (whole adult worm homogenate). |
| µl | (micro-litre). |
| µm | (micro-meter). |
| mm | (milli-meter). |
| MTT | ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)). |
| nm | (nano-meter). |
| r | (recombinant). |
| R.R. | (reference reagent). |

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## Summary.

This thesis reports two immuno-epidemiological studies of cellular immune responses to *Schistosoma haematobium* infection in humans. The first study was a cross sectional infection study. The study cohort consisted of 59 Gambians made up of two distinct age groups: children (12-16 years old) and adults (25-88 years old). The study examined three hypotheses: 1) protection against infection is associated with a Th2-type immune response, 2) Th1 and Th2 responses are dichotomous options in individuals and 3) cytokine production is affected by cross-reactive antigen.

The second, a re-infection study, was based in Zimbabwe. The study cohort consisted of 83 Zimbabwean children (6 to 15 years) recruited from two separate villages. One site had significantly lower prevalence of infection than the other, conferring an opportunity to examine the effects of transmission dynamics on the development of a protective immune response. The study addressed two major hypotheses: 1) an appropriate protective type of immune response develops faster in the high prevalence area compared to the low prevalence area and 2) individuals produce either IL-4 or IL-5 but not both.

PBMC from Gambian children produced more IFNγ on stimulation with antigen compared to PBMC from adults, whereas PBMC from Gambian adults had greater overall cellular immunological responses in response to PHA. Assuming that the adults have had past and continuing exposure to infection these observations could support the hypothesis that Th1-type responses are associated with susceptibility to infection.

To investigate the cytokine profiles that may be associated with protection or susceptibility to infection, peripheral blood mononuclear cells (PBMC) from the study cohort were isolated and cultured in the presence of schistosome egg antigen (SEA), whole adult worm homogenate (WWH) or phytohaemagglutinin (PHA). The levels of Interleukin (IL)-2, IL- 4, IL-5, IFNγ, TNFα, and GM-CSF released into the culture supernatants were measured. The statistically significant differences between the cellular responses of PBMC from infected and uninfected individuals when they were cultured with either PHA, WWH or SEA, minus the equivalent values for culture of PBMC without stimulant (net data) were as follows. Infected individuals, irrespective of age, had higher peripheral blood eosinophil counts ($p<0.003$) and produced more IFNγ on stimulation with SEA ($p<0.02$) and WWH ($p<0.02$) than uninfected individuals. PBMC from uninfected individuals produced more IL- 4 on stimulation with PHA ($p<0.001$) than infected individuals. No significant differences in levels of net IL-2, GM-CSF, IL-5, TNFα or cell proliferation were found between PBMC of infected and uninfected individuals.

Th1 and Th2 responses, as indicated by levels of IFNγ and IL-4 respectively, were not found to be dichotomous options. Although as a group, infected individuals and children produced more IFNγ and less IL-4 than uninfected individuals and adults, on a personal level high IFNγ production did not necessitate low IL-4 production.

Cross-reactive antigen was found to affect cell proliferative responses ($p<0.001$), GM-CSF, ($p<0.001$), IFNγ ($p<0.001$), IL-2 ($p<0.001$), IL-4, ($p<0.001$) and TNFα, ($p<0.001$). These results confirm that cytokine producing cells can be antigen-

specific and have the ability to recognise similar epitopes on different stages of the parasite.

If it is assumed that a degree of resistance to *S.haematobium* infection is associated with Th2-type responses, a number of results might have been expected from the re-infection study in Zimbabwe. Children from the high prevalence area might produce more Th2-type cytokines, and less Th-1 type cytokines than children from the low prevalence area. Since older children had been exposed for longer, it could be expected that they would produce more Th2-type cytokine than their younger colleagues. If resistance to infection was acquired faster in the high prevalence area, it might be expected that a difference between cytokine production might be expected in one area but not the other.

To investigate the cytokine profiles that may be associated with protection or susceptibility to infection, whole heparinised blood from the study cohort was cultured in the presence of SEA, WWH, Cercarial antigen (CERC) or phytohaemagglutinin (PHA). The levels of IL-4, IL-5, IL-10, IFNγ, TNFα, and GM-CSF released into the culture supernatants were measured.

On stimulation with PHA, children from the low prevalence area produced more IL-4, ($p<0.001$) IFNγ, ($p<0.0005$) and GM-CSF, ($p<0.0005$) than children from the high prevalence area. On stimulation with SEA, children from the high prevalence area produced more GM-CSF, ($p<0.05$) and IL-10, ($p<0.05$) than children from the low prevalence area. More children who were infected before treatment produced IL-5, ($p<0.05$) on stimulation with PHA than children who were uninfected. On stimulation

with SEA, production of IL-4, ($p<0.05$) was associated with lack on infection before treatment whereas production of IFN$\gamma$, ($p<0.05$) on stimulation with WWH was associated with protection from re-infection.

Area was the dominant factor in determining cytokine profile. I would speculate that the cytokine production was of a Th0 type in the low prevalence area, compared to the high prevalence area, whereas in the high prevalence area, a pattern that could be described as either macrophage or Th2 type predominated.

Analysis of IL-4 and IL-5 production in the Zimbabwean study gave some support to the hypothesis that there is a subdivision of the Th2 subset in humans. When blood is incubated in the presence of antigen or mitogen, for 24 or 48 hours, the majority of people will produce either IL-4 or IL-5 but not both.

# Chapter 1.

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# General Introduction.

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**1.1 Biology of Schistosomes.**

*Schistosoma spp.*, are digenean trematodes of the order *Strigeata*. Several species of the family Schistosomatidae can infect humans, but only six have been found to be capable of maturation and oviposition (Mahmoud, 1992). Of these species, *Schistosoma haematobium*, *S.mansoni* and *S.japonicum* are considered to be of international public health significance. *S.mattheei*, *S.intercalatum* and *S.mekongi* also infect humans and are of public health significance in more restricted foci (Schmidt *et al.*, 1989).

*S.haematobium*, *S.japonicum* and *S.mansoni* have distinct life cycles and cause different symptoms, mainly because of the different preferred sites of development in the mammalian host of each type of adult worm. Adult *S.haematobium* can be found primarily in the veins of the urinary bladder plexus, whereas *S.mansoni* adults live principally in the portal veins draining the large intestine and *S.japonicum* inhabit the veins of the small intestine. The female worm lies within the gynaecophoric canal of the male worm (Schmidt *et al.*, 1989).

*S.haematobium* lay their eggs in the small venules surrounding the bladder, whereas *S.mansoni*, and *S.japonicum* lay their eggs in the venules surrounding the intestine. In order to continue the life cycle, the eggs must be excreted in either urine or faeces. Some eggs successfully pass through intervening tissue from venule to the lumen of the bladder or intestine, but many remain lodged in gut or bladder mucosae, or are swept away in the bloodstream and become lodged elsewhere in the body. The eggs induce a granulomatous response; comprised of an aggregate of inflammatory immune cells, primarily macrophages, eosinophils

and lymphocytes. Eggs which become trapped in the tissue die and the granuloma is replaced by scar tissue (Mahmoud, 1992). In cases of chronic infection, the gut or bladder wall can become thickened by scar tissue with large numbers of eggs lodged in the tissues (Mahmoud, 1992).

On excretion into water, the eggs hatch and the larval miracidia which are released, infect an appropriate species of snail; *Bulinus sp.* are intermediate hosts for *S.haematobium.* After a number of larval stages, the cercariae emerge from the snail. Humans become infected when they contact water-containing cercariae. The cercariae penetrate the skin shedding their tails in the process. Within 24 hours the schistosomula, as they are now called, are moved by the peripheral circulation to the heart. Larval worms migrate through the pulmonary capillaries to the left side of the heart and on to the liver sinusoids, where they undergo a three week period of development before migrating to the definitive site. A schematic representation of the life cycle of *S.haematobium*, *S.mansoni* and *S.japonicum* is in figure 1.1.

In recent times, schistosomiasis has become an increasingly important problem because of the proliferation of hydrological development schemes that have provided additional environments for the snail intermediate hosts. Mortality due to schistosomiasis is difficult to quantify because death certificates rarely identify schistosomiasis as the underlying cause of death. However, Koroltchouk and colleagues (1987) estimated mortality at 1 in 1000 infected adults. Morbidity caused by chronic schistosomiasis constitutes a major public health problem in affected areas. *S.haematobium* infection can lead to inflammation of the bladder, haematuria and pain on urination. In some cases this can lead to more severe

pathology in the urinary tract and bladder, including fibrosis and calcification resulting in functional abnormalities. In the most serious cases, renal failure may also result. *S.haematobium* infection has also been linked to bladder cancer; it has been estimated that primary prevention of urinary schistosomiasis could reduce the global rate of carcinoma of the bladder by 5000-10,000 cases per year (Koreoltchouk *et al.*, 1987).

A number of methods have been used to control schistosomiasis, including chemotherapy, sanitation, health education and vector elimination. Effective drug treatments are available: praziquantel and oxamniquine reduce intensity of infection and allow improvement in clinical status of patients with severe schistosomiasis (Cline *et al.*, 1982; Mott, 1982; Sleigh *et al.*, 1986). Population based chemotherapy has been the most successful method of control over large areas (Cline *et al.*, 1982; Mott, 1982; Sleigh *et al.*, 1986) but re-infection occurs quickly and regular treatment programs are expensive, difficult to sustain, and in danger of producing drug-resistant strains of schistosome. For these reasons; drug treatment has been considered unsuitable for long-term control (Cline *et al.*, 1982; Butterworth *et al.*, 1987; Liese, 1987; United Nations Development Programme/World Bank/World Health Organistaion Special Programme for Research and Training in Tropical Diseases, 1990).

**1.2 Epidemiology of schistosomiasis.**

The distribution of schistosomiasis differs from species to species and is linked to the distribution of suitable intermediate hosts (Schmidt *et al.*, 1989; Mahmoud, 1992). *S.japonicum* is found predominantly in Japan and China, whereas

*S.haematobium* and *S.mansoni* are prevalent in Africa and in parts of the Middle East. *S.mansoni*, but not *S.haematobium*, can be found in parts of Central and South America (Schmidt *et al.*, 1989; Mahmoud, 1992).

Humans resident in an endemic area are often exposed early in life. Prevalence and intensity of infection, as measured by urinary or stool egg counts, increase slowly, normally peaking in the 10-20 age group. During adult life, prevalence of infection drops and there is a large decrease in intensity of infection (Bradley *et al.*, 1973; Mahmoud, 1992). Worm burden within a community exhibits a negative binomial distribution pattern - most infected individuals harbour low intensity infection, but a few people are heavily infected - a feature common to many helminth infections (Mahmoud, 1992).

The differences among individuals and age-related differences in the prevalence and intensity of infection undoubtedly arise from several sources. Amongst these sources are genetic susceptibility, exposure to infected water and immunity to infection. Each of these is discussed below.

Host genetic factors probably influence an individual's capacity to control worm burden. Family studies in Brazil have indicated that intensity of infection with *S.mansoni* is associated with a single gene locus (Abel *et al.*, 1991). The same research group subsequently mapped this locus: 'SM1' (Marquet *et al.*, 1996), which their analysis indicated could account for 66% of the residual variation in infection intensity after controlling for water contact, age and sex. This observation may explain why some individuals appear to be predisposed to high

intensities of infection, but it does not explain why prevalence of infection in a community is lower in adults than in children in endemic areas.

The genetic region that contains SM1, contains several loci (CSF1R, D55393 and 5q31-q33) that encode for components of the immune system and could thus be candidates for determining resistance or susceptibility to infection (Marquet *et al.*, 1996). CSF1R mediates colony stimulating factor 1 (CSF1) which modulates cell survival, proliferation and differentiation, and has a primary role in regulating mononuclear phagocyte production. D55393 denotes a region that includes genes coding for GM-CSF, IL-3, IL-4, IL-5, immune regulatory factor 1 and IL-13. 5q31-q33 is linked with a locus regulating IgE levels and a locus that controls bronchial hyper-responsiveness in asthma. The results of Marquet *et al.* (1996) were generated assuming that genetic predisposition to resistance/ susceptibility to infection with *S.mansoni* was linked to a single gene. Marquet and colleagues (1996) pointed out that the actual genetic picture could be more complicated implying that a 'resistant' or 'susceptible' phenotype may involve all or any of the previously listed factors, and may also include factors not encoded by SM1. It is reassuring that some of the factors possibly encoded by SM1 coincide with factors implicated by other areas of research, discussed in section 1.3. However, SM1 will be of only limited utility in identifying which phenotype might be associated with resistance or susceptibility to schistosomiasis, until the physical region of the genome to which it is associated is more tightly defined. Even so, genetic factors can only be useful in identifying factors that affect predisposition of an individual to infection, they cannot explain the drop in prevalence of infection that occurs around puberty in populations.

The relationship between age and intensity of infection could be explained by a slow attrition of adult worms from earlier infections in older people, at the same time as a gradually acquired resistance to subsequent infection (Clarke, 1966). Alternatively, a slow reduction in exposure to infection with age at the same time as the gradual death of an existing adult worm population could result in the same pattern (Butterworth *et al.*, 1992).

Whilst changing levels of exposure is certainly a factor in determining levels of infection (Wilkins *et al.*, 1987), Butterworth and colleagues (1992) reviewed evidence from several studies and concluded that exposure alone does not explain the drop in intensity of infection that occurs with age. The key evidence supporting this conclusion is that, following treatment, re-infection rates are strongly dependent on age. For example, in a study of *S.haematobium* infection in The Gambia (Wilkins *et al.*, 1987; Hagan *et al.*, 1991a; Hagan, 1992a) adult women were as intensely exposed as children who became heavily infected after treatment, despite having significantly lower re-infection intensities of infection. Further evidence resulted from a study of an *S.mansoni* endemic area in Kenya, where the level of re-infection was highest in children aged between 8 and 12 years whereas, greatest exposure to infected water did not occur until the ages of 16 to 24 years. Even when water contact rates were taken into account, they did not explain the large differences in infection intensities between the different age groups (Butterworth *et al.*, 1988b).

Evidence from re-infection studies therefore indicates that exposure to infection alone cannot account for the relationship between age and infection. This suggests

that other factors, perhaps including an increase in the effectiveness of the immune response may be responsible for the observed reduction in prevalence and intensity of infection with age.

An immunological response could result in the death of larvae or adult worms. It might also affect the measurement of worm burden by egg counts in urine or faeces by altering female worm fecundity or the number of eggs that successfully traverse the tissue between the venule and bladder or intestine, dependent on species. The extent and type of immune response could depend on a number of factors. These include: current infection (Smithers *et al.*, 1965; Smithers *et al.*, 1967), genetic predisposition to infection (discussed above), age dependent physiological features and immunity acquired as a result of experience of infection.

There are some data to support the hypothesis that immunity might reduce worm fecundity. *S.bovis* and *S.haematobium* adult worm pairs in immuno-competent mice produced significantly less eggs than the same number of pairs in T-cell depleted mice (Agnew *et al.*, 1992). This anti-fecundity immunity could be transferred to a different animal by transfer of serum. This phenomena was not observed in similar experiments using *S.mansoni* (Agnew *et al.*, 1992; Wynn *et al.*, 1996). Adult *S.japonicum* worm pairs produced less eggs per day in mice immunised with recombinant 26kDa glutathione-S-transferase (GST), a vaccine candidate (Liu *et al.*, 1995a). Similar observations have been reported when pigs were vaccinated with either purified recombinant *S.japonicum* 26 glutathione-S-transferase (rSj26GST) or rSj26GST from *S.japonicum* using alum as an adjuvant (Liu *et al.*, 1995b). Agnew and colleagues (1996) considered the relationships

between both *S.mansoni* and *S.haematobium* parasite density in humans and levels of circulating parasite antigen, egg excretion and host age. They found that whilst *S.mansoni* egg output remained stable irrespective of host age or infection intensity, *S.haematobium* egg production was reduced with host age compared to infection intensity as measured by circulating antigen (Agnew *et al.*, 1996). Since most pathology is egg induced (Mahmoud, 1992;) a reduction in fecundity could be a valid objective of a future vaccine since it could significantly reduce disease.

These interesting results notwithstanding, most attention in immunity to schistosomes has focused on killing of larvae and adult worms. The classic dogma within this field is that of concomitant immunity, an idea first introduced by Smithers and Terry (1967). The essential evidence for this theory was that when adult schistosomes were implanted into Rhesus monkeys with no history of schistosome infection, the hosts had the same capacity to kill schistosomula as those in a control group that had become immune through repeated exposure to infection. Concomitant immunity, as originally proposed, has not been universally accepted (as reviewed (Hagan, 1992a)), because not all of the data are in accordance with it. For example, observations of young children (under 10 years old), showed that despite a net increase in worm burden, some adult worms still died (Wilkins *et al.*, 1978). Wilkins and colleagues (1984) suggested therefore that worm burdens in humans are in a dynamic state and that 'concomitant immunity' represents a balance between gain and loss of worms, mediated by the immune response. They showed that when they halted transmission by killing the snail intermediate host with molluscicides, intensity of infection in the study population dropped, implying that adult worms were gradually dying and that the

acquisition of new infections was necessary to maintain a stable adult worm population (Wilkins *et al.*, 1984). Concomitant immunity could not, therefore, be completely effective in killing new larval invasions.

Further evidence from re-infection studies indicates that adults from an endemic area did not lose their partial immunity to schistosome infection after they have lost their adult worm populations as a result of treatment (Hagan *et al.*, 1991). This result suggests that concomitant immunity could be at most a minor factor in a protective immune response in adults although it does not rule out concomitant immunity entirely. Hagan and colleagues (1993) proposed that TNFα could facilitate concomitant immunity. TNFα is produced by macrophages, which can be found in granulomas surrounding schistosome eggs. There is some evidence to suggest that it can enhance adult worm fecundity, whilst having direct toxic effects on schistosomula (Hagan *et al.*, 1993b).

It is possible that, instead of removing the source of immunity, treatment with praziquantel could result in an enhanced immune response. The death of adult worms could result in the exposure to the immune system of large amounts of adult antigen, which could effectively be considered equivalent to vaccination. Thus the treatment intervention not only removes the worm burden but could also be immunising individuals to different degrees, dependent on the level of the worm burden before treatment. Acquired immunity after treatment, could therefore be affected by history of exposure, number of treatments and by worm burden at the time of treatment.

Hope for a future vaccine is based on the assumption that immunity can be synthetically induced at an early age by vaccination. If resistance is intrinsically age rather than exposure related, the development of a vaccine might face unforeseen difficulties. Kloetzel and Da Silva (1967) provided evidence that egg count patterns were attributable to duration of infection rather than age. They studied a group of men in Brazil, who had recently moved into an area endemic for *S.mansoni*. Although the data lacked any statistical analysis, they did show clearly that egg counts declined after twenty years of exposure. Gryseels (1994a), however, had a number of criticisms of this work. The conclusions relied on fourteen immigrants, who had moved into the area more than twenty years previously and had a higher average age than more recent immigrants. Gryseels (1994a) also pointed out that past and current exposure patterns and socio-economic status were not documented and variation in either could have confounded interpretation of the data.

More recent studies to investigate the separate effects of age and history of exposure/ immunity on worm burdens in Burundi, (Gryseels *et al.*, 1987; Gryseels *et al.*, 1988; Gryseels *et al.*, 1989; Gryseels, 1991a; Gryseels *et al.*, 1991b) and in Senegal (Talla *et al.*, 1990b; Gryseels *et al.*, 1994b) have considered new foci of infection. In Senegal, (Talla *et al.*, 1990b; Gryseels *et al.*, 1994b) prevalence of infection was 100% in every age group over five years of age. However, intensity of infection, as measured by both egg counts and circulating antigen followed a typical age-related pattern, with peak intensities in the 10-15 year old age group suggesting that age was dominant over history of exposure in determining the shape of the age-intensity of infection curve. Supporting evidence came from

epidemiological studies in Burundi (Gryseels *et al.*, 1987; Gryseels *et al.*, 1988). In these studies, both prevalence and infection levels rose to a peak in older children and then declined in adults. In subsequent re-infection studies in Burundi (Gryseels *et al.*, 1989; Gryseels, 1991a; Gryseels *et al.*, 1991b), children were observed to return to pre-treatment levels of intensity of infection one to two years after treatment. In contrast, adults had very low re-infection intensities despite continued exposure.

Woolhouse (1995) commented that immunity in the new foci in Senegal studied by Gryseels and colleagues (Talla *et al.*, 1990b; Gryseels *et al.*, 1994b) may have developed at an accelerated speed because of high rates of transmission. The immune responses observed, might not therefore reflect those of a naive population. Gryseels (1995) replied that this hypothesis was not consistent with the observed convex age-egg count patterns in Senegal. If exposure to high levels of antigen hastened development of immunity, children would be the first to develop a protective immunity because children generally have the highest levels of water contact and would be exposed to the most antigen (Gryseels, 1995), suggesting that immunity to infection may be strongly age- rather than experience of infection- dependent. Water contact observations for the new foci in Senegal, however, are yet to be published.

Mathematical models have also been used in an attempt to examine the various factors that might contribute to the age-intensity of infection curve. If acquired immunity is a significant factor in determining the distribution of worm burden in a community, it could be predicted that immunity would develop faster in areas of

intense transmission (Fisher, 1934; Clarke, 1966). This idea has been explored using an age-structured immigration-death model (Woolhouse *et al.*, 1991). This model considered changes in the mean intensity of infection with age, as a function of rate of infection and parasite mortality. It predicted changes in the shape of the age-intensity of infection curve: an increase in the maximum intensity of infection and a decrease in the age of peak intensity of infection in areas of intense transmission, compared to areas of less intense transmission. This latter characteristic has been called 'peak shift' (Woolhouse, 1994) and has been observed in a comparative analysis of seventeen data sets of prevalence of *S.haematobium* infection in Zimbabwean school children, suggesting, in contrast to the results from new foci of infection, that acquired immunity to infection as a result of history of exposure is a significant factor in limiting schistosome infections in endemic areas.

There is therefore evidence for both immunity due to age (Gryseels *et al.*, 1987; Gryseels *et al.*, 1988; Gryseels *et al.*, 1989; Talla *et al.*, 1990b; Gryseels, 1991a; Gryseels *et al.*, 1991b; Gryseels *et al.*, 1994b) and due to history of exposure (Woolhouse, 1994). I would like to speculate that both factors may act concurrently in the development of resistance but that the evidence from the new foci of schistosomiasis (Gryseels *et al.*, 1987; Gryseels *et al.*, 1988; Gryseels *et al.*, 1989; Talla *et al.*, 1990b; Gryseels, 1991a; Gryseels *et al.*, 1991b; Gryseels *et al.*, 1994b) would suggest that age is the dominant factor.

**1.3 Immune responses to *Schistosome* infection.**

Evidence from epidemiological studies would suggest that at least a partial immunity to schistosomiasis is attained by adults in endemic areas. The relative proportions of immunity attributable to age-dependent physiological changes and to history of exposure to infection are difficult to quantify. However, it is reasonable to assume that some type of immunity accounts for the observed reduction in prevalence and intensity of infection amongst adults in endemic areas.

It was the generally held view until relatively recently, that schistosomes were most vulnerable to an effective attack from the immune response during the three hour transformation (McLaren, 1980) from cercariae to schistosomula (Smithers *et al.*, 1976; Butterworth *et al.*, 1992), because many of the defence mechanisms employed by the adult worm were not active in newly invading larvae. The surface membrane of the adult is composed of two closely opposed bilayers (McLaren *et al.*, 1977) which can be repaired and replaced with the rest of the outer tegument (Perez *et al.*, 1973; Kusel *et al.*, 1975). In contrast, schistosomula immediately after invasion of a host, have one lipid bilayer (McLaren *et al.*, 1977; McLaren, 1980; McLaren *et al.*, 1982). Adult worms can acquire a coating of host molecules (Smithers *et al.*, 1969; Clegg *et al.*, 1971; Goldring *et al.*, 1976) and their own surface antigens may resemble those of the host (Damian, 1967) whereas early schistosomula have yet to acquire this protective cloak (McLaren, 1980). Despite the adult's apparently superior protection, and the persistent survival of the adult worm in experimental hosts that are immune from infection with larvae (Smithers *et al.*, 1967) it has become increasingly clear from *in vitro* (McLaren, 1980; McLaren, 1989; Wilson *et al.*, 1989; Agnew *et al.*, 1993) and *in*

*vivo* (Agnew *et al.*, 1992; Wynn *et al.*, 1996) studies that all stages of parasite can be subject to immune attack. Immune responses to antigens derived from a number of life cycle stages have therefore been considered in immuno-epidemiological studies.

The types of immune response that protect adults/experienced individuals against schistosomiasis have been investigated using infection and re-infection studies which relate human immune responses to schistosome antigen. This approach has a number of advantages over the use of animal models. The human situation is difficult to reproduce in an animal. Although *S.mansoni* and *S.haematobium* can infect laboratory animals, they are parasites of humans, so the host-parasite combination in animals is artificial. In addition, inbred laboratory animals, although convenient, may not adequately reflect the immune response in a human population with a large genetic polymorphism (Demeure *et al.*, 1993). Mechanisms of protection also differ in quality and quantity in different experimental models (Butterworth *et al.*, 1988b; Demeure *et al.*, 1993) making it difficult to ascertain which, if any, of the mechanisms reported from animal studies might be applicable to humans (Butterworth *et al.*, 1988b).

However, many types of investigation that cannot be carried out in humans are possible using animals. For example, it is possible to investigate the immune responses of laboratory animals using cells removed from the definitive site: the granuloma, or an appropriate draining lymph node; animals can be experimentally infected in controlled conditions without concurrent infections; and pathology can be directly observed. Quantifying intensity of infection, by directly counting

numbers of adult worms, not possible in a human, can be carried out using an animal. The use of genetically altered animals, such as 'knock-out mice' could also be extremely useful in an investigation of the main factors contributing to resistance or susceptibility to schistosomiasis. Mechanisms shown to work in animals, can be tested subsequently using human cells and serum, to ascertain if the response found in animals adequately reflects that of humans.

Human studies also have disadvantages. Infection or re-infection studies demonstrate potential immune effector mechanisms using *in vitro* techniques and then correlate results with observed immunity to infection or re-infection. This methodology could potentially result in significant but non-causative associations. Butterworth and colleagues (1988b) suggest that this possibility could be avoided by first establishing a meaningful pattern of correlations and using them to devise new assays that are predictive of immunity to schistosomiasis. Although an awareness of the limitations of correlative results is useful, it would seem there would also be a danger of designing a study for which the results have already been ascertained, and by circular logic ascribing a positive result as evidence that the original correlation was causative and not just associative. Despite these limitations it is encouraging that a number of immuno-epidemiological studies have arrived at similar conclusions, despite difference with the details of the experimental design, and studying quite genetically different populations, from The Gambia (Hagan *et al.*, 1991), Kenya (Dunne *et al.*, 1992), and Brazil (Demeure *et al.*, 1993).

**1.3.1 Humoral Immunity.**

Accumulated evidence suggests that resistance to schistosomiasis is associated with IgE. Low levels of IgE that recognised either *S.haematobium* adult worm antigen or *S.haematobium* egg antigen in The Gambia was predictive of re-infection one year after treatment (Hagan *et al.*, 1991). A positive correlation was also reported between resistance and anti-larval IgE (Rihet *et al.*, 1991; Demeure *et al.*, 1993) and between specific anti-adult *S.mansoni* IgE and resistance in Kenya (Dunne *et al.*, 1992). In the Kenyan study, other IgE responses were not correlated with resistance. For example, anti-*S.mansoni* egg antigen IgE antibodies were present at high levels in young children, who were susceptible to infection (Dunne *et al.*, 1992). The presence in western blots of IgE antibodies that recognise Sm22 (a 22kDa molecule and *S.mansoni* vaccine candidate), correlated with resistance to re-infection (Dunne *et al.*, 1992). IgE that recognised both the native molecule and recombinant peptide correlated with resistance to re-infection (Butterworth, 1994).

IgE levels can explain variation in levels of resistance that remain after age has been taken into account using either multiple linear or logistic regression analysis (Hagan *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993). This result would suggest that these studies are observing a component of resistance to schistosomiasis that is experience rather than age dependent.

IgE could mediate the immunity to schistosomiasis via antibody-dependent cell-mediated cytotoxicity (ADCC) (Butterworth, 1994; Capron *et al.*, 1994). Hagan and colleagues (1991) suggested that cross-linking of IgE bound to the surface of

mast cells resulting in the release of chemotactic agents such as leukotriene-$B_4$, platelet-activating factor and cytokines would allow a rapid amplification of a cellular effector response. Support for this hypothesis comes from the demonstration that inflammatory cells with IgE receptors such as eosinophils, macrophages and platelets can kill larval schistosomes *in vitro* (Capron *et al.*, 1989).

Butterworth and colleagues (1992) speculated that IgA rather than IgE might mediate the marked immunity seen in adults because anti-parasite IgA has been observed to increase more during adult life, from 25 years onwards (Butterworth, 1994), rather than during late childhood as observed in the case of IgE responses (Butterworth *et al.*, 1992). A protective role for IgA is supported by studies of immune responses to the vaccine candidate, P28 glutathione-S-transferase. Serum IgA levels that recognised P28 were higher in serum from children considered to be resistant to *S.mansoni* than in serum from susceptible children (Auriault *et al.*, 1990; Grzych *et al.*, 1993).

Resistance to re-infection with *S.mansoni* has been reported to be associated with IgG reactivity to the 37 kDa antigen, schistosome glyceraldehyde-3P-dehydrogenase, which is present in both larva and adult worms (Dessein *et al.*, 1988; Goudot-Crouzel *et al.*, 1989). IgG1 and IgG3 purified from serum from children have demonstrated the ability to mediate eosinophil killing of schistosomula *in vitro* (Khalife *et al.*, 1986), but in a re-infection study in The Gambia, the ability of IgG from children's sera to recognise *S.haematobium*

antigens appeared to be unrelated to resistance or susceptibility to infection (Hagan *et al.*, 1987).

Antibodies specific to schistosome antigens have been found in the blood of young children (Butterworth *et al.*, 1992), so the susceptibility of young children to schistosomiasis cannot be due to a low overall immune response, such as might be expected of a totally naive population. Instead, a number of studies have observed correlations of IgG4 (Auriault *et al.*, 1990; Hagan *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993), IgG2 (Butterworth *et al.*, 1988a; Demeure *et al.*, 1993) and IgM (King *et al.*, 1989a; Butterworth *et al.*, 1992) with susceptibility to infection or re-infection. These observations have lead to the hypothesis that some antibody isotypes share antigenic specificity and compete for epitopes with effector antibodies such as IgE and IgA without sharing the ability to mediate anti-parasite effector mechanisms (Butterworth *et al.*, 1992).

Butterworth (1994) speculated that the balance between 'blocking' isotypes and effector antibodies might be more important in the development and achievement of immunity to schistosomiasis than the absolute presence or absence of particular antibody isotypes. Blocking antibody activity had previously been demonstrated in the rat model of schistosomiasis. Grzych and colleagues (1984) observed *in vitro* and *in vivo* that rat IgG2c monoclonal antibodies could block the effect of an IgG2a monoclonal antibody, with specificity for the same antigen.

Production of non-specific IgE as well as parasite-specific IgE may be up-regulated as a result of infection (Hagi *et al.*, 1990a; Hagan, 1993a). Some

authors have suggested that non-specific IgE could favour parasite survival by competing with specific IgE for Fc-receptors on effector cells such as mast cells, macrophages, eosinophils (Capron *et al.*, 1984; Pritchard, 1993a), platelets (Joseph *et al.*, 1983) and phagocytes (Joseph *et al.*, 1978). However, in an area endemic for *S.haematobium* in Somalia, total IgE levels were high in all age groups but patterns of specific IgE were consistent with acquisition of resistance to infection (Hagi *et al.*, 1990), suggesting that the effects of the specific antibody are dominant over any blocking by non-specific antibody.

Anti-parasite IgM antibodies have been observed to be high in young children from endemic areas and decline with age (Butterworth *et al.*, 1992), and resistance to infection has been associated with IgM that recognises SmW68, a 68Kd antigen derived from *S.mansoni* (King *et al.*, 1989a). IgM purified from children's serum could block eosinophil-dependent killing of schistosomula, mediated by IgG antibodies from the same sera (Khalife *et al.*, 1986).

Specific IgG4 has been correlated with a high intensity of infection before treatment (Dunne *et al.*, 1988; Dunne *et al.*, 1992) or with re-infection after treatment (Hagan *et al.*, 1991; Demeure *et al.*, 1993). From a study in an area endemic for *S.mansoni* in Kenya, Auriault and colleagues (1990) reported that IgG4 specific to the vaccine candidate, recombinant glutathione-S-transferase (P28) was higher in susceptible than in resistant groups. In this study, (Auriault *et al.*, 1990) a susceptible individual was defined as having a high intensity of infection and low level of exposure, and a resistant individual had a low intensity of infection and a high level of exposure. A similar pattern was also observed for

IgG4 that recognised two out of three synthetic peptides derived from the primary sequence of P28 (amino acids 115-131 and 140-153).

Hagan and colleagues (1991) suggested that the slow development of immunity to *S.haematobium* could be due to a slow maturation of the protective specific IgE response and the early production of anti-adult worm and anti-egg IgG4. Similar mechanisms have also been suggested in a number of other studies in which IgG4 could be interpreted as blocking the activity of both anti-larval and anti-adult *S.mansoni* (Rihet *et al.*, 1991; Dessein *et al.*, 1992; Dunne *et al.*, 1992; Demeure *et al.*, 1993). Evidence for competition between IgG4 and IgE has been reported in studies of allergic patients (Stanworth *et al.*, 1973; Aalberse *et al.*, 1983; Hussain *et al.*, 1992; Lambin *et al.*, 1993) and there is some evidence that specific IgG4 is competing for the same epitopes as specific IgE that recognise either *S.mansoni* schistosomula or adult worm antigens (Rihet *et al.*, 1992).

Rihet and colleagues (1992) measured anti-parasite IgE in both unfractionated serum and in a sample in which the IgE had been separated from other isotypes by immunopurification. They reported that specific IgG4 and IgE from immunopurified sera were significant explanatory variables for levels of specific IgE measured by ELISA in unfractionated sera. IgG4 correlated negatively and immunopurified IgE correlated positively with IgE in unfractionated sera. The authors suggested that the specificity spectra of IgE and IgG4 overlap, and further speculated on this basis that IgE and IgG4 could be produced by progeny of the same B-cell clones, possibly during clonal expansion by switching from IgM to either IgE or IgG4 as a result of IL-4 and helper T-cell control (Devey *et al.*,

1976; Ortolani *et al.*, 1984). Since IgE would presumably interfere with the IgG4 assay in this experiment, it would have been interesting to see whether using immunopurified IgG4 as well as immunopurified IgE would have increased their joint ability to predict unpurified IgE levels.

The same group subsequently observed that the opposing effects of IgE and IgG4 as predictive factors of re-infection were undissociable in the analyses (Demeure *et al.*, 1993). The strong correlation between IgE and IgG4 meant that the variation in infection re-intensities could not be explained by one or other isotype alone, but could when both were taken into account. A resistant person therefore had a high IgE titre, and a low IgG4 titre.

Three mechanisms for IgG4 blocking of IgE mediated immunity have been suggested (Demeure *et al.*, 1993). First, IgG4 could block the development either of hypersensitivity reactions, locally in the skin in the case of an anti-schistosomula response, or in the blood. Second, IgG4 could inhibit mast cell or basophil activation, or inhibit IgE-dependent cell cytotoxicity mediated by monocytes, eosinophils or platelets. There is some evidence for IgG4 interference with IgE-mediated mast cell degranulation (Hagan *et al.*, 1992b), possibly as a result of competition with specific IgE (Stanworth *et al.*, 1973; Iskander *et al.*, 1981; Hussain *et al.*, 1986) and IgG4 blocking of basophil activation in studies of schistosomiasis (Hofstetter *et al.*, 1982) and filariasis (Ottesen *et al.*, 1981). Third, IgG4 could block IgG1- and IgG3- mediated eosinophil killing of schistosomula as demonstrated by Khalife and colleagues (1986), although, since

there is very little evidence to support a role for IgG1 or IgG3 *in vivo*, this mechanism could well be an *in vitro* phenomenon only.

**1.3.2 Mechanisms of immune killing.**

A range of human immune responses have been found capable of killing schistosomula *in vitro* (Butterworth *et al.*, 1992). Antibodies and complement were found to effect some damage on schistosomula (Clegg *et al.*, 1972). Cytotoxic lymphocytes were generally considered to be inactive (Butterworth *et al.*, 1979) although there were some reports of lymphocyte-mediated killing (Ellner *et al.*, 1982). Effective killing of schistosomula was mediated by ADCC reactions by macrophages and platelets in the presence of IgE (Capron *et al.*, 1975), and neutrophils (McKean *et al.*, 1981). The most important effector cells, however, were considered to be eosinophils: highly active in killing schistosomula in the presence of antisera (Butterworth *et al.*, 1975; Butterworth *et al.*, 1977; Vadas *et al.*, 1979) and IgE (Capron *et al.*, 1984). An ability to kill schistosomula *in vitro* does not necessarily signify an active role *in vivo* but, there is considerable correlative evidence from immuno-epidemiological studies that eosinophils do have an *in vivo* role.

Numbers of circulating eosinophils in blood measured before treatment from people resident in areas endemic for *S.mansoni* (Sturrock *et al.*, 1983) and *S.haematobium* (Hagan *et al.*, 1985a; Hagan *et al.*, 1987a; Hagan, 1992a) correlated with expressions of immunity in re-infection studies. There is also evidence that antibody-dependent killing of schistosomula by human eosinophils was more effective when the eosinophils were recovered from people with

eosinophilia induced by a helminth infection, or other condition. (David *et al.*, 1980). These cells were considered to be in an activated state (Butterworth *et al.*, 1988b). Despite the association between pre-treatment eosinophilia and lack of re-infection (Sturrock *et al.*, 1983a; Hagan *et al.*, 1985; Hagan *et al.*, 1987; Hagan, 1992a), there is less evidence to support an ADCC mechanism of eosinophil action *in vivo*. Hagan and colleagues (1985) attempted to correlate ADCC killing of *S.haematobium* schistosomula *in vitro* with immunity to re-infection. They reported that when using eosinophils from children, levels of killing were 'barely detectable' and did not differ between re-infected and non-re-infected groups.

### 1.3.3 Cytokine responses to schistosome infection.

The work reported in this thesis is primarily concerned with the role of cytokines in immune responses to human schistosomiasis. Cytokines, as defined by Vilcek and colleagues (1994) are,

> "regulatory proteins that are secreted by white blood cells and a variety of other cells in the body, the pleiotropic actions of cytokines include numerous effects on the cells of the immune system and modulation of inflammatory responses."

Cytokine production may be transient and often, as implied in their role in inflammation, within a short action radius. The actions of cytokines can usually be attributed to an alteration of the pattern of gene expression in target cells. These alterations could result in a change in the rate of cell proliferation, alteration in cell differentiation state, or in a change in their function (Vilcek *et al.*, 1994). For example, a B-cell may be induced to change the antibody isotype it expresses.

The nature of the immune response to an antigen is therefore dependent to a large extent on the modulating influence of cytokines (Del Prete *et al.*, 1994). It is the opinion of these authors, that it is largely the preferential activation of one sub-population of CD4+ helper T-cells over another that determines the profile of cytokines released on stimulation with antigen and therefore the nature of the ensuing immune response. Two subpopulations of helper T-cells, type 1 (Th1) and type 2 (Th2) were identified from studies of murine CD4+ T cell clones (Mosmann *et al.*, 1986; Mosmann *et al.*, 1989b). The two subpopulations produced a different range of cytokines (Mosmann *et al.*, 1986; Mosmann, 1987). Cytokines produced by Th1-type clones include IL-2, IL-12, TNFβ and IFNγ and those produced by Th2 clones include IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Both Th1 and Th2 cells produce IL-3, TNFα and GM-CSF (Vilcek *et al.*, 1994; Locksley, 1997). A further T cell type Th0, considered to be a precursor of Th1 and Th2 types is capable of producing both Th1 and Th2-type cytokines (Del Prete *et al.*, 1994).

There is some evidence that T-helper cells occur as two subpopulations in humans as well as in mice. Cytokine profiles of a series of antigen- specific T-cell clones taken from the peripheral blood of healthy people showed that stimulation of blood with different antigens resulted in T-cell clones of either one type or another (Del Prete *et al.*, 1994). For example, T-cell clones specific for the purified protein derivative (PPD) of *Mycobacterium tuberculosis* secreted IL-2 and IFNγ but not IL-4 or IL-5 and were therefore considered to be equivalent to murine Th1 cells. Conversely, *Toxocara canis*-specific clones secreted IL-4 and IL-5, but

not IL-2 and IFNγ and therefore resembled murine Th2 cells. (Del Prete *et al.*, 1991).

It is likely, however, that the Th1/Th2 model of cytokine production is an over-simplification of the *in vivo* situation since it is now clear that many cells other than T-helper cells produce a wide spectrum of cytokines in abundant quantity (Gordon *et al.*, 1990). For example the cell types capable of expressing GM-CSF include helper T-cells, B lymphocytes, macrophages, mesothelial cells, keratinocytes, osteoblasts, uterine epithelial cells, synoviocytes, mast cells, fibroblasts, stromal cells, endothelial cells and various solid tumours (Rasko *et al.*, 1994). *In vivo*, it would seem likely that cytokines are produced by a number of cell types. However, the Th1/Th2 model has persisted as a conceptual framework, even in situations where the cellular source of the cytokines has not been determined, because the two ranges of cytokines tend to be detected together and have different and often antagonistic modulatory effects on the immune system (Cox *et al.*, 1992).

In mice, the Th1/Th2 dichotomy has become more than simply a useful conceptual framework. Studies have shown that it is possible to manipulate the immune response of a host and therefore its ability to expel gut helminth infections by application of cytokine or cytokine neutralising antibody (Urban *et al.*, 1991; Urban *et al.*, 1993; Else *et al.*, 1994; Locksley, 1997), possibly as a result of influencing the development of Th0 cells (Locksley, 1997). Such findings suggest the possibility of cytokine-profile influencing therapies or vaccines.

Generally, in mice, intracellular pathogens, such as *Leishmania major* (Cox *et al.*, 1992), viruses, bacteria, and yeast (Locksley, 1997) are controlled or killed by a Th1 type immune response. Cytokines produced by Th1 cells stimulate macrophage activation, immunoglobulin selection for IgG2a and IgG3, both isotypes that mediate ADCC and complement activation. A Th2 type response has been shown to be deleterious to these intracellular pathogens, since IL-4, IL-10 and IL-13 can down-regulate macrophage activation, even when IFNγ is present (Sher *et al.*, 1992).

Cytokines produced by Th2 cells, favour production and activation of mast cells and eosinophils, stimulate B-cell growth and differentiation, and induce isotype switching to IgE and IgG1. These are features associated with infection with helminths (Finkelman *et al.*, 1992; Locksley, 1997), so it might be expected that the human immune response to schistosome infection would be of a Th2-type. However, since the Th1/Th2 model of immunity is likely to be an oversimplification, it might be useful to consider what cytokines are likely to be involved in the regulation of those components of an immune response, currently considered to be important in schistosomiasis in humans: IgE, IgA, IgM, IgG2, IgG4 and eosinophilia.

IgE production (Finkelman *et al.*, 1990) and up-regulation of IgE receptor expression (Defrance *et al.*, 1987b) are induced primarily by IL-4, although IL-13 (Cocks *et al.*, 1993;) and IL-5 (Coffman *et al.*, 1987) are also involved in the promotion of the IgE response. These responses are inhibited by IFNγ (Snapper *et al.*, 1987) and to a lesser extent IFNα (Mond *et al.*, 1986; Rabin *et al.*, 1986). IgE

secretion from murine (Vilcek *et al.*, 1994) and human (Snapper *et al.*, 1987) cells is enhanced by IL-4 *in vitro* and IL-4 neutralising antibody or antibody that binds to the IL-4 receptor can inhibit polyclonal and antigen-specific primary and secondary IgE responses (King *et al.*, 1990). IL-4 dependent isotype switching to IgE may depend on the type of antigen that activated the B-cells. For example, IL-4 can induce IgE in an enriched B-cell population that contains T-cells, but when purified B-cells are pre-activated with *Staphylococcus aureus* IL-4 induces IgM production and switching to IgG instead of IgE (Yokota *et al.*, 1988). Since IgM may block an effective response to schistosomiasis, this may be an important subtlety to consider. IL-13 can induce IgE synthesis independently of IL-4 (Cocks *et al.*, 1993), although IL-4 and IL-13 may share the same signalling pathways (Punnonen *et al.*, 1993). IL-5 can synergise with IL-4 to promote IgE production (Coffman *et al.*, 1987), although IL-5 alone does not appear to be involved in the production of IgE antibodies (Sanderson, 1994).

IL-4 dependent IgE synthesis is inhibited by IFNγ (Snapper *et al.*, 1987). This attribute of IFNγ has been observed *in vivo:* preliminary clinical trials using hyper-IgE patients have indicated that IFNα or IFNγ can result in decreased levels of circulating IgE (King *et al.*, 1989b; Souillet *et al.*, 1989). IgE bound to the low affinity receptor for IgE, CD23, on B-cells, can present antigen to T-cells. Since CD23 expression is enhanced by IL-4 (Defrance *et al.*, 1987b) and IL-13 (McKenzie *et al.*, 1993; Punnonen *et al.*, 1993) antigen presentation is also enhanced. IFNα and IFNγ can block the IL-4 enhancement of CD23, (Mond *et al.*, 1986; Rabin *et al.*, 1986) thus limiting antigen presentation through this mechanism.

IgA levels can be increased by TGFβ, IL-2, IL-5, IL-10 (Mosmann, 1994) and IL-6 (Beagley *et al.*, 1989). TGFβ can selectively augment IgA synthesis (Lebman *et al.*, 1990), presumably by inducing isotype switching since it generally inhibits secretion of all immunoglobulins, including IgA by cells that have already switched (Mosmann, 1994). IL-2 and IL-5 can enhance TGFβ induced IgA production by LPS stimulated B-cells (Sonoda *et al.*, 1989; Lebman *et al.*, 1990) and IL-10 synergises with TGFβ in inducing IgA secretion by anti-CD40 activated B-cells (Defrance *et al.*, 1992). Although IL-5 has been considered to be of primary importance in enhancing IgA levels (Sanderson, 1994), some studies have suggested that IL-6 (Beagley *et al.*, 1989) and IL-2 (Sonoda *et al.*, 1989) may be of greater importance than IL-5.

Production of the putative blocking antibodies, IgM, IgG2 and IgG4, may be determined by a number of cytokines. IL-2 (Matsui *et al.*, 1989) and IL-5 (Takatsu *et al.*, 1988; Matsui *et al.*, 1989) can together, but not separately, up-regulate IgM secretion (Matsui *et al.*, 1989). IL-5 can however, in the absence of IL-2, enhance numbers of murine IgM-producing B-cells (O'Garra *et al.*, 1989). IL-4 and IL-10 can independently induce activated B-cells to produce larger amounts of IgM (Rousset *et al.*, 1992). IgG4 synthesis can be induced by either IL-4 or IL-13 (Rothman *et al.*, 1988; Zurawski *et al.*, 1994). The cytokines that modulate IgG2 are less clearly defined, because most studies have concentrated on murine IgG subclasses and these may not be entirely equivalent to human IgG2. However, murine IgG2b is down modulated by IL-4 and IFNγ and upmodulated by TGFβ (Vilcek *et al.*, 1994).

Eosinophilia is a T-cell-dependent phenomenon (Sanderson *et al.*, 1985) controlled primarily by IL-5 (Sanderson, 1992; Sanderson, 1993). Eosinophil levels tend to be independent of the levels of other leukocytes and, in the murine model, IL-5 can induce eosinophil production in bone marrow suspension cultures without inducing the production of other cell types (Sanderson *et al.*, 1985; Sanderson *et al.*, 1988; Sanderson, 1990). IL-3 and GM-CSF, although they can produce eosinophils independently of IL-5 (Clutterbuck *et al.*, 1989), also stimulate production of other cell types (Campbell *et al.*, 1988). The details of the interactions between IL-3, GM-CSF and IL-5 are not clear from *in vitro* experiments because they appear to depend to a large extent on the precise mixture of cells and the culture conditions into which the cytokines were added (Sanderson, 1994). *In vivo* experiments however, provide clear evidence of the pivotal role of IL-5. A transient eosinophilia has been observed in mice treated with IL-5 (Sanderson, 1994) and mice infected with *Trichinella spiralis* developed eosinophilia, which dropped to levels below that of control animals on administration of anti-IL-5 antibody (Coffman *et al.*, 1989).

In summary (table 1), by extrapolation from the humoral responses and effector cells considered to be important in the immune response to schistosomiasis, it would seem reasonable to predict that IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFNα, IFNγ, TGFβ and GM-CSF might have some role in modulating the immune response during infection. IL-2 can enhance IgA production (Mosmann, 1994) and in the presence of IL-5, can up-regulate IgM production (Matsui *et al.*, 1989). IL-3 has a role in increasing eosinophil production (Clutterbuck *et al.*, 1989). IL-4 and IL-13 can both increase levels of IgE (Defrance *et al.*, 1987a; Finkelman *et*

*al.*, 1990) and induce IgG4 synthesis (Rothman *et al.*, 1988; Zurawski *et al.*, 1994). IL-4 can also induce activated B-cells to produce larger amounts of IgM (Rousset *et al.*, 1992) and down regulate murine IgG2b production (Vilcek *et al.*, 1994). IL-5 synergises with IL-4 to promote IgE production (Sanderson, 1994), although on its own, it does not appear to be capable of influencing IgE production (Sanderson, 1994). IL-5 can enhance IgA secretion (Lebman *et al.*, 1990; Sanderson, 1994), although it is not clear if it is the dominant cytokine controlling the IgA response (Sonoda *et al.*, 1989). IL-5 is considered to be the cytokine primarily responsible for the up-regulation of eosinophil production (Sanderson, 1992; Sanderson, 1993). It is interesting that both IgA and eosinophilia are influenced by the same cytokine, because IgA can induce eosinophils to degranulate and eosinophils are frequently found at mucosal surfaces where IgA is the most abundant immunoglobulin (Sanderson, 1994). Another function of IL-5 is the up-regulation of IgM production, which requires the presence of IL-2 (Matsui *et al.*, 1989), although it can independently enhance the numbers of murine IgM producing cells (O'Garra *et al.*, 1989). IL-6 can enhance IgA production, and may play a more important role than IL-5 in this respect (Beagley *et al.*, 1989). IL-10 can also enhance IgA production (Mosmann, 1994), in the presence of TGFβ (Defrance *et al.*, 1992) and can induce activated B-cells to produce larger amounts of IgM (Rousset *et al.*, 1992). IFNγ inhibits IgE synthesis (Snapper *et al.*, 1987) and can induce reduced levels of circulating IgE in hyper-IgE patients (King *et al.*, 1989b) it can also down regulate production of IgG2b (Vilcek *et al.*, 1994). IFNγ is a potent inhibitor of IL-4, in that it can block the response of resting B-cells to IL-4 (Noelle *et al.*, 1984; Rabin *et al.*, 1986). IFNα can also down regulate IgE production (Souillet *et al.*, 1989).

TGFβ is probably responsible for promoting B-cells to switch isotypes and produce IgA (Lebman *et al.*, 1990; Mosmann, 1994) and can up regulate production of IgG2b (Vilcek *et al.*, 1994). GM-CSF can up-regulate eosinophil production (Clutterbuck *et al.*, 1989).

**Table1.1: A summary of cytokine modulation of IgA, IgE, IgG2, IgG4, IgM and eosinophil production.**

| | IgA | IgE | IgG2 | IgG4 | IgM | Eosinophils |
|---|---|---|---|---|---|---|
| IL-2 | ↑ | | | | ◆ | |
| IL-3 | | | | | | ↑ |
| IL-4 | | ↑ | ↓ | ↑ | ↑ | |
| IL-5 | ↑ | ❖ | | | ✪ | ↑ |
| IL-6 | ↑ | | | | | |
| IL-10 | ⊙ | | | | ↑ | |
| IL-13 | | ↑ | | ↑ | | |
| IFNα | | ↓ | ↓ | | | |
| IFNγ | | ↓ | | | | |
| TGFβ | ↑ | | ↑ | | | |
| GM-CSF | | | | | | ↑ |

↑ can act alone to increase level of antibody

↓ can act alone to decrease levels of antibody

◆ acts only in the presence of IL-5, ❖ synergises with IL-4,

✪ acts only in the presence of IL-2, ⊙ acts only in the presence of TGFβ

It is very difficult to predict from an extrapolation of the results of studies on humoral responses and effector cells whether a Th1-type or a Th2-type cytokine

response would be protective against a schistosome infection. The classic Th2-type cytokines: IL-4, IL-5, IL-10 and IL-13 all have a role in up-regulating the production of both protective and putative blocking antibodies. IFNγ, a Th1-type cytokine can reduce the response of B-cells to IL-4 and may down-regulate IgG4 production, a blocking antibody, at the same time as reducing levels of protective IgE and IL-2, can enhance production of both IgA (a protective isotype) and IgM (a blocking isotype).

Butterworth (1994) speculated that it might be the balance between different antibody isotypes rather than the presence or absence of any particular isotype that differentiates between a protective and a non-protective immune response. The same may be true of cytokine responses, with the additional complication that combinations of cytokines can have very different effects from cytokines on their own.

To illustrate this point further, consider a hypothetical population of B-cells and T-helper cells stimulated with schistosome antigen, in micro-environments that included either IL-2 or IL-5. Evidence from *in vitro* experiments discussed above, suggests that B-cells with the capacity to produce IgA would probably be up-regulated. According to Butterworth and colleagues (Auriault *et al.*, 1990; Butterworth *et al.*, 1992; Grzych *et al.*, 1993; Butterworth, 1994) this could be considered a protective response. Thus, the same protective response could have been triggered by either a Th1-type cytokine or a Th2-type cytokine. However, some B-cells may be influenced by both IL-2 and IL-5 at the same time. This combination of cytokines could up-regulate the production of IgM an antibody

with blocking potential. Whilst it seems reasonable that the immune response would have negative feedback mechanisms, this indicates that whilst the Th1/Th2 model can be useful in some instances, particularly in mice, it is likely to be an over-simplification of the *in vivo* immune response in humans and it may thus also be more appropriate to consider the net effect of a given cytokine or mixture of cytokines, rather than attempting to classify a response as either Th1 or Th2 type *per se*.

In the murine model, Sher and colleagues (1991) observed that Th2-type responses were associated with egg production and pathology. Vaccination with IL-12, which up-regulated the Th1 response, was shown to inhibit pathology in mice infected with *S.mansoni* (Sher *et al.*, 1991). Conversely, a Th1-type profile, as characterised by IFNγ production and macrophage activation was linked to host protective responses. Smythies and colleagues (1993) noted a Th1 mediated protective response in mice. Multiple vaccination with attenuated cercariae, have resulted in a protective Th2-type immune response, with the added benefit that a protective humoral response was also stimulated using this method (Wynn *et al.*, 1996). Further protection was achieved by using a multiple vaccination protocol using IL-12 as the adjuvant, in which a Th1-type protection with both cellular and humoral components was achieved. It would appear therefore that either a strong Th1 or a Th2 response can protect against challenge infections in mice (Wynn *et al.*, 1996). In other helminth infections in mice, Th1 responses are not the only means of protection. Th2-type responses can also play a role in resistance in for example, *Heligomosomides polygyrus* (Urban *et al.*, 1991), *Nippostrongylus*

*braziliensis*, *Trichuris muris* and *Strongyloides venezuelensis* infection (Finkelman *et al.*, 1992).

The cellular immune responses to schistosomiasis in humans have been studied directly in a number of different ways. Some research groups have concentrated on determining the dominant phenotype of parasite-specific T-cell clones (Hirayama *et al.*, 1994; Couissinier-Paris *et al.*, 1995), whereas others have attempted to correlate cellular parameters, such as cytokine production or cell proliferation *in vitro* on stimulation with parasite antigen with either, infection (Araujo *et al.*, 1994), re-infection (Roberts *et al.*, 1993) or pathology (Williams *et al.*, 1994). Another approach has attempted to measure schistosome-modulated cellular responses by comparing cellular parameters before and after treatment (Abdel-Salam *et al.*, 1981; Grogan *et al.*, 1996).

Couissiner-Paris and Dessein (1995) measured IL-2, IL-4 and IFNγ produced by T-cell clones from three adult Brazilian men considered to be resistant to infection with *S.mansoni* and one adult European man, considered susceptible to infection. T-cell clones from the susceptible individual produced more IFNγ and less IL-4 than T-cell clones from the resistant individuals, although all of the clones produced some IL-2, IL-4 and IFNγ. The authors concluded that a Th0/2 phenotype characterised resistance whereas a Th0/1 phenotype characterised susceptibility. All of the T-cell clones produced IL-4 and IFNγ and therefore technically had a Th0 phenotype, however, this should not detract from the main conclusion that cells from resistant individuals presented a more Th2-biased cytokine profile then the susceptible individual.

From one individual, Hirayama *et al* (1994) produced four CD4+ T-cell clones, specific to *S.mansoni* antigen. Each of the four clones produced a different range of cytokines. One was considered to have a Th0 phenotype, because it produced all of the cytokines measured: IL-2, IL-4, IL-5, IFNγ, GM-CSF and TNFα. The second clone was considered to have a Th1 phenotype because it produced GM-CSF, IFNγ and TNFα. It also produced very small amounts of IL-5, but no IL-2 or IL-4. The third clone was characterised as a Th-2 cell type because it produced high levels of IL-4, IL-5 and GM-CSF but low levels of IFNγ and TNFα. The authors classified their fourth clone as 'Th3', because it produced IL-2, IL-5, GM-CSF, IFNγ and TNFα but no detectable IL-4. Cytokine levels were measured by sandwich ELISA and the presence of cytokine mRNA was verified using a reverse transcriptase polymerase chain reaction (RT-PCR).

T-cell cloning experiments have an advantage over *in vitro* stimulation of heterogeneous cell populations, in that it is possible to classify individual cell-types. It could be argued, however, that the phenotype of individual cells is less important than the overall balance of cytokines produced by all cell types since the function of a cytokine is not dependent on the type of cell that produced it. It is possible to interpret the results from Couissiner-Paris and Dessein (1995), in terms of a Th1/Th2 dichotomy. However since Hirayama and colleagues (1994) could not identify a dominant T-cell phenotype, and one of their clones did not conform to Th1, Th2 or Th0 phenotypes, this study could be considered further evidence that the Th1/Th2 model (which can be considered to include the Th0 phenotype) has limited utility in explaining human immunity to schistosomiasis.

In a study in an *S.mansoni* endemic area in Kenya, Roberts and colleagues (1993), measured cell proliferation, IL-2, IL-4, IL-5 and IFNγ production by PBMC on stimulation with *S.mansoni* antigen and mitogen and compared the results with resistance to re-infection. IL-5 and cell proliferation were significant determinant factors for post-treatment re-infection intensities. After controlling for age, sex and exposure to infection in a multiple linear regression model, proliferation of PBMC taken from 14-35 year old residents of the endemic area, was negatively correlated with re-infection intensity (this was not reported to be log transformed). This relationship was statistically significant when PBMC were stimulated with either adult or schistosomulum antigen. They also reported that on stimulation with adult and schistosomulum antigens, cell proliferations were higher 3 months after treatment than before treatment, and had dropped to pre-treatment levels again one year post treatment. IL-5 was measured in the culture supernatants of PBMC taken three months post-treatment and stimulated with egg antigen. In this one instance, IL-5 correlated positively with age ($p<0.02$), and associated with low intensity of infection ($p<0.05$). However, analysis of a repeat assay set, did not confirm the latter association, and a multiple linear regression analysis did not dissociate the effects of age, infection and IL-5.

In order to elucidate the interactions between cytokines, Roberts and colleagues (1993) correlated different cytokines measured in culture supernatants. They reported negative correlation co-efficients between levels of IFNγ (a Th1-type cytokine) and IL-5 (a Th2-type cytokine) detected in both 2- and 4- day culture supernatants from cells stimulated with adult worm antigens, schistosomula and egg antigens. These correlations were observed pre-treatment, three months after

treatment and one year after treatment. These observations imply a cross-regulatory relationship between Th1 and Th2 helper T-cell subsets. Although only the data for the three months post-treatment were presented, from this data it is possible to see that any combination of cytokine and time at which culture supernatants were harvested were correlated. For example, any combination of IFNγ and IL-5 measured in culture supernatants harvested at either two or four days post incubation was considered acceptable for analysis. In this study, peak levels of both cytokines occurred at four days post incubation. There were therefore four opportunities at each bleed and for each antigenic stimulation for IFNγ to negatively correlate with IL-5, however there is no record of correction for multiple tests. Since significant results are reported at 95% confidence limits, one would expect one in twenty correlations to be significant by simple chance and thus the possibility of Type I errors cannot be entirely ruled out.

Williams and colleagues (1994), compared cellular responses of Brazilian patients presenting with different manifestations of disease caused by *S.mansoni*. They concluded that patients with *S.mansoni*-related pathology produce more Th2-type cytokines on stimulation with poke weed mitogen and ionomycin or egg antigens, but produce more IL-5 and IFNγ on stimulation with worm antigens and therefore have a Th0-like profile.

Their rational for these conclusions was as follows. *In vitro* stimulation of PBMC with mitogen indicated that the Th2 cell population was expanded as a result of schistosome infection. PBMC from acutely infected ($p<0.001$), and hepatosplenic patients ($p<0.005$), produced significantly more IL-4 than uninfected controls.

More IL-5 was produced by PBMC from 'acute' patients ($p<0.01$) and those with intestinal disease ($p<0.001$) than by PBMC from the control group. These results were linked to an expansion of numbers of IL-4 producing cells. The percentage of IL-4 producing cells was higher in acutely infected patients ($p<0.05$) and hepatosplenic patients ($p<0.005$) than in uninfected controls, although the absolute difference in percentage was small ($\approx$1.5%). These authors reported that while both egg and adult worm antigens could induce Th2-type responses, egg antigen failed to trigger Th1 cytokine production, except during the acute stages of infection. It is difficult to interpret the results of stimulation with antigen, because the authors were unable to detect any cytokine in culture supernatants. The results rely entirely on cytokine mRNA measured RT-PCR. Although, in most cases, mRNA does correlate with secreted protein, RT-PCR is semi-quantitative at best and cannot distinguish between cells that are producing cytokine and those that are synthesising cytokine without secreting it (Mosmann *et al.*, 1989a). Given this qualification, PBMC from acute patients produced more mRNA encoding for IL-5 ($p<0.01$) and IFNγ ($p<0.02$) on stimulation with adult worm antigen, and more mRNA encoding for IL-2 ($p<0.05$) on stimulation with egg antigen. PBMC from hepatosplenic patients produced more mRNA encoding for IL-4 ($p<0.02$) on stimulation with egg antigen and IFNγ ($p<0.02$) on stimulation with adult worm antigen. Only background levels of cytokine mRNA was produced by patients with intestinal schistosomiasis.

Although this study produced some interesting results, an analysis of their conclusions should be qualified by some consideration of the methodology. The authors reported that their control group of uninfected volunteers were from

endemic areas and from North America. This could mean two things: either that the control group was a mixture of people who had been exposed but remained uninfected (i.e. uninfected volunteers from an endemic area), and people who were uninfected and had never been exposed i.e. uninfected volunteers from North America; or the control group was comprised entirely of individuals who had never been exposed to schistosomiasis.

Assuming the control group consists entirely of people with no history of infection or exposure, the paper states that PBMC from these people have lower Th2 type responses, on stimulation with mitogen, than people with a current infection or schistosomiasis related pathology. One interpretation of this finding could be that adults with experience of disease have a higher Th2 response than adults with no experience of disease. This interpretation suggests that a Th2 response is related to experience rather than necessarily to pathology. Indeed, Williams and colleagues (1994) report that acutely infected patients produced more IL-4 on stimulation with a mitogen than patients with intestinal disease. This implies that as the disease progresses, from acute to chronic, the Th2 response, as characterised by IL-4 produced on stimulation with mitogen, is reduced. This result is only significant to within 95% confidence limits and given the limited method of statistical analysis used, there may be an unacceptable risk that this observation could have occurred by chance.

Although patients and controls were classified into four groups, the authors did not use a test suitable for multiple groups such as parametric, or Kruskal-Wallis ANOVA (Siegel *et al.*, 1988a) and made no attempt to correct for the probability

that an apparently significant result had occurred by chance due to the number of tests carried out (Rice, 1989). The difference in absolute terms ranged from 0 to 1.5% and is reliant on 2 out of 5 patients. In such a case, a more stringent analysis might require a value of $p<0.01$ before the results are considered to be statistically significant.

Grogan and colleagues (1996), investigated the changes in cellular immune parameters that occurred with treatment for *S.haematobium*. Proliferation of PBMC stimulated with adult worm antigen and IL-4 produced on stimulation with both adult worm and egg antigen were significantly greater five weeks post treatment compared to pre-treatment levels ($p<0.001$ in each case). Cultures of PBMC were depleted of either CD4+ or CD8+ T-cells. IL-4 production was reduced to below the cut off level of the IL-4 assay in CD4+ depleted cells, but was not affected by removal of CD8+ cells. Levels of IL-4 increased after treatment, but levels of IL-5 did not (Grogan *et al.*, 1996). The authors interpreted this as implying that distinct cells produced these cytokines. There is some evidence from other studies to support this conclusion (Jung *et al.*, 1995; Sewell *et al.*, 1996).

Grogan and colleagues (1996) interpret their results as implying that chronic infection with *S.haematobium* was associated with a reversible down-regulation of T-cell proliferative responses and IL-4 release, and that CD4+ T-cells were the target of this down-modulation. They also discuss the possibility that antigen released by treatment, could have up-regulated the IL-4 and proliferative responses. They reject this second possibility because egg antigen-specific

responses were up-regulated, despite the eggs remaining intact after treatment. Although this could be due to worm antigen cross-reacting with egg antigen, T-cell responses to egg antigen, unlike adult worm antigen, were non-proliferative. As further evidence for rejecting the possibility that the release of adult worm antigen on treatment could up-regulate cellular responses, the authors note that T-cell proliferative responses to schistosomal antigen can be sustained for up to one year post treatment (Barsoum *et al.,* 1982). However, if people are effectively vaccinated by treatment, could the effect equally last for up to one year post treatment?

Barsoum and colleagues (1982), also report that people who had been treated a year and a half prior to the start of the study, did not show as much of an elevated response one month after re-infection, compared to patients who were treated for the first time. Therefore, whilst down-regulation of responsiveness as suggested by Grogan and colleagues (1996) is possible, their justification for rejecting possible up-regulation of responses by drug treatment is deficient. I consider it reasonable to propose that antigen release might contribute, at least in part, to the effect of treatment on cellular immune responses.

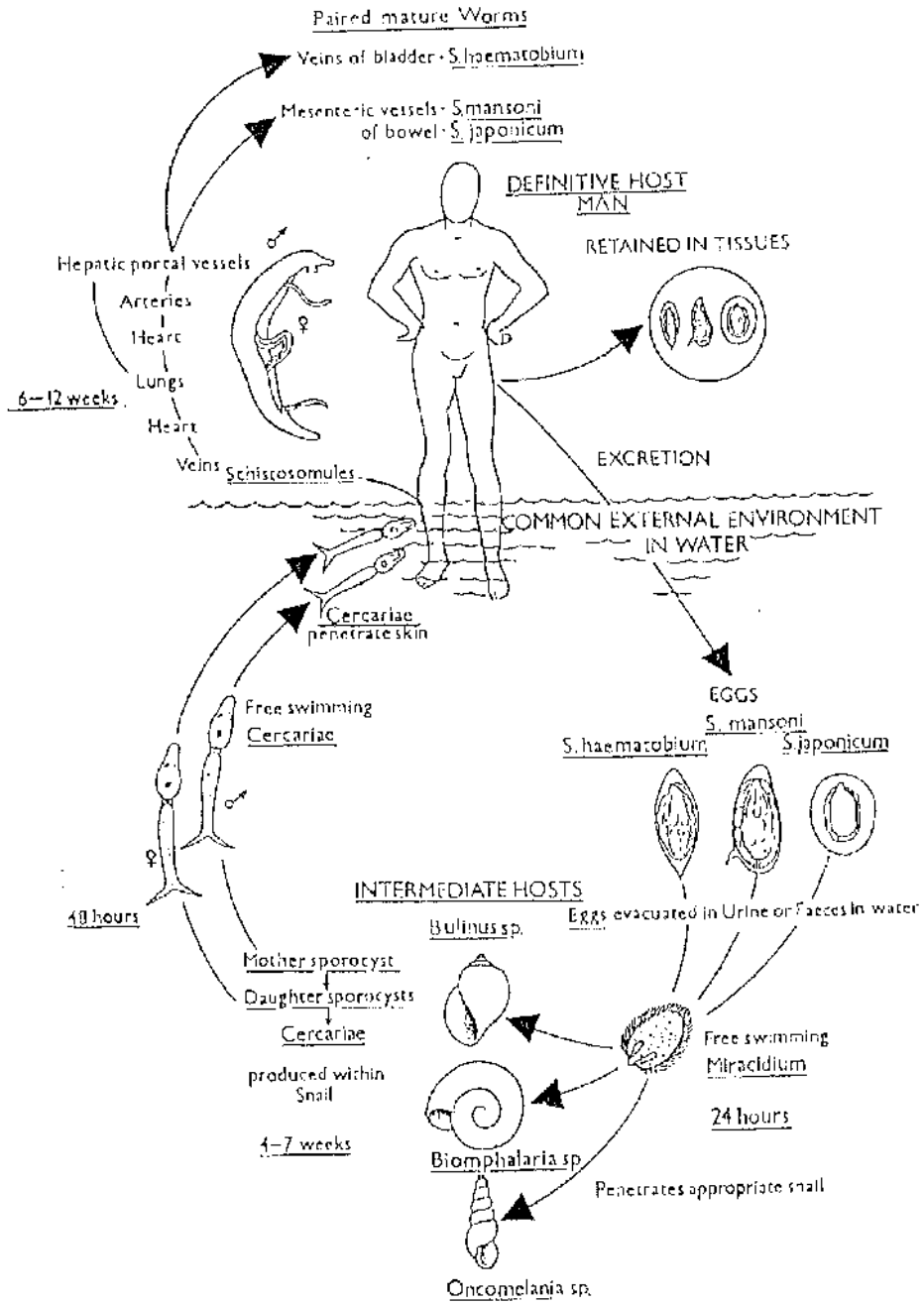
Grogan and colleagues (1996) also conclude that a deficiency in IFNγ and IL-2 release was not restored by chemotherapy, because no detectable IL-2 production was observed pre-treatment, and IFNγ production to parasite antigens was only detectable in 21% of people and these levels did not rise after treatment. However, an alternative interpretation might imply that the lack of significant difference between IL-2 and IFNγ before and after treatment is not evidence for

Th1 cell deficiency, but simply that production of these cytokines is not the dominant effect of stimulation with schistosome antigen.

In summary, cell proliferation on stimulation with either adult or schistosomulum antigen was negatively correlated with re-infection intensity (Roberts *et al.*, 1993) and the Th2 cell population, as characterised by IL-4 and IL-5 production on stimulation with mitogen expanded as a result of schistosomiasis infection (Williams *et al.*, 1994). Proliferation of PBMC stimulated with adult worm antigen and IL-4 produced on stimulation with both adult worm and egg antigen increased after treatment either as a result of release from down-modulation by adult worms, (Grogan *et al.*, 1996) or as a result of release of antigen at treatment. Cellular immunity to schistosomiasis is therefore not as clearly understood as the humoral response. The primary aim of this thesis is to attempt to elucidate further the role of cytokines in the immune response to schistosomiasis, by associating cytokines produced on *in vitro* stimulation with mitogen and *S.haematobium* antigens with prevalence of infection (chapter 3) and re-infection (chapter 4).

**Figure 1.1**

**A Schematic representation of the life cycle of *S.haematobium*, *S.mansoni* and *S.japonicum*.**



# Chapter 2.

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## Development of assays for the analysis of cytokine production in human peripheral blood.

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**2.1 Introduction.**

A variety of methods are available for the measurement of cytokines. The choice of a protocol for the investigation of cytokine responses in humans must depend to some extent on the hypothesis being tested and on the sample type and quantity. Resources and time available in the field are also determining factors.

Cytokine production can be measured in a variety of complex milieu including saliva and blood; in supernatants from heterogeneous populations of cells cultured *in vitro*, for example peripheral blood mononuclear cells (PBMC), spleen cells or triturated tissue; or in homogeneous cell cultures such as T-cell clones. For practical purposes however, in situations where there is a systemic circulation of cytokine producing cells, peripheral blood is the preferred source of cells.

Until relatively recently, it was assumed that CD4+ T-cells were the dominant cytokine producers (Mosmann, 1987). It is now evident that many populations of lymphocytes such as CD8+ T-cells, lymphokine activated killer cells and B-lymphocytes, together with other cell types such as mast cells and eosinophils not only produce cytokines but do so in quantity (Gordon *et al.*, 1990). It can be argued that a clearer insight into *in vivo* cytokine activity may be obtained by assessing the overall cytokine production by heterogeneous cell populations rather than homogeneous sub-populations.

In many population-based studies of helminth infections the aim is to investigate the immune responses to infection. The immune response is studied in relation to

resistance to infection rather than to aspects of pathology that may occur at tissue sites from which routine sampling for cells would be impossible. Studies of pathology can be problematic since cytokines are generally locally acting and have a short half-life. Circulating cytokines are usually only detectable in cases of severe pathology such as cerebral malaria. The typical action of cytokines is autocrine or paracrine rather than endocrine. Bearing these restrictions in mind, the present study was designed to allow an assessment of cytokine production in relation to infection, rather than pathology. In this context, PBMC or whole peripheral blood was used, not only because it is the only practicable source of cells, but because the argument that it will yield information about whether an individual is infected or not is reasonable, whereas the argument that peripheral blood truly reflects local tissue pathology is not. Cellular immune responses that can be detected systemically, as reflected by cytokine production by peripheral blood, may be an important factor in assessing and designing any vaccine candidates, which will probably also act at a systemic rather than local level.

The argument for studying cytokines produced by cells in the peripheral blood is further strengthened by a consideration of schistosome biology. Adult worms live in the blood, so immune cells in contact with adult worm antigens are likely to move freely in the systemic circulation. Likewise, eggs, the principle direct cause of pathology, are shed into the bloodstream before lodging in a variety of tissue locations, allowing systematically circulating immune cells considerable exposure to egg antigens.

**2.2 Detection of cytokines.**

Both bioassays and immunoassays can be used for detecting and measuring cytokines in complex milieu. The results from either assay type should be qualified by a consideration of the method employed. The accuracy of the assays may be influenced by a variety of factors including the *in vivo* or *in vitro* absorption and utilisation of cytokines that are produced.

Neither bioassays nor immunoassays give any indication of the frequency of cytokine producing cells nor of the anatomical site of their production *in vivo*. Investigating newly synthesised cytokines at a clonal level or studying individual secreting cells could give some insight into these aspects. A number of techniques are available to measure cytokine production in single cells. These include the haemolytic plaque assay, the cell blot assay and ELIspot (a modification of the enzyme linked immuno-sorbant assay (ELISA)). Visualisation of intracellular cytokine markers by flow cytometry can also demonstrate the phenotype of cytokine secretion by cells (Lewis, 1991). These questions were not considered in this study.

Bioassays detect functionally active cytokine. However, they are time consuming and rely on the availability of permissive cell lines. They are not necessarily specific in that they do not exclusively measure the activity of a single cytokine. Given the complexity of the concurrent messages a target cell line may receive, care must be taken to ensure that adequate controls are included.

Ascertaining the concentration of cytokine in a sample using a bioassay can be more time consuming than using an ELISA. It is necessary to culture cell lines in the presence of sample and neutralising cytokine antibody as well as the sample alone at a variety of concentrations. Target cell lines need to be maintained and for assays based on cell proliferation therefore appropriate facilities for $^{3}H$ thymidine use are required. Thus several factors make bioassays unsuitable as a field method or for screening large numbers of samples.

Immunoassays, whether radio-, fluoro- or enzyme-linked are usually specific to individual cytokines. Such assays can be readily performed on large numbers of samples; a considerable bonus for population based studies. However, they do not necessarily detect biologically functional cytokine since the monoclonal antibodies on which they are based may bind fragments of inactive cytokine. It is useful to screen large numbers of samples using ELISA and consider results from both ELISA and bioassay for a smaller subset of samples. Although sensitive, ELISAs are not as sensitive as RT-PCR which detects cytokine mRNA even when relatively few cells in the sample are producing the cytokine being studied.

RT-PCR tends to be more sensitive than either bioassay or ELISA because the mRNA signal is amplified many times before the detection stage. For example, RT-PCR can be used to detect cytokine mRNA in a single cell whereas a minimum of $1x10^{6}$ cells $ml^{-1}$ are required for short term culture to produce cytokine at concentrations measurable by ELISA (Brenner *et al.*, 1991). However, RT-PCR is semi-quantitative at best and cannot distinguish between cytokine producing cells

and cells that are synthesising cytokine mRNA but not secreting cytokine protein. In most cases however, transcribed mRNA correlates well with secreted protein (Mosmann *et al.*, 1989a).

**2.3 Development of cytokine immunoassays.**

A sandwich ELISA protocol was received from Pharmingen, on purchase of their anti-human cytokine antibody pairs. The protocol gave a range of concentrations for the use of capture and detection antibodies. On testing this range of concentrations it became clear that there were problems with the protocol. These problems included low overall absorbance and a high level of absorbance in wells containing a negative control as compared to wells containing cytokine standards. Wells prepared in an identical way produced markedly different absorbance readings. Over a series of trials, therefore, the method was honed until the results achieved as determined by absorbance readings and reproducibility, were considered acceptable. A number of changes were made.

Using the original protocol, across a standard curve ranging from $512uml^{-1}$ to $0.0625uml^{-1}$ $OD_{405}$ ranged from 0.01 to 0 after background was subtracted. Using the improved protocol, $OD_{405}$ ranged from 1.999 to 0 after background was subtracted. Overall absorbance was increased by altering ABTS substrate (2,2'azino-bis(3-ethyl benzthiazoline-sulfonic acid) concentration until a stronger colour reaction was attained thirty minutes after addition of the substrate. By preparing the ABTS substrate immediately before use, rather than using substrate prepared in

advance and stored frozen as suggested by Pharmingen's protocol, optical density readings in negative control wells reduced from $OD_{405}$ 0.11-0.14 to 0.066-0.07.

It was important for the cytokine ELISAs to have a low well to well variability because replicate tests on each sample were not possible due to the low volume of sample supernatants from the field studies. Reproducibility was measured in terms of the standard deviation between absorbance levels of 24 wells processed in an identical manner. Standard deviation was measured using four concentrations of cytokine standard: $8uml^{-1}$, 16 $uml^{-1}$, 32 $uml^{-1}$ and 64 $uml^{-1}$ and a mean of these standard deviations was considered for each ELISA protocol. A standard deviation of $\leq 0.03$ was considered acceptable, which resulted in the ELISA having a minimum accuracy of 0.01 $uml^{-1}$. Well to well variation was brought to within acceptable bounds by increasing the volume of the first antibody from 50µl to 100µl and using a newly serviced multi-well pipette, (figure 2.1). The acceptable standard deviation value ($\leq 0.03$) was derived using Equation 2.1.

**Equation 2.1**

The equation was designed to find the sample size required to find a significant difference using an ANOVA (Sokal *et al.*, 1995). It was used to estimate standard deviation assuming a sample size of 1. As ANOVA was not used to analyse the data, this should be considered an estimate only.

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left\{t_{\alpha[v]} + t_{2(1-P)[v]}\right\}^2$$

-where

**n** = number of replications

$\sigma$ = true standard deviation

$\delta$ = the smallest true difference that it is desired to detect.

**v** = degrees of freedom of the sample deviation with *a* groups and *n* replications per groups

**P** = desired probability that a difference will be found to be significant (if it is as small as $\delta$). This is the intended power of the test.

$\mathbf{t_{\alpha[v]}}$ **and** $\mathbf{t_{2(1-P)[v]}}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of $\alpha$ and 2(1-P), respectively.

Two types of standard curve have been used (figures 2.2 and 2.3) in this study. For The Gambian study (chapter 3), a linear regression was used. This had the disadvantage that only the linear portion of the graph could be employed. This restricted the practical sensitivity of the assay. For the Zimbabwean study (chapter 4), the standard curve was described using a sigmoidal equation (figure 2.3). This allowed the utilisation of all parts of the standard curve, and so effectively improved the sensitivity of the assay. The sensitivity of the assays as used in The Gambian study was below $1 \text{uml}^{-1}$ in each case, and in the Zimbabwean study was below $0.0625 \text{ uml}^{-1}$. Both equations adequately describe the relationship between standard concentration and optical density as evidenced by correlation co-efficients of >0.9. Standard curves were run on each plate.

**The revised cytokine protocol as used in both field studies (Chapters 3 and 4)**

**Primary mAb**

1)Dilute purified anti-cytokine capture monoclonal antibody (mAb) to 1μgml$^{-1}$ in coating buffer. Add 100μl to wells of an enhanced protein binding ELISA plate (Immulon IV, Dynatech).

2)Cover plate and incubate overnight at 4°C.

3)Wash 2x with PBS-Tween. For each wash, fill wells with PBS-Tween and allowed to stand for 1 minute prior to aspirating or dumping, pound plate on paper towels as a final step in the drying process.

**Blocking**

4)Add 200μl of PBS/10% FCS to each well.

5)Cover plate and incubate at room temperature for 2 hours.

6)Wash 2x with PBS/Tween 20.

**Samples**

7)Add standards and samples at 100μl per well (diluted in RPMI media/heat inactivated human serum at 10% for the Gambian study and 50% for the Zimbabwean, as appropriate for the different type of sample supernatant). Standards ranging from 512 to 0.0625 units ml$^{-1}$ are appropriate.

8)Cover and incubate overnight at 4°C.

9)Wash 4x with PBS/Tween.

**Secondary mAb**

10)Dilute biotinylated anti-cytokine (detecting) mAb to 2μgml$^{-1}$ in PBS/10% FCS. Add 100μl per well.

11)Cover and incubate at room temperature for 1.5 hours.

12)Wash at least 6x with PBS/Tween.

**Avidin-peroxidase** 13)Dilute avidin-peroxidase to 0.4ugml$^{-1}$ in PBS/10% FCS. Add 100µl per well.

14)Cover and incubate at room temperature for 30 minutes.

15)Wash at least 8x with PBS/Tween 20.

**Substrate** 16)Prepare ABTS substrate immediately prior to use. Protect it from light. Add 10µl of Hydrogen peroxidase per 10ml of substrate. Immediately add 100µl per well and allow to develop at room temperature in the dark.

17)Stop the colour reaction by adding 100µl of SDS/DMF.

18)Read the plate at OD 405 nm.

**Solutions** Coating buffer.

0.1M sodium hydrogen carbonate ($NaHCO_3$) pH8.2

Phosphate Buffered Saline Solution (PBS).

4.25g sodium chloride (NaCl)

14.48g di-sodium hydrogen orthophosphate ($Na_2HPO_4$)

0.78g anhydrous sodium dihydrogen orthophosphate ($NaH_2PO_4$)

1 L double distilled water ($H_2O$)

PBS/Tween 20.

Add 0.5ml of polyoxyethlenesorbitan monolaurate (tween-20) to 1L PBS from above.

Substrate buffer.

Make up a 0.5mgml$^{-1}$ solution of 2,2'Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), in 0.1M citric acid in dd$H_2O$. Adjust the pH to 4.35 with sodium hydroxide (NaOH) pellets. (Add 10µl of 30% hydrogen peroxidase ($H_2O_2$) per 10ml of substrate solution just before use.)

SDS/DMF.

200ml N,N-dimethyl formamide (DMF)

80g sodium dodecyl sulphate (lauryl sulphate ) (SDS)

220ml double distilled water ($H_2O$)

**2.4 Comparison of an IL-4 immunoassay and bioassay.**

As previously discussed, bioassays detect biologically functional cytokine but immunoassays detect the epitopes on the cytokine protein recognised by the component antibodies of the ELISA. Therefore ELISAs do not necessarily measure biologically active cytokine. Although it was not possible to measure all of the samples using a bioassay, IL-4 was measured in a sub-set of samples from The

Gambian study in order to investigate the hypothesis that the IL-4 measured by the ELISA was predominantly biologically active (see figure 2.4a and b).

IL-4 was detected using a sandwich ELISA as previously described (section 2.3), and a bioassay. The bioassay was based on the proliferation of the C.Th.4S cell line, generously provided by W. E. Paul (Huli *et al.*, 1989) and has been described elsewhere (Wadhwa *et al.*, 1995). Briefly, C.Th.4S cells require IL-4 for proliferation. Cells were cultured in the presence of IL-4 standard at various concentrations, in the presence of sample diluted to 1:8, 1:16, 1:32 and 1:64 or in the presence of sample and neutralising IL-4 antibody. The IL-4 international standard 88/656 (1000iu per ampoule) (NIBSC) was used to calibrate both assays (figures 2.4a and 2.4b). Medium alone was included as a negative control. Proliferation was measured using $^{3}H$ thymidine incorporation.

Using Spearman's Rank correlation, the relationship between the results of the ELISA and bioassay correlated to a significance of $p<0.001$ and an $r^2$ of 75% ($n=65$). When only samples positive for IL-4 were considered, the correlation co-efficient had a significance of $p<0.008$ and an $r^2$ of 38% ($n=17$). Although the bioassay and the immunoassay intrinsically measure different attributes of the cytokine, the results of one are significantly correlated with the results of the other (figure 2.4b).

**2.5 Development of field culture techniques.**

The following method was used successfully in The Gambian study (see Chapter 3). PBMC were obtained by ficoll-hypaque gradient centrifugation of heparinised peripheral blood (Boyum, 1968) under sterile conditions using lymphoprep, according to the manufacturer's instructions. Briefly, approximately 20ml of whole heparinised blood was gently layered on top of 10ml lymphoprep (Nycomed) in a 50ml centrifuge tube and centrifuged at 800g for 20 minutes. After centrifugation, the PBMC formed a distinct band at the sample/medium interface. PBMC were transferred using a plastic pasteur pipette to approximately 10ml of sterile culture media and centrifuged at 350g for 15 minutes to wash them. Culture media consisted of RPMI 1640, supplemented with 2.4mM sodium hydrogen carbonate, 2mM glutamine, 50mg $L^{-1}$ gentamycin, 100 000 units $L^{-1}$ nystatin, 1x non-essential amino acids, 1mM sodium pyruvate, 30mM Hepes and 0.5mM ß- mercaptoethanol. The resulting pellet of PBMC was re-suspended, washed twice more and used in lymphocyte proliferation assays and for short term culture in the presence of various stimuli as described in Chapter 3.

For proliferation studies and short term culture, PBMC were adjusted to a concentration of 1 x $10^6$ cells $ml^{-1}$ in culture media supplemented with 10% heat inactivated normal AB plasma generously donated by the West of Scotland Blood Donation Service. Cells were incubated for 48 hours in a humidified incubator at 37° C and 5% $CO_2$/air .

### 2.5.1 Use of Cryopreserved PBMC.

The same method was employed in the Zimbabwean study in 1994. The study site, the Burma Valley in the Eastern Highlands of Zimbabwe, was an overnight drive away from the laboratory in Harare so PBMC were cryopreserved in culture media described above, supplemented with 20% foetal calf plasma and 5% DMSO. Cells were frozen at a rate of 1°C per minute in liquid nitrogen vapour until they reached -120°C. PBMC were stored in liquid nitrogen, and transferred to Glasgow via Harare for subsequent *in vitro* cell culture.

Sterile conditions in the field were not ideal. Blood was processed in a chipboard 'hood'. The hood and equipment was swabbed with methylated spirits once an hour and bathed in ultra violet light when not in use. Flaming of containers was not possible. The equipment for ficol-gradient centrifugation was also less than ideal. The purpose bought equipment: 50ml centrifuge tubes and lymphoprep, remained impounded by customs for the duration of the field trip in 1994. Leucoprep tubes, universal tubes, and lymphoprep (some unfortunately past their sell by date) were utilised instead.

### 2.5.2 Initial Testing of Cell Viability.

**Materials and Methods.**

On return to Glasgow, 120 vials of stored cells from thirty individuals picked randomly from the cohort were defrosted by placing the cryovials in a universal tube and standing in a 37°C water bath. Viable cells were identified by trypan blue exclusion and counted using a haemocytometer. For each sample 1 x $10^6$ cells were

cultured in 1 ml cultures in the presence of either Schistosome Egg Antigen, (SEA) Whole adult Worm Homogenate (WWH) phytohaemagglutinin (PHA) or without stimulant. Supernatants were harvested at 24, 48, 72 and 96 hours post culture and tested for IL-4, IL-5, IL-10, GM-CSF, IFNγ, and TNFα using sandwich ELISAs.

*S.haematobium* antigens were purchased in freeze dried form from the Schistosome Biological Supply Programme, Theodore Bilharz Research Institute, Giza, Egypt. They were reconstituted in sterile phosphate buffered saline. Some preparations did not arrive pre-homogenised. In these cases, antigen was ground in a homogeniser with a ≈100µl of PBS. Dissolved antigen was separated from undissolved antigen by centrifuging the sample at centrifugation for 10 minutes at 800g. This process was repeated three times. The final concentrations were measured using a BCA Protein Assay Kit (Pierce), according to the manufacturer's instructions.

As outlined below, difficulties were encountered in detecting cytokines from the cryopreserved cells in view of which a number of pilot experiments were constructed in an attempt to isolate the source of the problem. Viability of stored cells from Zimbabwe was tested under a variety of culture conditions. The media recipe, concentration of cells, fungicide and serum supplement were altered.

In Glasgow, PBMC separated by ficol gradient centrifugation from blood taken from two volunteers were used as positive controls for the effects of cell preparation with out-of-date reagents and cryopreservation. These were prepared as described above. Briefly, cells were washed three times in RPMI 1640 and re-suspended at a

concentration of $1x10^6$ cells$ml^{-1}$. They were cultured for 48 hours with either 10 % heat inactivated human AB plasma, foetal calf serum (FCS) batch number 228 (Labtech) or Lot number 40Q7354F (Gibco). They were either stimulated with PHA at a variety of concentrations or cultured without stimulant.

Two different recipes for the culture medium were tested: one as described previously used without nystatin, and the other as described by Williams and colleagues (1994). It consisted of RPMI 1640 supplemented as before except $100uml^{-1}$ Penicillin and $100ugml^{-1}$ Streptomycin were used instead of Gentamycin, and the 2-mercaptoethanol concentration was reduced from 0.5mM to 50μM. One set of cells was prepared and cultured using each recipe. Neither preparation contained fungicide.

Two concentrations of PBMC were compared. Cells were cultured at $1x10^5$ or $1x10^6$ $ml^{-1}$for 48 hours, stimulated with $10\mu gml^{-1}$ PHA or not stimulated. Half of the supernatant from cultures initiated at $1x10^5$ $cell ml^{-1}$ was tested at the original concentration. The other half was concentrated by freeze drying and reconstituting at ten times the original concentration in an attempt to detect very low concentrations of cytokine. All supernatants were tested for IL-4, IL-5, IL-10, IFNγ, GM-CSF and TNFα.

The possibility that fungicide in the medium might have affected cell growth in cytokine products was investigated. Culture media was supplemented with either nystatin, fungizone or left without fungicide.

**Results.**

When cryopreserved cells from Zimbabwe were defrosted, cell death as measured by trypan blue exclusion ranged from 40% to 100%. When live cells were cultured with either variant of culture medium, at either concentration of cells, with or without fungicide or with any serum supplement no measurable cytokine was produced, even in response to PHA.

Cytokines were produced by fresh PBMC (figure 2.5). IL-4 and IFNγ were detected in supernatants from cultures supplemented with FCS, but not in supernatants from cultures supplemented with hAB plasma. IL-5 was detected in only one supernatant which was taken from a culture of PBMC supplemented with FCS supplied by GIBCO and stimulated with $10\mu gml^{-1}$ of PHA. IL-10 was not detected in supernatants from cultures supplemented with FCS, but was detected in supernatants from cultures supplemented with hAB plasma. GM-CSF was detected in all supernatants. TNFα was detected in some cultures supplemented by each type of FCS and some cultured with hAB plasma.

**Discussion.**

This series of trials suggests that either culture media would be acceptable, and that $1x10^{-6}$ is a reasonable density at which to culture cells but that although some cells were alive, as defined by ability to exclude trypan blue, they no longer had the ability to produce cytokine under the conditions tested here. Preferential death of antigen presenting cells, which are larger and more delicate than T-cells is one possible explanation, although it is surprising that PHA stimulation, which does not depend

on antigen presentation should fail to illicit a cytokine response. The fungicides, necessary for culture in The Gambia, could be removed when culturing in the laboratory in Glasgow.

The presence and amount of detectable cytokine produced by fresh PBMC from volunteers in Glasgow, was influenced by the culture conditions. For example IL-10, a Th2-type cytokine was produced when the fresh PBMC were cultured with human AB plasma, but was not produced when they were cultured with either batch of FCS. Conversely, IFNγ, a Th1-type cytokine, was produced when PBMC were cultured with FCS, but not produced when cultured with Human AB plasma. Different types of heterologous plasma can therefore bias cytokine production, making it difficult to compare studies in which PBMC are cultured in heterologous plasma.

**2.5.3 Development of a Whole Blood Stimulation Method.**

The limitations identified during the field trip in 1994 indicated that ficol gradient centrifugation of blood was not a practicable method to use under the field conditions in Zimbabwe, because under the conditions used here cryo-preserved cells produced no cytokine.

Field conditions placed a number of restrictions on any new methods. Cell culture equipment such as a sterile hood and incubator were available only in Harare, but blood could be transported to Harare overnight. The method had to be carried out by one operator, processing up to 80 samples per day. A maximum of 10mls of

blood was available per person. A whole blood stimulation method was therefore developed for use in the field trip in 1995 because it involved less manipulation of sample, was a faster technique, did not require serum supplements and was a more economical use of blood. Whole blood stimulation has been successfully used in other studies (Kirchner *et al.*, 1982; Elsasser-Beile *et al.*, 1991).

In advance of the 1995 field session, the whole blood stimulation method was established in Glasgow, using heparinised blood taken from four volunteers. From each individual 2ml of blood was diluted in culture media (using the recipe of Williams and colleagues (1994)) at dilutions of 1/2, 1/4, 1/5, 1/8, and 1/10. Blood was cultured in the presence of either $10ugml^{-1}$ PHA or without stimulation. Blood was removed 24, 48, 72, and 96 hours post incubation and cells and debris were separated by centrifugation for 10 minutes at 800g. The supernatants were removed and stored at -20°C. Supernatants were then tested for IL-10, GM-CSF, IFNγ and TNFα production using sandwich ELISAs (see section 2.3).

The results for cytokine production with time and at different concentrations of whole blood for GM-CSF, IFNγ, IL-10 and TNFα are shown in figures 2.6, 2.7, 2.8, 2.9. In each case more cytokine was produced by cultures containing PHA than blood cultures without stimulant.

Markedly more GM-CSF was produced at 48 hours than at other time points and the culture that contained blood diluted to 1/4 with culture media produced the most GM-CSF with dilutions of 1/5 and 1/8 also producing acceptable levels, but cultures

containing blood diluted to 1/2 and 1/10 produced negligible amounts of cytokine (figure 2.6). The low concentrations of GM-CSF produced by blood cultured at a dilution of 1/2 could be due to an inhibitory factor produced at an effective concentration at this level of dilution or alternatively high levels of cytokine consumption.

IFNγ production ranged at 24 hours from 5 $uml^{-1}$ in the case of the 1/8 dilution to 70$uml^{-1}$ in the case of the 1/2 diluted culture. The 1/2 dilution produced 180$uml^{-1}$ by 48 hours, rising to 200$uml^{-1}$ by 72 hours before reducing to 160$uml^{-1}$ by 96 hours. Cytokine produced by other dilutions peaked at 72 hours. The 1/4 dilution produced about half the amount of IFNγ compared with the 1/2 diluted culture at 48 hours reached the same level as the 1/2 dilution by the 72 hour time point and then reduced to a lower level (figure 2.7).

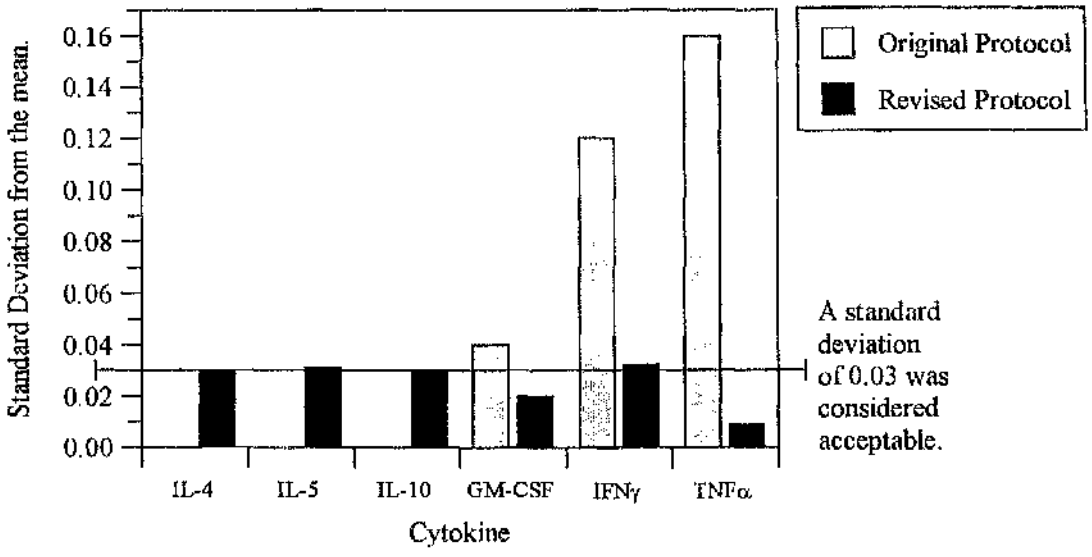
IL-10 production remained fairly stable across the four time points. The 1/2 dilution produced between 7 and 11 $uml^{-1}$, the 1/4 between 3 and 6 $uml^{-1}$ with cultures of blood at 1/5, 1/8 and 1/10 dilutions producing very low levels of cytokine (figure 2.8).

TNFα production was highest at 24 hours. Blood cultured at a 1/2 dilution produced 79$uml^{-1}$, at least double the amount produced in other cultures (figure 2.9).

**Discussion.**

These data clearly demonstrate that a whole blood stimulation assay was a practicable method of assessing cytokine production. A 1/2 dilution of blood was chosen because in most cases, this produced the largest amount of cytokine. Antigen stimulated cultures tend to produce less cytokine than mitogen stimulated culture, so a high concentration was considered desirable. High dose inhibition factors were thought to be less likely to be a problem in an antigen-driven system. Supernatants were harvested at 24, 48 and 72 hours post culture because peak production of cytokine was passed by 72 hours and no additional information could be gleaned by taking an additional sample after 96 hours.

**Figure 2.1. A comparison of the accuracy of two protocols for cytokine sandwich ELISAs.**




The standard deviation between wells was measured using either the original or the revised protocol. In each case, standard deviation was measured using 24 wells containing one of four concentrations of cytokine standard: $8uml^{-1}$, $16uml^{-1}$, $32uml^{-1}$ and $64uml^{-1}$. A mean of these standard deviations was considered for each ELISA protocol. All cytokines were measured using sandwich ELISAs using Pharmingen antibody pairs.

Standard deviation is not shown for IL-4, IL-5 or IL-10 using the original protocol. Optical density readings for IL-4 and IL-5 were so low as to render accurate reading impossible using the original protocol. An IL-10 ELISA was standardised later than the others, so the improved protocol was tested in the first instance and found to be acceptable.

**Figure 2.2. Typical standard curves as produced in the Gambian study (Chapter 3).**

The best fit line was fitted using a linear regression described by the equation $y = A + Bx$

where x = log (concentration + 1), y = log (Optical Density +1), A & B = constants.

The sensitivity for each assay was < 1uml$^{-1}$ (approximately 100pg ml$^{-1}$) and the accuracy was within 0.01uml$^{-1}$ (approximately 1pg ml$^{-1}$).

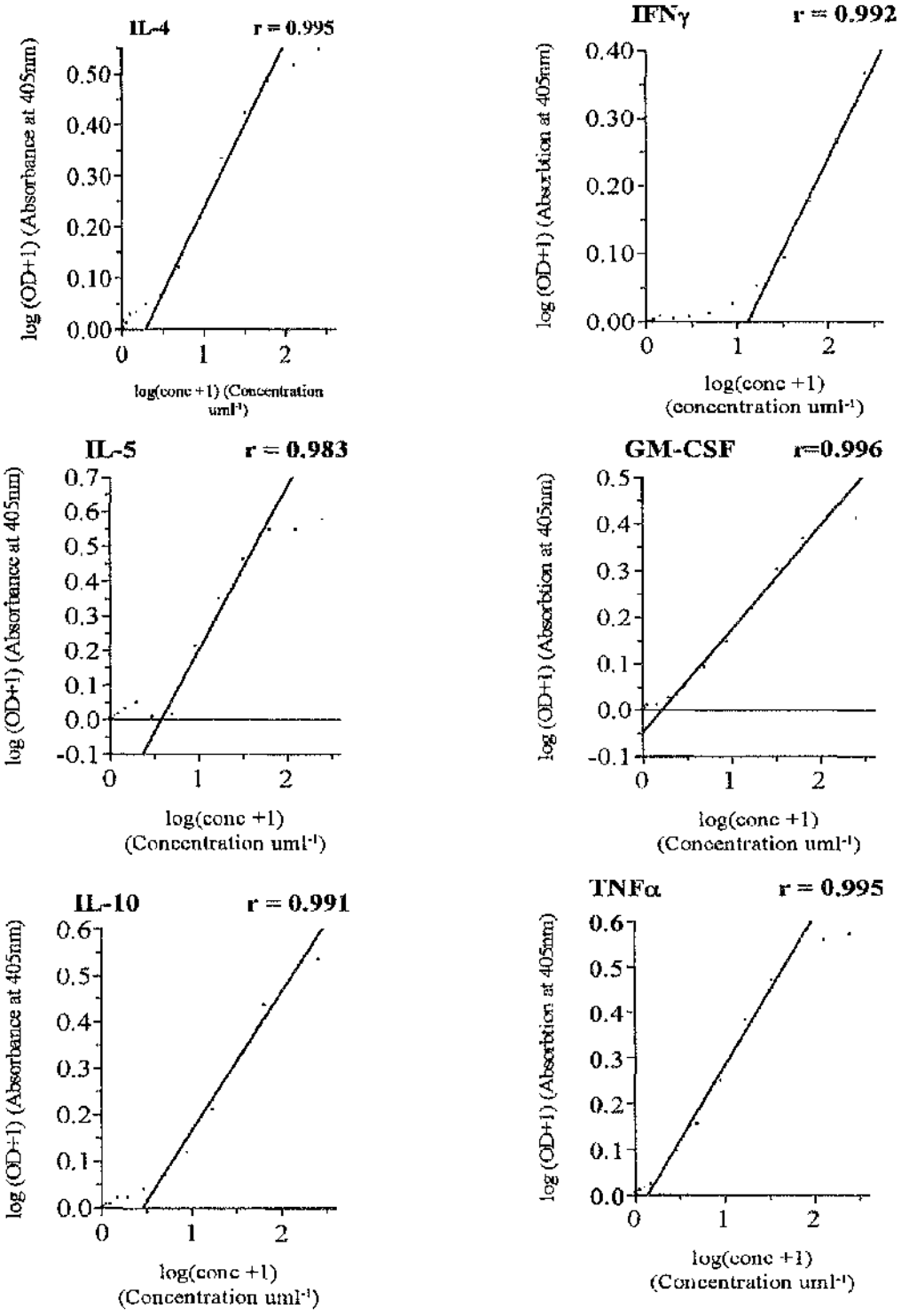
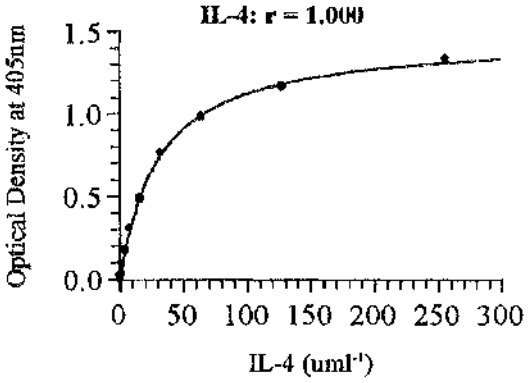


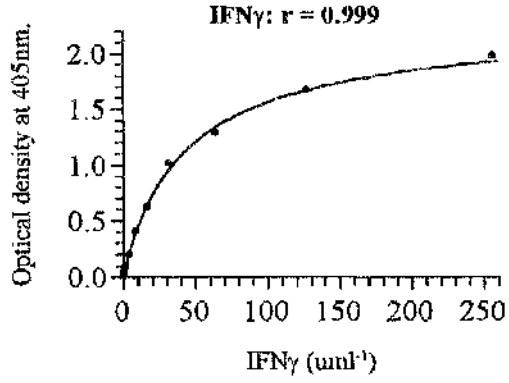
**Figure 2.3 Typical standard curve as produced in the Zimbabwean study (Chapter 4)**

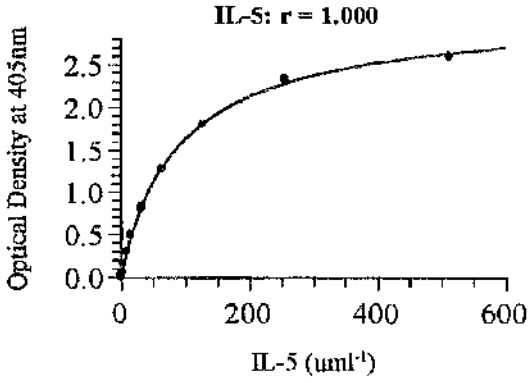
A sigmoid curve expressed by equation of the form:

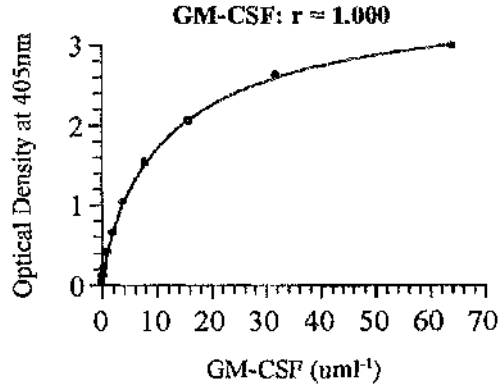
$$y = D + \frac{(A-D)}{\left(1+{}^{x}/_{C}\right)^{B}}$$

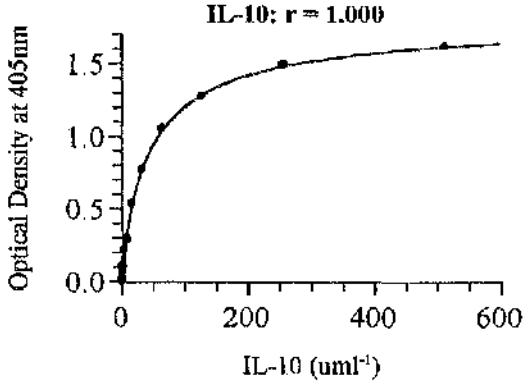
where x = log (concentration),
y = log (Optical Density),
A, B, C & D = constants.
-was used to describe the relationship between optical density and concentration of cytokine. The sensitivity for each assay was below 0.0625uml$^{-1}$ (approximately 62.5 pgml$^{-1}$) and the accuracy was within 0.01uml$^{-1}$ (approximately 1pg ml$^{-1}$).



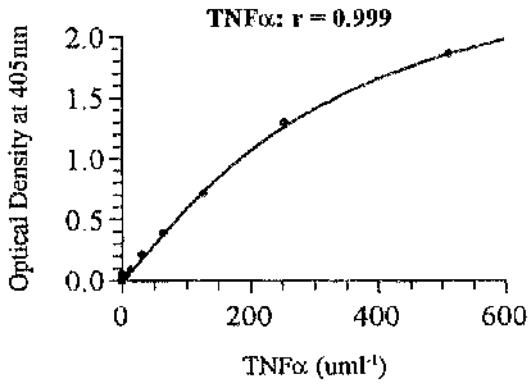



**Figure 2.4a. Standard curve for a CTh4s proliferation assay for the measurement of IL-4.**

A standard curve for an IL-4 bioassay. The graph also demonstrates its specificity for IL-4 by means of a neutralising antibody.

**Figure 2.4b. IL-4 measured by bioassay correlated with IL-4 measured by sandwich ELISA.**

A comparison of IL-4 measured using a sandwich ELISA as described in section 2.3 and a bioassay based on the proliferation of the C.Th.4S cell line described in section 2.4. IL-4 was produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x $10^6$ cells$ml^{-1}$ in 1ml aliquots, for 48 hours in the presence of 10$\mu g ml^{-1}$ PHA, SEA or WWH. IL-4 measured by bioassay correlated with IL-4 measured by sandwich ELISA using a Spearman's Rank correlation. $p<0.001$, $r^2 = 75\%$, $n=65$

**Figure 2.4a**

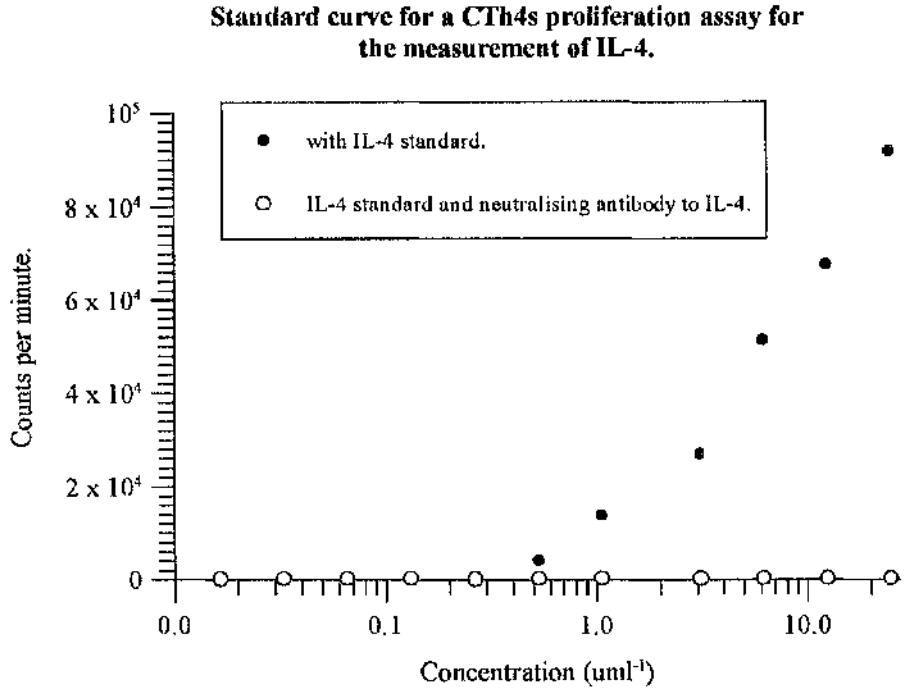



**Figure 2.4b**

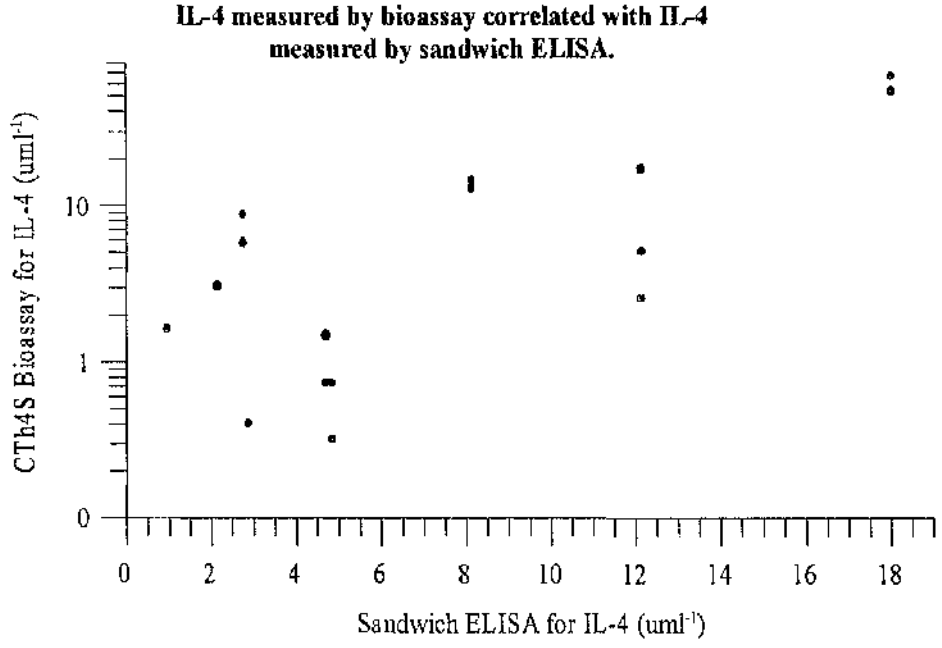


**Figure 2.5. The influence on cytokine production by PBMC of different plasma supplements in RPMI.**

Cytokines were produced by fresh PBMC cultured in RPMI at a concentration of 1 x $10^6$cellsml$^{-1}$ supplemented with either FCS or hAB plasma and stimulated with PHA at a variety of concentrations. Six different cytokines were measured in culture supernatants using sandwich ELISAs as described in section 2.3

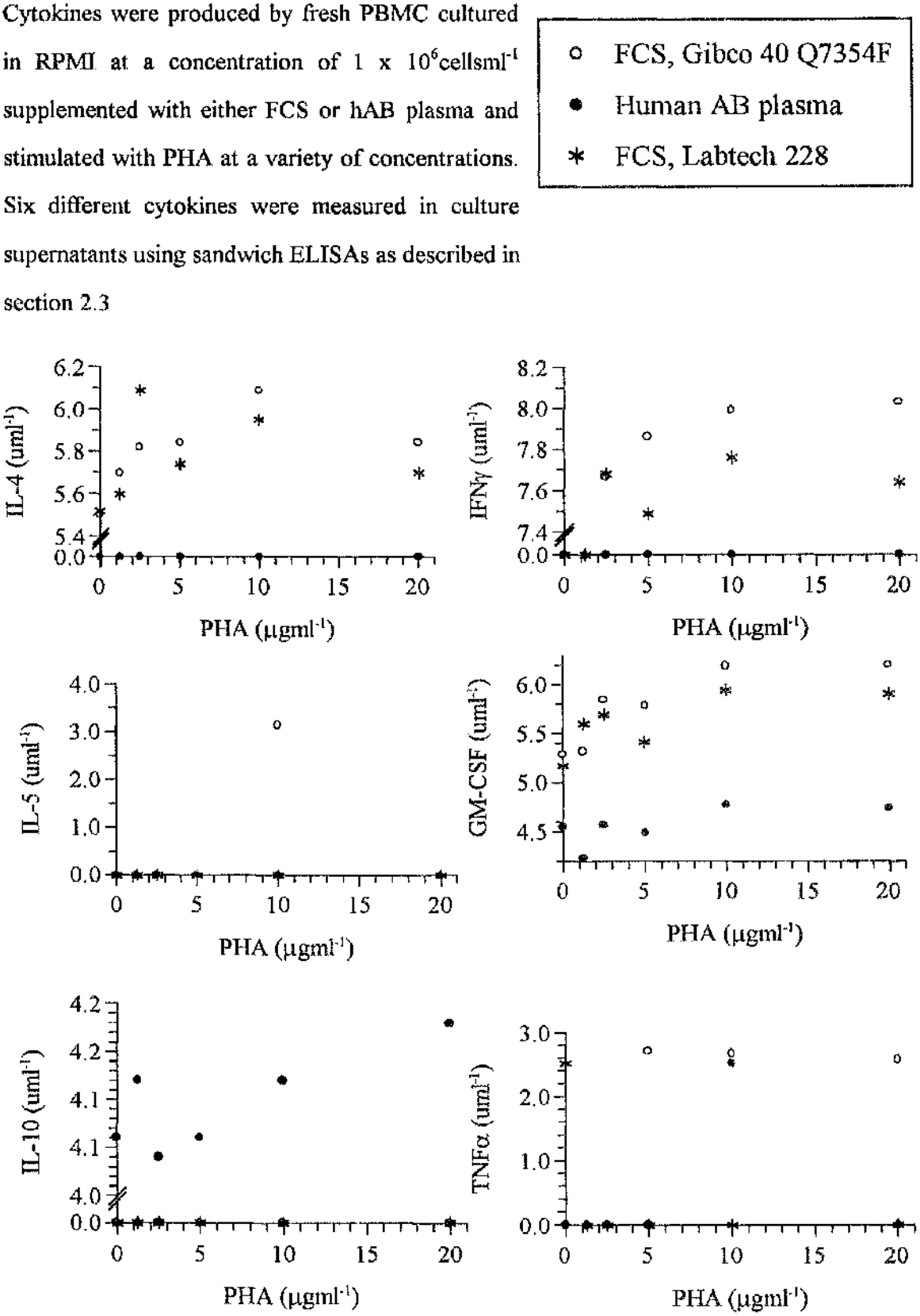


**Figure 2.6. Net production of GM-CSF by whole blood at different dilutions and time points.**

A comparison of net GM-CSF produced by whole blood collected from volunteers (n = 4 for each point) from the University of Glasgow and cultured in 1ml aliquots, for 24, 48, 72 or 96 hours in the presence of 10μgml$^{-1}$ PHA. GM-CSF concentration was determined using a sandwich ELISA as described in section 2.3, which has a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. These background levels were always <1.30uml$^{-1}$ (median control value = 0.00 uml$^{-1}$).

**Figure 2.7. Net production of IFNγ by whole blood at different dilutions and time points.**

A comparison of net IFNγ produced by whole blood collected from volunteers (n = 4 for each point) from the University of Glasgow and cultured in 1ml aliquots, for 24, 48, 72 or 96 hours in the presence of 10μgml$^{-1}$ PHA. IFNγ concentration was determined using a sandwich ELISA as described in section 2.3, which has a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. These background levels were always <9.3uml$^{-1}$ (median control value = 0.00 uml$^{-1}$).

**Figure 2.6. Net production of GM-CSF by whole blood at different dilutions and time points.**

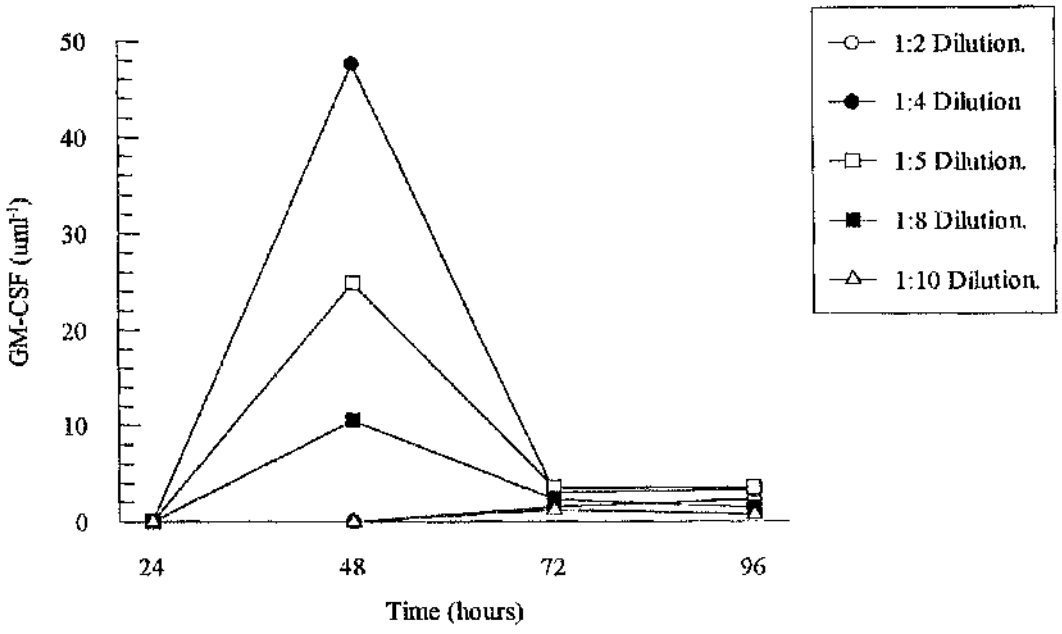



**Figure 2.7. Net production of IFNγ by whole blood at different dilutions and time points.**

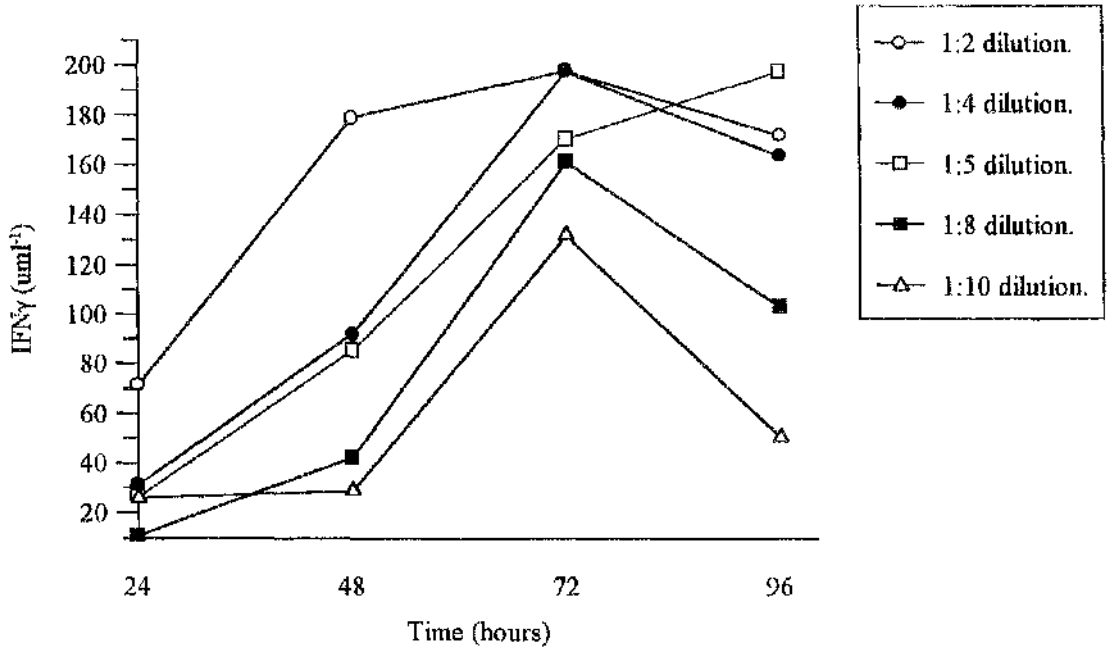


**Figure 2.8. Net production of IL-10 by whole blood at different dilutions and time points.**

A comparison of net IL-10 produced by whole blood collected from volunteers (n = 4 for each point) from the University of Glasgow and cultured in 1ml aliquots, for 24, 48, 72 or 96 hours in the presence of 10μgml$^{-1}$ PHA. IL-10 concentration was determined using a sandwich ELISA as described in section 2.3, which has a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. These background levels were always <1.9uml$^{-1}$ (median control value = 0.00 uml$^{-1}$).

**Figure 2.9. Net production of TNFα by whole blood at different dilutions and time points.**

A comparison of net TNFα produced by whole blood collected from volunteers (n = 4 for each point) from the University of Glasgow and cultured in 1ml aliquots, for 24, 48, 72 or 96 hours in the presence of 10μgml$^{-1}$ PHA. TNFα concentration was determined using a sandwich ELISA as described in section 2.3, which has a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. These background levels were always <34.48uml$^{-1}$ (median control value = 0.00 uml$^{-1}$).

**Figure 2.8. Net production of IL-10 by whole blood at different dilutions and time points.**

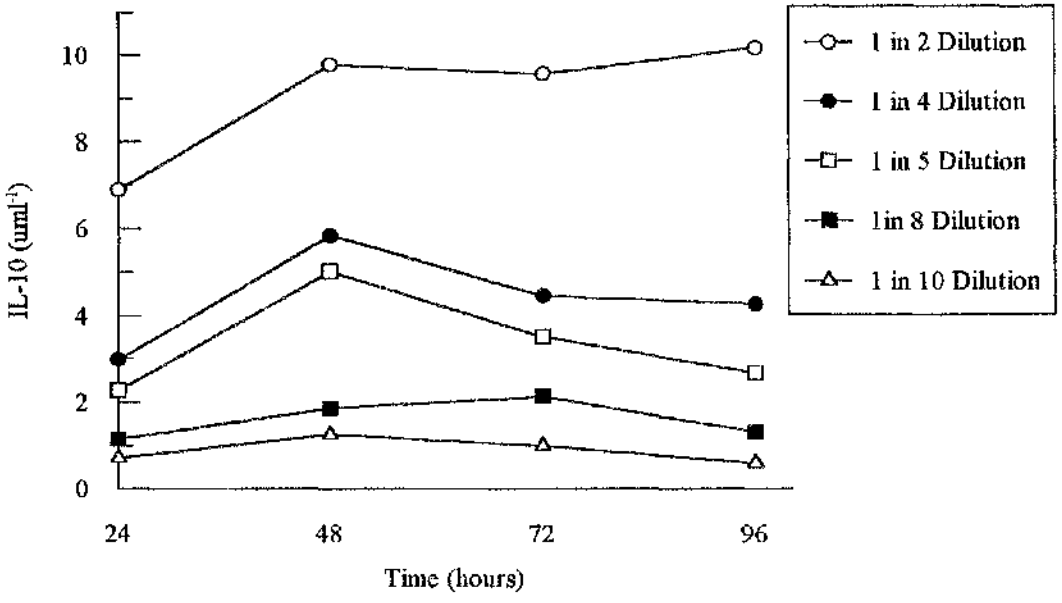
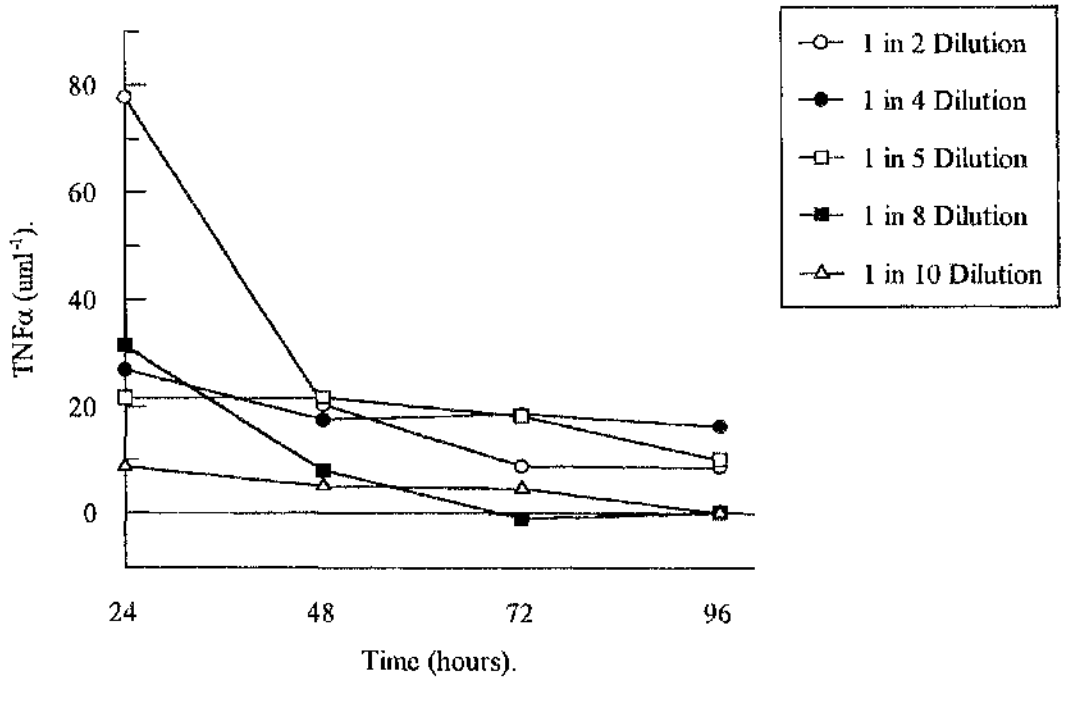



**Figure 2.9. Net production of TNFα by whole blood at different dilutions and time points.**



# Chapter 3.

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## Cytokine production in response to *Schistosoma haematobium* infection in Gambian individuals.

---

**3.1 Introduction.**

Despite the progress which has been reported on attempts to develop a vaccine which would protect against schistosome infection (Dunne *et al.*, 1995; Wynn *et al.*, 1995), there is still some controversy regarding the particular types of immune responses that will need to be induced when any vaccine candidates are used in humans. Studies on murine schistosomiasis indicate that Th1-type responses with the production of IFNγ and macrophage activation, but no humoral component to the immune response, are associated with protection in animals which have been immunised once with attenuated cercariae (Wynn *et al.*, 1996) and with some vaccine candidates such as glutathione-S-transferase and triose-phosphate isomerase (Dunne *et al.*, 1995). Responses of the Th-2 type appear to be a feature of egg-induced pathology in mice (Sher *et al.*, 1991). Triple vaccination of mice with attenuated cercariae however improved protection and changed the nature of the protective response from a Th1 to a Th2 type (Wynn *et al.*, 1996). Furthermore, protection has been observed to improve if IL-12 is administered as an adjuvant along with radiation attenuated cercariae in the triple vaccination model. Mice vaccinated three times with cercariae and IL-12, display predominantly Th1 type cytokines and although they retain a humoral response, the antibodies produced are also associated with a Th1-type pattern of immunity.

Studies of reinfection after chemotherapy in humans have demonstrated that resistance is associated with eosinophilia (Sturrock *et al.*, 1983; Hagan *et al.*, 1985; Hagan *et al.*, 1987; Hagan, 1992) and with the production of parasite-specific IgE (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993). The production of eosinophils is largely dependent on IL-5

(Sanderson, 1992; Sanderson, 1993), and the production of IgE is largely dependent on IL-4 (Defrance *et al.*, 1987a; Finkelman *et al.*, 1990). IL-4 and IL-5 are cytokines classically associated with Th2-type responses (Vilcek *et al.*, 1994). The protective effects of IgE may be influenced by the presence of IgG4 (Hagan *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993), IgM (King *et al.*, 1989a; Butterworth *et al.*, 1992) and IgG2 (Butterworth *et al.*, 1988a; Demeure *et al.*, 1993) antibodies which appear to have the capacity to block the activities of protective antibody classes. IgG4 production is up-regulated by both IL-4 and IL-13 (Lebman *et al.*, 1988; Rothman *et al.*, 1988; Zurawski *et al.*, 1994). IgM is largely modulated by IL-5 (O'Garra *et al.*, 1989), IL-2 (Matsui *et al.*, 1989), IL-4 and IL-10 (Rousset *et al.*, 1992) and IgG2 may be down modulated by IL-4 and IFNγ and upmodulated by TGFβ (Vilcek *et al.*, 1994).

There have been relatively few studies of human cytokine responses to schistosomiasis, and a clear picture has yet to emerge. Cell proliferation on stimulation with either adult worm or schistosomulum antigens has been negatively correlated with re-infection intensity whereas IL-5 was associated with low intensity of infection (Roberts *et al.*, 1993). More IL-4 and IL-5 mRNA was measured in patients with greater degrees of pathology attributed to *S. mansoni* infection (Williams *et al.*, 1994). Proliferation of PBMC stimulated with adult worm antigens and IL-4 production on stimulation with both adult worm and egg antigens increased after treatment either as a result of release from down-modulation by adult worms, or as a result of boosted immune reactivity due to release of antigen at treatment (Grogan *et al.*, 1996).

In the light of evidence from the murine model that suggests that either Th1 or Th2-type responses (Wynn *et al.*, 1996) could provide protection, it is important to clarify this issue in humans. In this chapter, results are reported from studies of the production of cytokines in response to stimulation with schistosome antigens and mitogen by peripheral blood mononuclear cells from 59 Gambians, resident in an area where *S. haematobium* is endemic. Three hypotheses have been examined:

1. that protection against infection is associated with a Th2-type immune response.
2. that Th1 and Th2 responses are dichotomous options in individuals.
3. that cross reactive antigens in Whole adult Worm Homogenate (WWH) and Soluble Egg Antigens (SEA) result in related secretion of cytokine to the two antigenic types.

**3.2 Methods.**

59 subjects, 32 children (12 to 16 years) and 27 adults (25 to 88 years) were recruited from the villages of Misera and Diabugu Basillah in the Upper River Division, The Gambia. Forteen adults were male and 13 were female. Of the children, 27 were male and 5 female. A comprehensive re-infection study, including water contact observations, has been previously carried out in the village of Madina which is also in the upper river division of The Gambia (Wilkins *et al.* 1987). The area is endemic for *S.haematobium* infection, but *S.mansoni* is rare (Hagan, pers. communication). Transmission for *S.haematobium* is restricted to the wet season, which is between June and November. Water contact is predominantly from water holes. The area is also endemic for malaria (predominantly *falciparum* malaria), although again transmission is principally restricted to the wet season. The study was carried out in April 1994, during the

dry season so the prevalence of infection with malaria was likely to be low. Intestinal helminth infections, including *Ascaris spp.* are also endemic. Urine and blood samples were obtained from all participants. All were offered treatment with 40mg $kg^{-1}$ praziquantel.

Intensity of infection was determined by counting *S. haematobium* eggs stained with Lugol's iodine in 10ml of urine after filtration through 20µm pore 25mm diameter polycarbonate filters (Nucleopore Corporation). Lugol's iodine consists of 5g Iodine, 1g Potassium iodide in 100ml water. Absolute eosinophil counts were determined in a Fuchs-Rosenthal cytometer, after dilution of blood in a Phloxin B-based stain (Speirs, 1952). Peripheral blood mononuclear cells (PBMC) from the study population were obtained by Ficoll-hypaque gradient centrifugation of total heparinised peripheral blood (section 2.5) (Boyum, 1968). People were transported from the village to the MRC laboratories in Basse, where samples were taken. PBMC were then used in lymphocyte proliferation assays and for short term culture in the presence of various stimuli as described in section 2.1.

**Proliferation Studies.**

For proliferation studies, 1 x $10^6$ cells $ml^{-1}$ in 0.1ml aliquots were incubated with *S.haematobium* antigens: soluble egg antigen (SEA), whole adult worm homogenate (WWH) or phytohaemagglutinin (PHA) at a final concentration of 10µg $ml^{-1}$ or without stimulant (see section 2.1.2). At 5 days, 6 hours post incubation, proliferative responses were assayed using a commercially available kit (Promega) according to the manufacturers instructions. Briefly, plates were pulsed with 15µl dye (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

(MTT). Four hours later a 100µl solublisation agent (proprietary recipe) was added. Plates were incubated at 37°C overnight and optical densities were read at 570nm.

**Short Term Culture for the production of cytokines.**

1ml volumes of PBMC at a concentration of 1 x $10^6$ cells $ml^{-1}$ were stimulated with SEA, WWH or PHA at a concentration of 10µg $ml^{-1}$ in culture, or without stimulant. Forty-eight hours post incubation, culture supernatants were removed, and centrifuged for 5 minutes at 12 000 gravity to ensure removal of all cell fragments and nystatin and stored at -20°C for use in ELISAs or bioassays.

**Detection of Cytokine.**

The concentrations of IL-4, IFNγ, TNFα, IL-5 and GM-CSF were each measured using a sandwich ELISA. Genzyme kits were used for IFNγ and IL-4 Pharmingen antibodies were used to measure TNFα, IL-5 and GM-CSF concentrations using protocols described in section 2.3. In addition, IL-4 produced on stimulation with SEA was also measured using a sandwich ELISA using Pharmingen antibodies as described in section 2.3. IL-2 concentration was measured using a bioassay based on proliferation of the CTLL cell line (Gillis *et al.*, 1978; Wadhwa *et al.*, 1995). The international standards (I.S.) or reference reagents (R.R.) from National Institute for Biological Standards and Control (NIBSC) were used to calibrate the cytokine assays. The following standards were used : IL-4: NIBSC 88/656 I.S. (1000 international units (iu) per ampoule), IL-5: NIBSC 90/586 R.R. (5000 units (u) per ampoule), TNFα: NIBSC 87/650 I.S. (40 000iu per ampoule), GM-CSF:

NIBSC 88/646 I.S. (10 000iu per ampoule), IL-2: NIBSC 86/502 I.S. (13203iu per ampoule), IFNγ: Genzyme kit standard.

**Data analysis.**

Data were analysed using non-parametric statistics on SPSS for Windows version 6. The two-tailed Mann-Whitney U Rank Comparison was applied to comparisons of two groups. Statistical analyses were based on mean rank data because mean values are inappropriate for non-parametric data and $\log_{10}x$ transformation failed to normalise the data.

The results refer to net and gross levels of cytokine or proliferation. Net levels of cytokine or proliferation refer to cytokine or proliferation produced as a result of culture in the presence of a stimulant, such as antigen or mitogen, minus cytokine or proliferation produced with no stimulation. This allows the influence of individual variations in baseline cytokine or proliferation levels to be minimised. Gross levels of cytokine or proliferation refer to total proliferation or cytokine produced.

**Examination of the three hypotheses.**

**Hypothesis 1: protection against infection is associated with a Th2 type immune response.**

This hypothesis was examined by comparing differences in levels of cytokine produced by PBMC from either infected and uninfected individuals or children and adults using a Mann-Whitney U test.

**Hypothesis 2: Th1 and Th2 responses are dichotomous options in individuals.**

If Th1 and Th2 responses are dichotomous options, one would expect to find relatively low amounts of IFNγ in individuals with relatively high amounts of IL-4, and *vice versa*. It follows that the relative amounts of IL-4 and IFNγ, may be responsible for driving an immune response in a particular direction, rather than the absolute amount of cytokine. If this is the case it might be expected that ratios between IL-4 and IFNγ might fall into two distinct clusters, hypothetically depicted in figure 3.1, or be negatively correlated.

**Hypothesis 3: Cytokine responses to WWH are related to the cytokine responses to SEA and *vice versa*, as a result of cross-reactive antigen.**

Cross-reactive antigens in SEA and WWH may result in the cytokines produced in response to one life-cycle stage being associated with the cytokine produced in response to another as cells specifically sensitised to antigens from one life cycle stage may also respond to shared or similar epitopes in the antigens from another life cycle stage. WWH and SEA will have cross-reactive antigen, because WWH will contain female worms with eggs. However it is useful to test the hypothesis in this instance when the answer is already clear, to test the methodology for potential future use with larval and adult antigen and for testing stage-specificity of effect of possible recombinant vaccine candidates. Cross reactive antigens have been suggested as a possible medium for effecting concomitant immunity (Hagan *et al.*, 1993).

In order to investigate this hypothesis potential correlations between cellular responses produced on stimulation with SEA and cellular responses produced on

stimulation with WWH were sought. A positive correlation between the cellular response produced on stimulation with the two antigens would suggest that cross reactive antigens may affect the production of cytokine. However, an alternative interpretation of such a result, would be that PBMC from some individuals produce more cytokine than others, irrespective of the stimulant used. If this second possibility was correct, cytokine produced on stimulation with antigen would be expected to correlate not only with cytokine production to the other antigen, but also with cytokine production to the non specific mitogen PHA. Therefore, to test the hypothesis, cytokine produced in response to the SEA was partially correlated with cytokine produced on stimulation with WWH controlling for cytokine produced on stimulation with PHA using a Kendall non-parametric correlation coefficient.

A minimum sample size of 10 was accepted as a valid comparison. Where a large proportion of the individuals produced no cytokine in response to one or both antigens, a significant positive correlation could be the result of one or two outlying data points. For this reason, a correlation was only accepted as supporting the hypothesis if a simple majority of data points were non-zero $uml^{-1}$ for both antigens.

**3.3 Results.**

**Intensity of infection.**

Children (16 years and less) had a higher intensity of infection than adults (over 25 years) (figure 3.2)[1]. 1 adult and 29 children were positive for *S.haematobium*

[1] $p<0.0001$, n=59: 32 children, 27 adults, U= 50.0, W= 428.0, Z = -6.1912

infection. 26 adults and 3 children were negative for *S.haematobium* infection. The burden of infection was therefore predominantly in the children. Peak infections were in those aged 13 to 14 years.

### 3.3.1 Hypothesis 1: protection against infection is associated with a Th2 type immune response.

A summary of statistical comparisons between two groups for proliferative responses and the production of six cytokines is shown in tables 3.1 and 3.2. Eosinophil levels were higher in children than in adults[2] and higher in infected than uninfected individuals (figure 3.3)[3].

**Cytokines produced by PBMC on stimulation with PHA.**

PBMC from adults produced more IFNγ on stimulation with PHA than PBMC from children when net[4] and gross data were analysed[5]. However, when infected and uninfected individuals were compared, this difference was no longer significant.

PBMC from adults and uninfected individuals produced more IL-4 on stimulation with PHA than PBMC from children and infected individuals respectively when net data were analysed (figure 3.4)[6]. These observations were significant when IL-4 was measured using the Genzyme Kit ELISA. When gross data were analysed PBMC from adults and uninfected individuals produced more IL-4 on stimulation

[2] $p<0.0004$, n=59: 32 children, 27 adults, U=200.5, W=578.5, Z=-3.5222
[3] $p<0.003$, n=59: 29 uninfected, 30 infected, U=238.5, W=673.5, Z=-2.9794
[4] $p<0.05$, n=45: 21 children, 24 adults, U=165.0, W=396.0, Z= -1.9794
[5] $p<0.05$, n=45, 24 adults and 21 children, U=166.0, W=397.0, Z= -1.9567

with PHA than PBMC from children and infected individuals when IL-4 was measured with either the Genzyme Kit ELISA [7,8] or Pharmingen antibody pairs as described in section 2.3[9]

PBMC from adults and uninfected individuals produced more IL-2 on stimulation with PHA than PBMC from children[10] or infected individuals[11] respectively when gross, but not when net data were analysed.

There was no significant difference between IL-5, TNFα or GM-CSF production by PBMC from adults compared to children or infected individuals compared to uninfected individuals when cultured with PHA for 48 hours.

**Cytokines produced by PBMC on stimulation with SEA or WWH.**

PBMC from children showed increased IFNγ production compared to adults following stimulation with SEA and WWH. These observations were unchanged whether net (SEA[12], WWH[13]) or gross (SEA[14], WWH[15]) data were analysed. PBMC from infected individuals had produced more IFNγ on stimulation with

[6] adults vs. children: p<0.0001, n=45, 21 children and 24 adults, U=85.0, W=316.0, Z=-3.7994 and uninfected vs. infected p<0.0007, n=45, 23 uninfected, 22 infected, U=103.0, W=356.0, Z=-3.4059

[7] adults vs. children: p<0.0002, n=45, 24 adults and 21 children, U=86.0, W=317.0, Z= -3.7768

[8] uninfected vs. infected: p<0.0007, n=45, 22 infected and 23 uninfected, U=104.0, W=357.0, Z= -3.3833

[9] adults vs. children: p<0.005, n=59, 32 children and 27 adults, U=250.0, W=992.0, Z=-2.7807 and uninfected vs. infected p<0.0063, n=59, 29 uninfected, 30 infected, U=255.5, W=1049.5, Z=-2.7331

[10] adults vs children: p<0.01, n=39, 19 adults, and 20 children, U=102.0, W=468.0, Z=-2.48

[11] infected vs uninfected: p<0.02, n=39, 17 infected and 22 uninfected, U=107.0, W=260.0, Z= -2.2726

[12] p<0.04, n=45, 21 children and 24 adults, U=166.0, W=569.0, Z= -2.0641

[13] p<0.04, n=45, 21 children and 24 adults, U=166.0, W=569.0, Z= -2.0353

[14] p<0.01, n=45, 24 adults, 21 children, U=141.0, W=594.0, Z=-2.7418

[15] p<0.003, n=45, 24 adults and 21 children, U=131.5, W=603.5, Z= -2.9776

WWH[16] (figure 3.5) and SEA[17] (figure 3.6) over background levels (net data) than the PBMC from uninfected individuals. These differences remained significant when gross data were analysed (SEA[18] and WWH[19]).

When gross data were analysed, PBMC from children produced more IL-4 than PBMC from adults, measured using Pharmingen antibody pairs as described in section 2.3, on stimulation with, SEA,[20] although this significant difference was the result of low levels of IL-4 produced by four children (Appendix 2a and 2b), Conversely, when net IL-4, as measured using the Pharmingen antibody pairs, was analysed there was no significant difference between IL-4 produced by PBMC from children compared to adults, however, there was a significant difference between infected and uninfected individuals[21], however, uninfected individuals (who were predominantly adult) produced more IL-4 than infected individuals (who were predominantly children). This difference between infected and uninfected individuals, was also due to very low levels of IL-4 production by a few individuals (appendix 2c and 2d).

There was no significant difference between IL-2, IL-5, TNFα or GM-CSF production by PBMC from adults compared to children or infected individuals compared to uninfected individuals when cultured with SEA or WWH for 48 hours.

[16] $p<0.02$, n=45, 23 uninfected and 22 infected, U=154.5, W=604.5, Z= -2.3265
[17] $p<0.02$, n=45, 23 uninfected and 22 infected, U=153.0, W=606.0, Z= -2.3953
[18] $p<0.003$, n=45, 23 uninfected and 22 infected, U=130.0, W=629.0, Z= -3.0322
[19] $p<0.001$, n=45, 23 uninfected and 22 infected, U=122.0, W=637.0, Z= -3.2306
[20] $p<0.05$, n=59, 27 adults and 32 children, U=353.0, W=731.0, Z= -2.0197
[21] $p<0.05$, n=59, 29 uninfected and 30 infected, U=361.5, W=826.5, Z=-1.984

**Proliferation of PBMC on stimulation with SEA or WWH.**

PBMC from adults proliferated more than PBMC from children, without stimulation[22] and stimulation with PHA[23], SEA[24] and WWH[25]. On stimulation with PHA[26] and WWH,[27] PBMC from uninfected individuals proliferated more than PBMC from infected individuals, but there were no significant differences when PBMC were stimulated with SEA or were cultured without stimulation. Proliferation on stimulation with mitogen or antigen was not significantly different between adults and children or between infected and uninfected individuals when net values were analysed.

### 3.3.2 Hypothesis 2: Th1 and Th2 responses are dichotomous options in individuals.

No clustering was observed in cytokine produced on stimulation with SEA, WWH or PHA or when the cohort was split into infected and uninfected or child and adult (figure 3.7a and b) suggesting that Th1 and Th2 responses during schistosomiasis infection (as indicated by capacity to produce IFNγ and IL-4 respectively) are not dichotomous options. IL-4 was measured using the Genzyme Kit ELISA and a sandwich ELISA using Pharmingen antibody pairs as described in section 2.3.

[22] $p < 0.0043$, n=59, 27 adults and 32 children, U=244, W=997.5, Z= -2.8529
[23] $p < 0.0002$, n=59, 27 adults and 32 children, U=186.0, W=1056.0, Z= -3.7429
[24] $p < 0.003$, n=59, 27 adults and 32 children, U=239.5, W=1002.5, Z= -2.9288
[25] $p < 0.001$, n=59, 27 adults and 32 children, U=219.0, W=1023.0, Z= -3.2408
[26] $p < 0.0009$, n=59, 30 infected and 29 uninfected, U=215.0, W=1090.0, Z= -3.3358
[27] $p < 0.04$, n=59, 30 infected and 29 uninfected, U=298.0, W=1007.0, Z= -2.0773

### 3.3.3 Hypothesis 3: Cytokine responses to WWH were related to cytokine responses to SEA and *vice versa* as a result of cross reactive antigen.

Cell proliferative responses[28], GM-CSF[29], IFNγ[30], IL-4, measured using the Genzyme kit ELISA,[31] and TNFα[32] produced by SEA correlated with the response induced on stimulation with WWH, controlling for the response to PHA (figure 3.8). This suggests that cross reactive epitopes in the antigen from the two life cycle stages have some effect on cytokine production. No such correlation was found in the case of IL-5, probably because of low overall IL-5 production. IL-4 measured using the Pharmingen antibody pairs was significant, but the majority of samples contained undetectable levels of cytokine, so the result cannot be considered to be evidence supporting the hypothesis.

[28] N=59, $p<0.001$
[29] N=59, $p<0.001$
[30] N=45, $p<0.001$
[31] N=45, $p<0.001$
[32] N=59, $p<0.001$

**3.4 Discussion.**

**Hypothesis 1: protection against infection is associated with a Th2-type immune response.**

Both net and gross data have been analysed in this chapter. Net cytokine production takes background cytokine levels into account, so it is less affected by variation due to factors other than the immune response to the stimulant in question than gross cytokine production. Sources of such variation could include concurrent infection, maturity of the immune system or MHC. Net data has been used by others, (Grogan *et al.*, 1996) although Roberts *et al* (1993) preferred gross data.

PBMC from children or infected individuals produced more IFNγ (a Th1-type cytokine) than PBMC from adults or uninfected individuals when cultured with either SEA or WWH. This observation was statistically significant whether gross or net data were analysed. PBMC from children produced more IL-4 (measured using Pharmingen antibody pairs) on stimulation with SEA than PBMC from adults when gross, but not net data, was analysed. Conversely, PBMC from uninfected people produced more IL-4 (measured using Pharmingen antibody pairs) than infected people.

These observations could be interpreted to mean that children produced cytokine (whether Th1 or Th2-type) in response to schistosome antigen, whereas adults did not. However, the statistically significant difference between IL-4 produced when cultured with SEA, did not remain statistically significant when background levels were removed (net data) and the comparison of infected and uninfected people

contrasted strongly with the comparison of children and adults. This was probably due to the very small number of people whose PBMC produced detectable levels of IL-4 (appendix 1). I would like to propose therefore that the overriding immune response exhibited by children and infected individuals to schistosome antigens could be considered comparable to a murine Th1-type response.

The data obtained using PHA as stimulant contrasted strongly with those using *S.haematobium* antigens. PBMC from adults or uninfected individuals produced more IL-4 on culture with PHA than children or infected individuals when net or gross data were considered. When gross data were analysed, PBMC from adults or uninfected individuals produced more IL-2 on stimulation with PHA than children or infected individuals. IFNγ production on stimulation with PHA considering either net or gross data, was also higher in adults compared to children although the significance of the difference between uninfected and infected individuals was not significant.

Gross levels of cell proliferation were generally higher in adults or uninfected individuals compared to children or infected individuals. When stimulated with PHA and WWH both the differences in proliferative responses between adults and children, and infected and uninfected individuals were statistically significant. When cultured with SEA or cultured without stimulant, only the difference between adults and children was significant. These differences in proliferative response could be attributed to differences in spontaneous cell proliferation observed between adults and children, since whilst differences in gross proliferation were

very significant ($p<0.004$) no significant differences were observed when net proliferative responses were considered.

As discussed above, PBMC from adults or uninfected individuals tended to produce more cytokine and proliferate more when cultured in the presence of PHA than PBMC from children or infected individuals. Since these observations were due to mitogen, rather than antigen stimulation, they could represent some difference other than schistosome infection, the most likely being age since in this study uninfected individuals roughly corresponds with adults and infected individuals with children.

It is reasonable to assume that the adults in this study have had past and continuing exposure to schistosomiasis because the people have a similar lifestyle to residents of the village of Madina, in the same area, where detailed water contact observations have been carried out (Wilkins *et al.* 1987, Hagan *et al.*, 1991). Given this assumption, the adults in this study are likely to have been exposed to infection for many more years than the children and have gained some degree of protection against infection since they have no detectable worm burden. The differences in immune response between children and adults could therefore be due to age related factors or reflect number of years of experience of *S.haematobium*.

Evidence from new foci would support the hypothesis that resistance was related to age rather than experience (Gryseels *et al.*, 1987; Gryseels *et al.*, 1994b) and observations of peak shift as predicted by mathematical models (Woolhouse *et al.*, 1991) and differences in the immune responses of children resident in a high

transmission area compared to a low transmission area (chapter 4), suggest that experience is a factor.

Irrespective of whether experience of infection or age causes resistance, evidence primarily based on serological studies has suggested that Th2 responses play a predominantly protective role in schistosomiasis in humans. IgE and eosinophilia, hallmarks of Th2-type responses, have been associated with resistance to reinfection, although IgG4, the secretion of which is also elicited by the Th2-type cytokines IL-4 and IL-13 (Lebman *et al.*, 1988; Rothman *et al.*, 1988; Zurawski *et al.*, 1994), was associated with susceptibility to reinfection (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992). However, the evidence from this present study for a protective Th2-type response rests on the greater net production by PBMC of adults and uninfected individuals of IL-4 when cultured with the mitogen PHA. Taken in the context of the other significant differences observed between either children and adults or infected and uninfected individuals, these results could be interpreted in another way. In particular, the observation that IFNγ (a Th1-type cytokine), produced on stimulation with PHA, was also higher in adults compared to children is pertinent. The overall greater response by PBMC from adults to PHA could be due to longer experience of infection generally, or some other aspect of their greater maturity; for example, a different hormonal environment. Alternatively, since the adults group is also predominantly uninfected, these greater non-specific responses could represent a protective strategy. In this case the response would be more comparable to a murine Th0, rather than a Th2-type response. However, if the adults are assumed to have greater protection from infection than children, and they are still exposed, the results would suggest that

this protection has more to do with producing less IFNγ in response to antigenic stimulation than a positive Th2-type response.

Exploiting the background of the age-prevalence and age-intensity profiles from communities in endemic areas, most immuno-epidemiological studies have attempted to identify immune responses associated with resistance and/or susceptibility to infection. Williams and colleagues (1994) employed an alternative strategy. They examined cytokine production by PBMC from *S.mansoni*-infected individuals in relation to their degree of pathology arising from their infection. Williams and colleagues (1994) conclude that *S.mansoni*-related pathology stemmed from the failure of patients to down-regulate Th2-type responses which predominated when PBMC from these individuals were stimulated *in vitro* with mitogens or egg antigen. Therefore, using a different approach, these authors have further confirmed the importance of an understanding of Th2 responses in determining protection from or the generation of pathology by schistosomiasis in humans.

In the murine model, Sher and colleagues (1991) found that Th2-type responses were primarily associated with the production of eggs by adults worms and therefore with the induction of pathology. In support of these findings, vaccination with IL-12, which favours the priming of Th1 cells was shown to inhibit pathology in mice sensitised with eggs of *S. mansoni* (Wynn *et al.*, 1995). Host protective responses have been linked to a Th1-type cytokine profile, primarily IFNγ production and macrophage activation, in mice vaccinated once with attenuated cercariae (Smythies *et al.*, 1993).

It should be noted that Smythies and colleagues (1993) studied not only a different species of host, but also a different species of schistosome from that considered in this chapter. Among other differences, *S.mansoni* occupies the intestinal and mesenteric veins, whereas *S.haematobium* is found in the blood vessels surrounding the bladder.

Th2 type responses have been associated with protection in mice, when vaccinated three times (Caulada-Benedetti *et al.*, 1991). This vaccination protocol successfully induced a humoral as well as a cellular protective immune response and provided enhanced protection against subsequent challenge. Triple vaccination may mimic the natural situation better than the single vaccination model because people are naturally exposed on numerous occasions. However, the use of IL-12 as an adjuvant resulted in triple vaccinated mice displaying a Th1-type response with both cellular and humoral components. This protocol provided the most effective protective response of the three investigated (Wynn *et al.*, 1996). It might therefore be possible to provide protection by inducing either a strong Th1 or a strong Th2-type response. Eliciting a Th1-type response may have the advantage of avoiding any exacerbation of Th2-induced pathology. It would be interesting to see the effects of using Th2-inducing cytokine such as IL-4 or IL-13 as an adjuvant for triple vaccination of mice.

In other helminth infections in mice, such as *Nippostrongylus braziliensis*, *Trichuris muris* and *Strongyloides venezuelensis* (Finkelman *et al.*, 1992), there is evidence that Th2-type responses can play a key role in resistance (reviewed in

Finkelman *et al.*, 1992). Supporting evidence for a role for Th2-type responses in protection against schistosome infections in humans has come from studies of *S. mansoni* in which IL-5 production (a Th2-type cytokine) correlated positively with age and was associated with low intensity of infection (Roberts *et al.*, 1993). The current study found no direct relationship between infection levels and IL-5 production, indeed very little IL-5 was produced (appendix 1). Although Roberts and colleagues (1993) demonstrated evidence for IL-5 mediated protection, they found no significant relationship between IL-4 and protection. These differences between their study and the current investigation may reflect the different time points at which cell culture supernatants were harvested in the two studies and/or differences between the two parasite species. The parasites would primarily stimulate the lymph nodes draining different sites.

**Hypothesis 2: Th1-type and Th2-type responses are dichotomous options in individuals.**

The results suggest that Th1 and Th2 responses as indicated by levels of IFNγ and IL-4 respectively are not dichotomous options. Although as a group, individuals infected with *S.haematobium* and children produce more IFNγ and less IL-4 than uninfected individuals and adults, this does not mean than at an individual level high IFNγ production necessitates low IL-4 production or *vice versa*. Del Prete and colleagues (1994) have reviewed the evidence that IFNγ or IL-4 administered at a susceptible moment of a T-cell's development may direct it towards a Th1 or Th2 route respectively. However, once a T-cell is mature, IL-4 is unable to induce a Th1-type cell to switch to a type 2 phenotype. I would speculate therefore that the T-cells in this study were mature cells of defined phenotype. It may also

indicate that resistance to infection is not modulated by a switch from the dominance of one T-cell subset to another. It would lend further support to the hypothesis that levels of IL-4 production remain relatively stable, whereas IFNγ levels change.

**Hypothesis 3: Cytokine response to WWH is related to the cytokine response to SEA and *vice versa* as a result of cross reactive antigen.**

Cross-reactive antigens have been suggested as a mechanism for concomitant immunity. Concomitant immunity, a concept first introduced by Smithers and Terry (1965) is regarded as a state of resistance to infection due to a current burden of adult worms. In principle, concomitant immunity suggests that the adult worms elicit an immune response that they are themselves unaffected by, but is effective in protecting the host against incoming larvae. Hagan and colleagues (1993b) suggest that cross reactive antigens released from adult worms and present on the larval stages may explain how a response that originates with the adult worms might be targeted at larvae and that the agent of this type of immunity maybe TNFα. TNFα was singled out as a prime suspect because of its capacity to induce granuloma formation and increase female worm fecundity whilst at the same time being directly toxic to schistosomula.

The results of the current study suggest that cross-reactive antigens affect cell proliferation and the production of IL-4, GM-CSF, IFNγ and TNFα. No partial correlation between IL-5 produced in response to SEA and WWH, controlling for PHA, was observed, possibly because of low levels of IL-5 production overall.

These results confirm that cytokine producing cells can be antigen-specific and have the ability to recognise similar epitopes on different stages of the parasite.

**Potential improvements which could be made to the study design.**

Diagnosis of schistosomiasis was ascertained on the basis of a single urine sample. It was logistically impossible to acquire repeat urine samples on subsequent days. For this reason the intensity of infection data is not as robust as I would wish and may have resulted in some false negative diagnoses. For the purposes of this study however, this is not a serious flaw because the test served to show that the greatest burden of worms was carried by children. The population could be split into juvenile and adult groups, which roughly approximated to infected and uninfected individuals respectively. Since the conclusions of this study are not altered by the consideration of either child versus adult, or infected versus uninfected this confirms the validity of the approach.

Culture supernatants were harvested only after 48 hours. Different cytokines are produced at varying time points during *ex vivo* culture and stimulation, so it is possible that peak production of some of the cytokines occurred earlier or later. This does not invalidate statistically significant differences in levels of cytokine between groups. However, no conclusions should be drawn from the lack of a statistically significant result, or even the low overall production of a cytokine, for this reason.

PBMC were harvested in heterologous, rather than autologous human plasma despite laboratory observations that plasma could markedly effect the cytokine

production of PBMC in culture (see chapter 2). Although autologous plasma would have been a preferred option, it was logistically impracticable. In each analysis however, conclusions have been drawn from comparative and not absolute levels of cytokine. Since the same batch of plasma was used throughout, the overall conclusions should remain unaffected.

Cytokines were detected using a single measurement, based on one well of an ELISA plate. Although duplicate or triplicate measurements would have been preferable, there was insufficient supernatant to do this. However, the well-to-well variability of each ELISA has been reduced to a minimal level where this approach was considered acceptable (chapter 2).

**Figure 3.1. Hypothetical clustering of IL-4 and IFNγ.**

A hypothetical example of clusters of cytokine produced by infected and uninfected individuals, as might be expected if the ratio of IL-4 to IFNγ correlated with infection status.

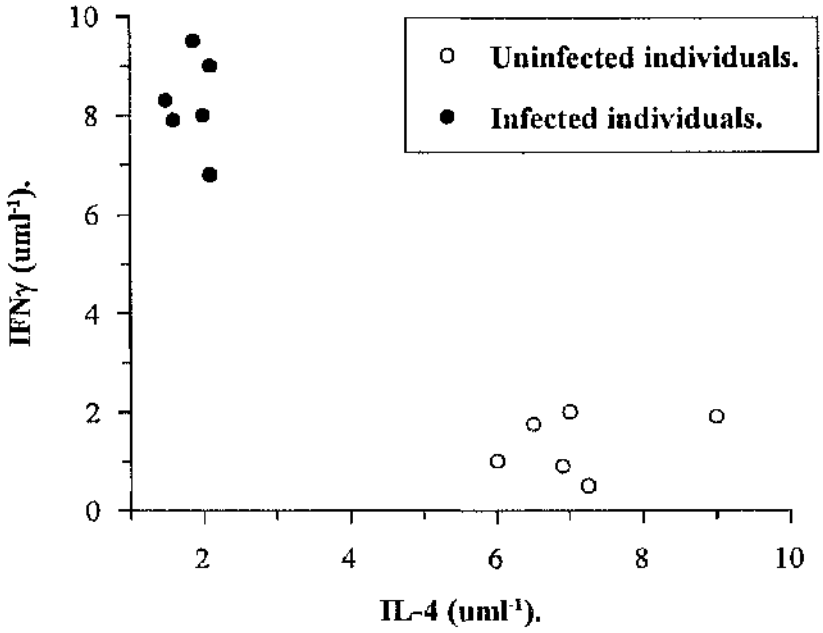


**Table 3.1**

**Summary of net data comparing the cellular responses of PBMC from children with PBMC from adults and comparing the cellular responses of PBMC from uninfected and infected individuals on stimulation with SEA, WWH, PHA or without stimulation.**

A summary of statistical comparisons between people who were infected or uninfected based on examination of filtered urine samples, or children (16 years or less) and adults (over 25 years) for proliferative responses of PBMC and the production of six cytokines by their PBMC. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 1uml-1 and an accuracy of 0.01uml-1, IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturers instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line.

PBMC were separated from blood taken before treatment from Gambian people and cultured at a concentration of 1 x 106 in 0.1 ml aliquots for 5 days for the proliferation assays and in 1ml aliquots for 48 hours for the production of cytokine. Stimulants (all at 10μgml-1 ) were either PHA, SEA or WWH. All comparisons were made using Mann -Whitney U Rank test of net data. Net data are either proliferative responses, or cytokine concentration produced by PBMC cultured with either mitogen or antigen minus the level of proliferation or cytokine produced in PBMC cultured without stimulant. Full statistical details are given in the text where appropriate and in appendix 1.

**Table 3.1**

**Summary of net data comparing the cellular responses of PBMC from children with PBMC from adults and comparing the cellular responses of PBMC from uninfected and infected individuals on stimulation with SEA, WWH, PHA or without stimulation.**

| | Comparison of cellular responses of PBMC from children with PBMC from adults on stimulation with: | | | Comparison of cellular responses of PBMC from infected people with PBMC from uninfected people. | | |
|---|---|---|---|---|---|---|
| **Cellular response.** | **SEA** | **WWH** | **PHA** | **SEA** | **WWH** | **PHA** |
| **PBMC Proliferation** | . | . | . | . | . | . |
| **IL-2** | . | . | . | . | . | . |
| **IL-4 (Genzyme)** | . | . | +++ (a>c) | . | . | +++ (u > i) |
| **IL-4 (as in section 2.3)** | | | | + (u > i) | | |
| **IL-5** | . | . | . | . | . | . |
| **TNFα** | . | . | . | . | . | . |
| **GM-CSF** | . | . | . | . | . | . |
| **IFNγ** | + (c>a) | + (c>a) | + (a>c) | + (i > u) | + (i > u) | . |

. = $p > 0.05$ + = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

c > a = PBMC from children produced more cytokine/proliferated more than PBMC from adults.

a > c = PBMC from adults produced more cytokine/proliferated more than PBMC from children.

i > u = PBMC from infected individuals produced more cytokine/proliferated more than PBMC from uninfected individuals.

u > i = PBMC from uninfected individuals produced more cytokine/proliferated more than PBMC from infected individuals.

**Table 3.2a.**

**Summary of gross data comparing the cellular responses of PBMC from children with PBMC from adults on stimulation with SEA, WWH, PHA or without stimulation.**

A summary of statistical comparisons between children (16 years or less) and adults (over 25 years) for proliferative responses of PBMC and the production of six cytokines by their PBMC. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 1uml-1 and an accuracy of 0.01uml-1. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturers instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line.

PBMC were separated from blood taken before treatment from Gambian people and cultured at a concentration of 1 x 106 in 0.1 ml aliquots for 5 days for the proliferation assays and in 1ml aliquots for 48 hours for the production of cytokine. Stimulants (all at 10μgml-1 ) were either PHA, SEA or WWH. All comparisons were made using Mann -Whitney U Rank test of gross data. Full statistical details are given in the text where appropriate and in appendix 1.

**Table 3.2a.**

**Summary of gross data comparing the cellular responses of PBMC from children with PBMC from adults on stimulation with SEA, WWH, PHA or without stimulation.**

| | **Comparison of cellular responses of PBMC from children with PBMC from adults on stimulation with:** | | | |
|---|---|---|---|---|
| **Cellular response** | **media alone** | **SEA** | **WWH** | **PHA** |
| **PBMC Proliferation** | + (a>c) | + (a>c) | +++ (a>c) | +++ (a>c) |
| **IL-2** | . | . | . | ++ (a>c) |
| **IL-4 (Genzyme)** | . | | . | +++ (a>c) |
| **IL-4 (as in section 2.3)** | | + (c>a) | | ++ (a>c) |
| **IL-5** | . | . | . | . |
| **TNFα** | . | . | . | . |
| **GM-CSF** | . | . | . | . |
| **IFNγ** | . | + (c>a) | ++ (c>a) | + (a>c) |

. = $p > 0.05$ + = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

c > a = PBMC from children produced more cytokine/proliferated more than PBMC from adults.

a > c = PBMC from adults produced more cytokine/proliferated more than PBMC from children.

**Table 3.2b.**

**Summary of gross data comparing the cellular responses of PBMC from infected individuals with PBMC from uninfected individuals on stimulation with SEA, WWH, PHA or without stimulation.**

A summary of statistical comparisons between people who were infected or uninfected on the bases of examination of urine for *S.haematobium* eggs for proliferative responses of PBMC and the production of six cytokines by their PBMC. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 1uml-1 and an accuracy of 0.01uml-1. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturers instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line.

PBMC were separated from blood taken before treatment from Gambian people and cultured at a concentration of 1 x 106 in 0.1 ml aliquots for 5 days for the proliferation assays and in 1ml aliquots for 48 hours for the production of cytokine. Stimulants (all at 10μgml-1 ) were either PHA, SEA or WWH. All comparisons were made using Mann -Whitney U Rank test of gross data. Full statistical details are given in the text where appropriate and in appendix 1.

**Table 3.2b.**

**Summary of gross data comparing the cellular responses of PBMC from infected individuals with PBMC from uninfected individuals on stimulation with SEA, WWH, PHA or without stimulation.**

| | **Comparison of cellular responses of PBMC from infected people with PBMC from uninfected people on stimulation with:** | | | |
|---|---|---|---|---|
| **Cellular response** | **media alone** | **SEA** | **WWH** | **PHA** |
| **PBMC Proliferation** | . | . | + (u > i) | +++ (u > i) |
| **IL-2** | . | . | . | + (u > i) |
| **IL-4 (Genzyme)** | . | . | . | +++ (u > i) |
| **IL-4 (as in section 2.3)** | | | | ++ (u > i) |
| **IL-5** | . | . | . | . |
| **TNFα** | . | . | . | . |
| **GM-CSF** | . | . | . | . |
| **IFNγ** | . | ++ (i > u) | ++ (i > u) | . |

. = $p > 0.05$ + = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

i > u = PBMC from infected individuals produced more cytokine/proliferated more than PBMC from uninfected individuals.

u > i = PBMC from uninfected individuals produced more cytokine/proliferated more than PBMC from infected individuals.

**Figure 3.2. *S.haematobium* intensity of infection in the study group.**

Intensity of infection was determined before treatment in Gambian individuals in the cohort by counting *S.haematobium* eggs in 10ml of urine after filtration through 20μm filters. Intensity of infection was higher in children (under 16 years) than in adults (over 25 years). $p<0.0001$, n=59, 32 children, 27 adults, U=50.0, W=428.0, Z=-6.1912. The statistical significance of this difference was determined using a Mann-Whitney U comparison.

**Figure 3.3. A comparison of peripheral blood eosinophil counts in *S.haematobium* infected and uninfected individuals in The Gambia.**

A comparison of absolute eosinophils counts determined in a Fuchs-Rosenthal cytometer, after dilution of blood in a Phloxin B-based stain. Eosinophil levels were higher in individuals infected with *S.haematobium* compared to uninfected individuals. Infection status was as determined by the presence of *S.haematobium* eggs in urine. The statistical significance of this difference was determined using a Mann-Whitney U comparison. $p<0.003$, n=59 29 uninfected individuals, 30 infected individuals, U=238.5, W=673.5, Z=-2.9794.

**Figure 3.2. *S.haematobium* intensity of infection in the study group**

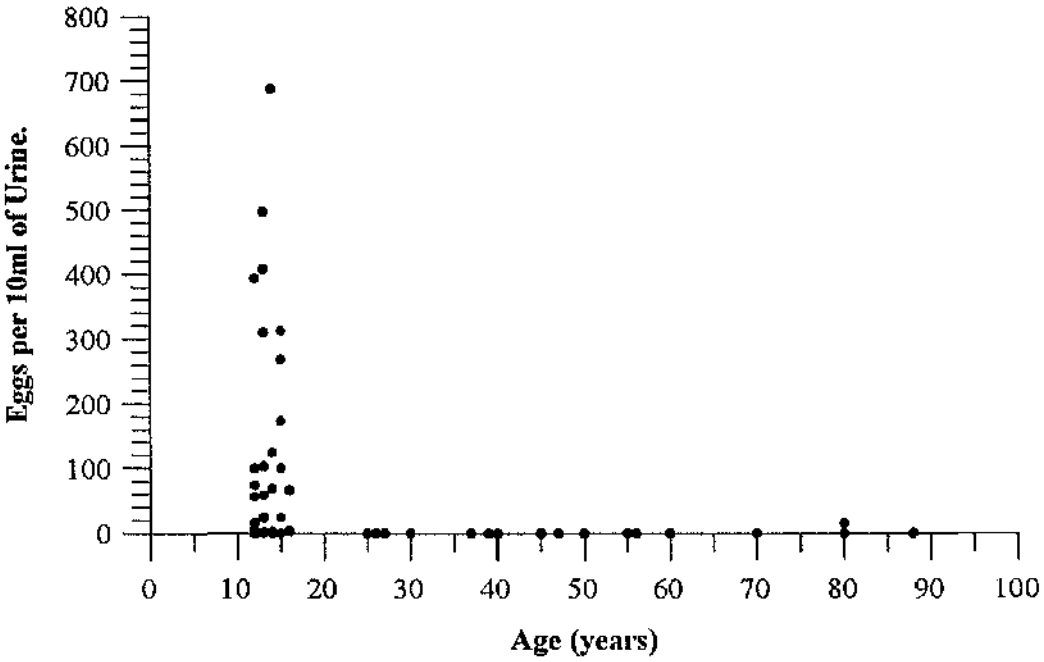



**Figure 3.3. A comparison of peripheral blood eosinophil counts in *S.haematobium* infected and uninfected individuals in The Gambia.**

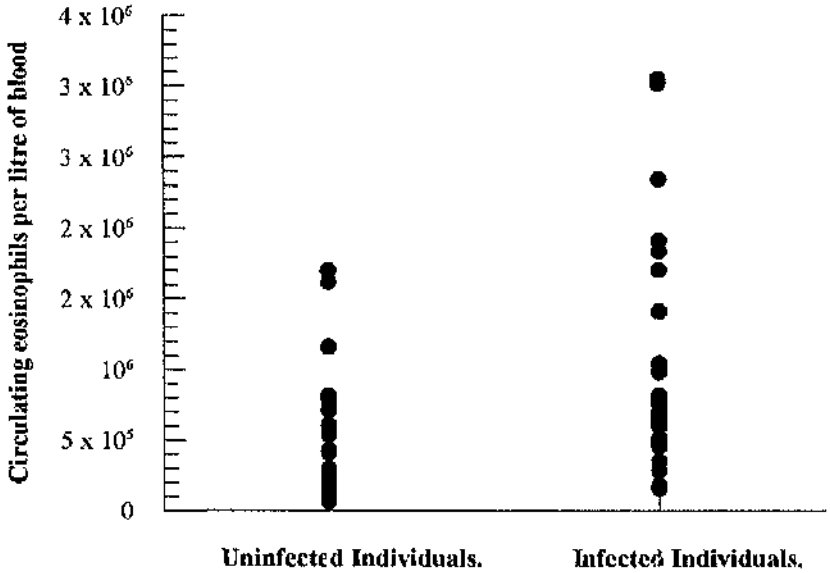


**Figure 3.4. A comparison of IL-4 produced by PBMC incubated with PHA from *S.haematobium* infected and uninfected individuals from The Gambia.**

A comparison of net IL-4 concentration produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x 106 cellsml-1 in 1ml aliquots, for 48 hours in the presence of 10µgml-1 PHA. IL-4 concentration was determined using a Genzyme sandwich ELISA kit. Net data are the amount of cytokine produced by PBMC when cultured with PHA minus levels produced by PBMC cultured without stimulant. Background levels were less than the sensitivity of the assay. Net IL-4 production was higher in individuals uninfected with *S.haematobium* compared to infected individuals. Infection status was determined by the presence of *S.haematobium* eggs in urine. The statistical significance of this difference was determined using a Mann-Whitney U comparison. $p<0.0007$, $n=45$, 23 uninfected individuals, 22 infected individuals, $U=103.0$, $W=356.0$, $Z=-3.4059$.

**Figure 3.4. A comparison of IL-4 produced by PBMC incubated with PHA from *S.haematobium* infected and uninfected individuals from The Gambia.**

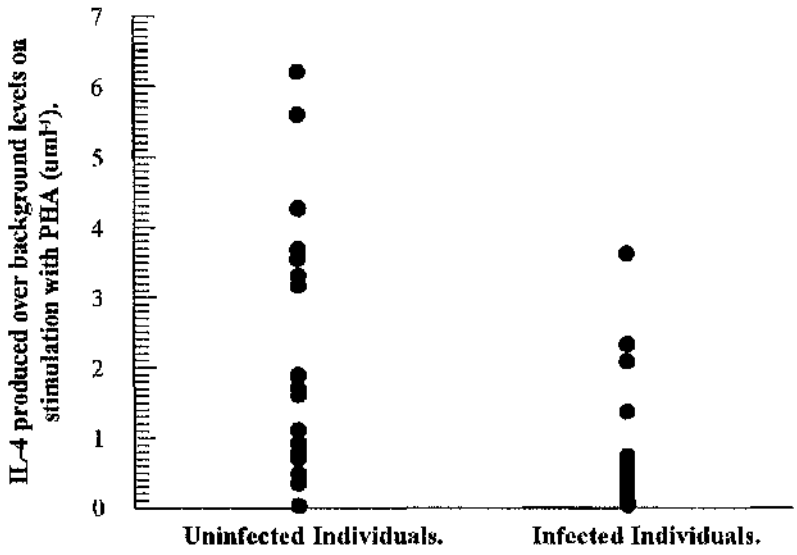


**Figure 3.5. A comparison of IFNγ produced by PBMC incubated with WWH from *S.haematobium* infected and uninfected individuals from The Gambia.**
A comparison of net IFNγ concentration produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x 106 cellsml-1 in 1ml aliquots, for 48 hours in the presence of 10μgml-1 WWH. IFNγ concentration was determined using a Genzyme sandwich ELISA kit. Net data are the amount of cytokine produced by PBMC when cultured with WWH minus levels produced by PBMC cultured without stimulant. Background levels were less than the sensitivity of the assay. Net IFNγ production was higher in individuals infected with *S.haematobium* compared to uninfected individuals. Infection status was determined by the presence of *S.haematobium* eggs in urine. The statistical significance of this difference was determined using a Mann-Whitney U comparison. $p<0.02$, n=45, 23 uninfected individuals, 22 infected individuals, U=154.5, W=604.5, Z=-2.3265.

**Figure 3.6. A comparison of IFNγ produced by PBMC incubated with SEA from *S.haematobium* infected and uninfected individuals from The Gambia.**
A comparison of net IFNγ concentration produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x 106 cellsml-1 in 1ml aliquots, for 48 hours in the presence of 10μgml-1 SEA. IFNγ concentration was determined using a Genzyme sandwich ELISA kit. Net data are the amount of cytokine produced by PBMC when cultured with SEA minus levels produced by PBMC cultured without stimulant. Background levels were less than the sensitivity of the assay. Net IFNγ production was higher in individuals infected with *S.haematobium* compared to uninfected individuals. Infection status was determined by the presence of *S.haematobium* eggs in urine. The statistical significance of this difference was determined using a Mann-Whitney U comparison. $p<0.02$, n=45, 23 uninfected individuals, 22 infected individuals, U=153.0, W=606.0, Z=-2.3953.

**Figure 3.5. A comparison of IFNγ produced by PBMC incubated with WWH from *S.haematobium* infected and uninfected individuals from The Gambia.**

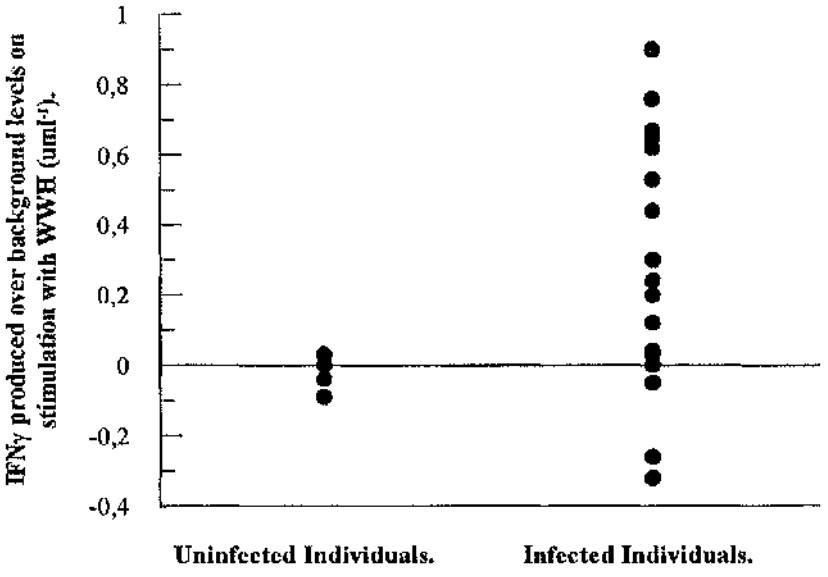



**Figure 3.6. A comparison of IFNγ produced by PBMC incubated with SEA from *S.haematobium* infected and uninfected individuals from The Gambia.**

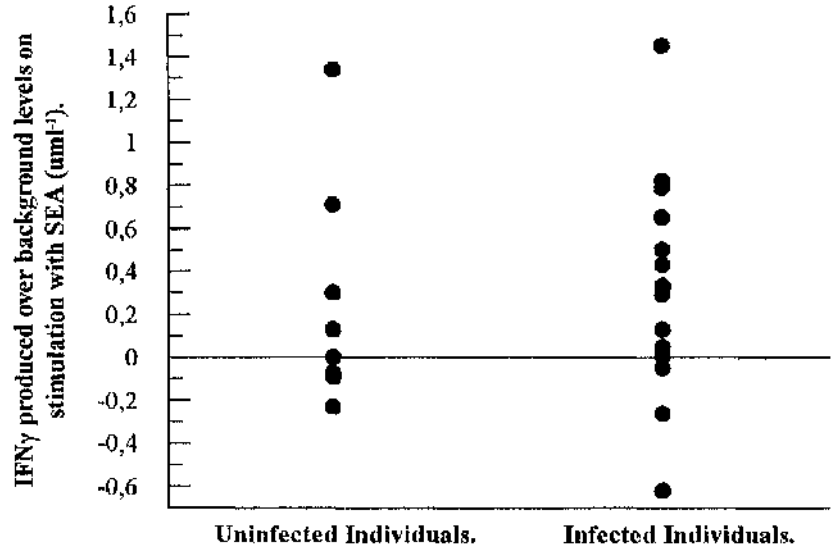


**Figure 3.7a A comparison of levels of IL-4 (measured using a Genzyme Kit ELISA) and IFNγ in *S.haematobium* infected and uninfected individuals from The Gambia on stimulation of PBMC with PHA, SEA or WWH.**

If Th1 and Th2 responses are dichotomous options, one would expect to find relatively low amounts of IFNγ in individuals with relatively high amounts of IL-4, and *vice versa.* It follows that the relative amounts of IL-4 and IFNγ may be responsible for driving an immune response in a particular direction, rather than the absolute amount of either cytokine. If this is the case, it might be expected that ratios between IL-4 and IFNγ might fall into two distinct clusters, hypothetically depicted in figure 3.1, or be negatively correlated. No clustering or negative correlation was observed in cytokine produced with SEA, WWH or PHA.

Net IFNγ produced on culture with either WWH, SEA or PHA was plotted against net IL-4 produced on culture with either WWH, SEA or PHA respectively. Both cytokines were produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x 106 cellsml-1 in 1ml aliquots, for 48 hours in the presence of 10μgml-1 of either WWH, SEA and PHA. IFNγ and IL-4 concentrations were determined using a Genzyme sandwich ELISA kit. Net data are the amount of cytokine produced by PBMC when cultured with SEA minus levels produced by PBMC cultured without stimulant. Infection status was determined by examination of 10ml urine for *S.haematobium* eggs.

**Figure 3.7a. A comparison of levels of IL-4 (measured using a Genzyme ELISA Kit) and IFNγ in *S.haematobium* infected and uninfected individuals from The Gambia on stimulation of PBMC with PHA, SEA or WWH.**

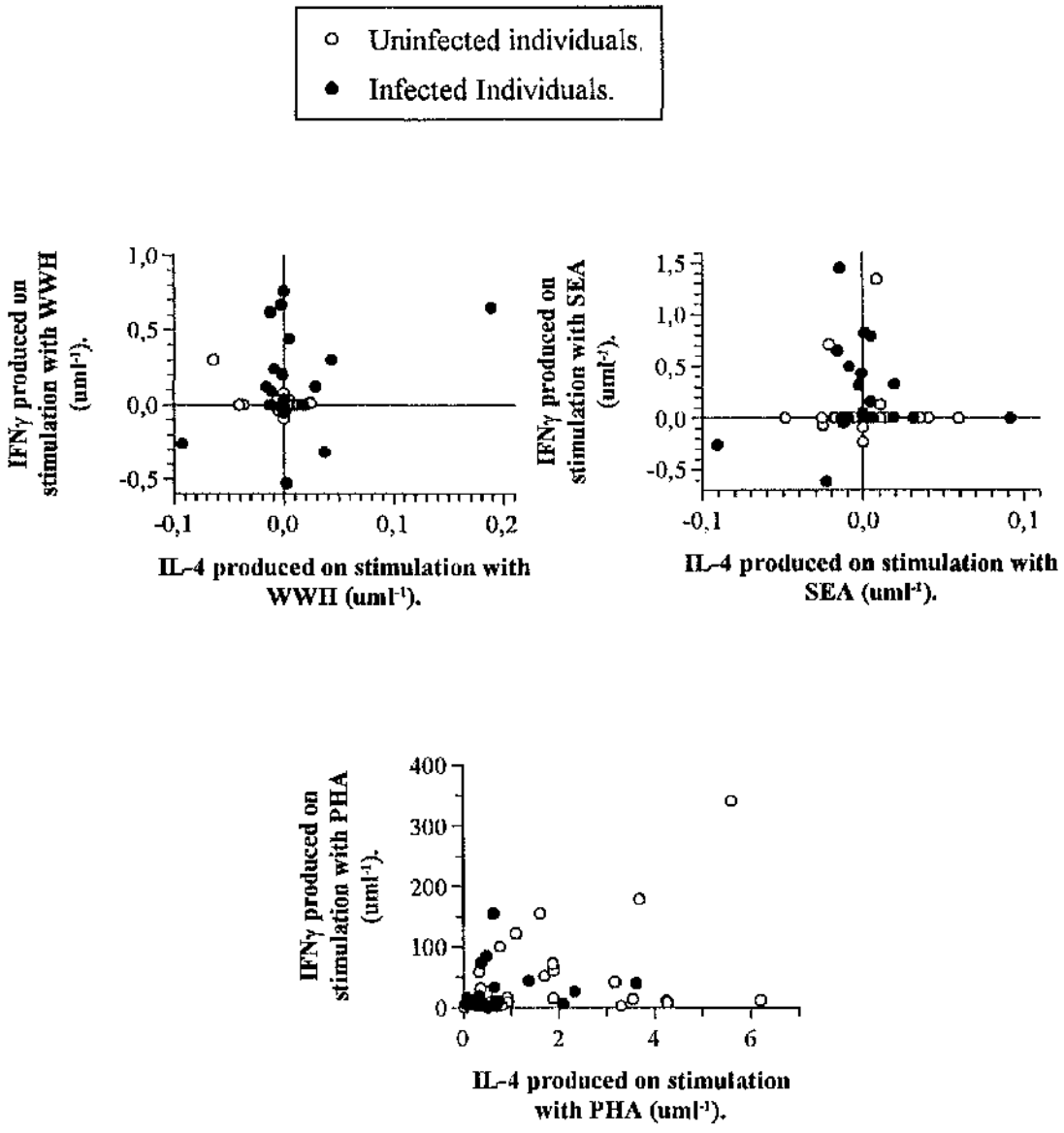


**Figure 3.7b A comparison of levels of IL-4 (measured using a Sandwich ELISA using Pharmingen antibody pairs as described in section 2.3) and IFNγ in *S.haematobium* infected and uninfected individuals from The Gambia on stimulation of PBMC with PHA, SEA or WWH.**

If Th1 and Th2 responses are dichotomous options, one would expect to find relatively low amounts of IFNγ in individuals with relatively high amounts of IL-4, and *vice versa*. It follows that the relative amounts of IL-4 and IFNγ may be responsible for driving an immune response in a particular direction, rather than the absolute amount of either cytokine. If this is the case, it might be expected that ratios between IL-4 and IFNγ might fall into two distinct clusters, hypothetically depicted in figure 3.1, or be negatively correlated. No clustering or negative correlation was observed in cytokine produced with SEA, WWH or PHA.

Net IFNγ produced on culture with either WWH, SEA or PHA was plotted against net IL-4 produced on culture with either WWH, SEA or PHA respectively. Both cytokines were produced by PBMC collected before treatment from Gambian individuals and cultured at a concentration of 1 x 106 cellsml-1 in 1ml aliquots, for 48 hours in the presence of 10μgml-1 of either WWH, SEA and PHA. IFNγ and IL-4 concentrations were determined using a sandwich ELISA using Pharmingen antibodies as described in section 2.3. Net data are the amount of cytokine produced by PBMC when cultured with SEA minus levels produced by PBMC cultured without stimulant. Infection status was determined by examination of 10ml urine for *S.haematobium* eggs.

**Figure 3.7b. A comparison of levels of IL-4 (measured using a Sandwich ELISA using Pharmingen antibody pairs as described in section 2.3) and IFNγ in *S.haematobium* infected and uninfected individuals from The Gambia on stimulation of PBMC with PHA, SEA or WWH.**

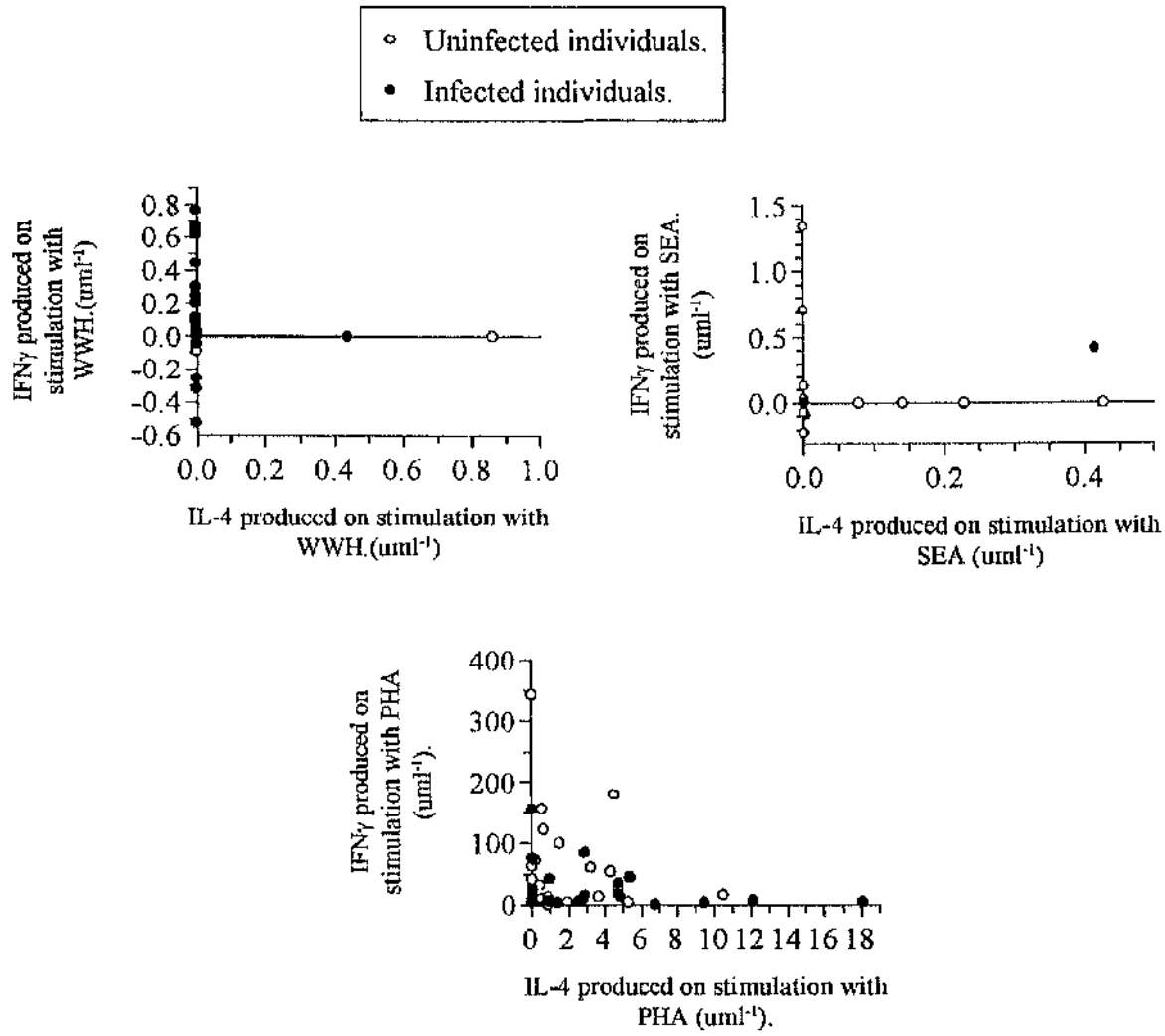
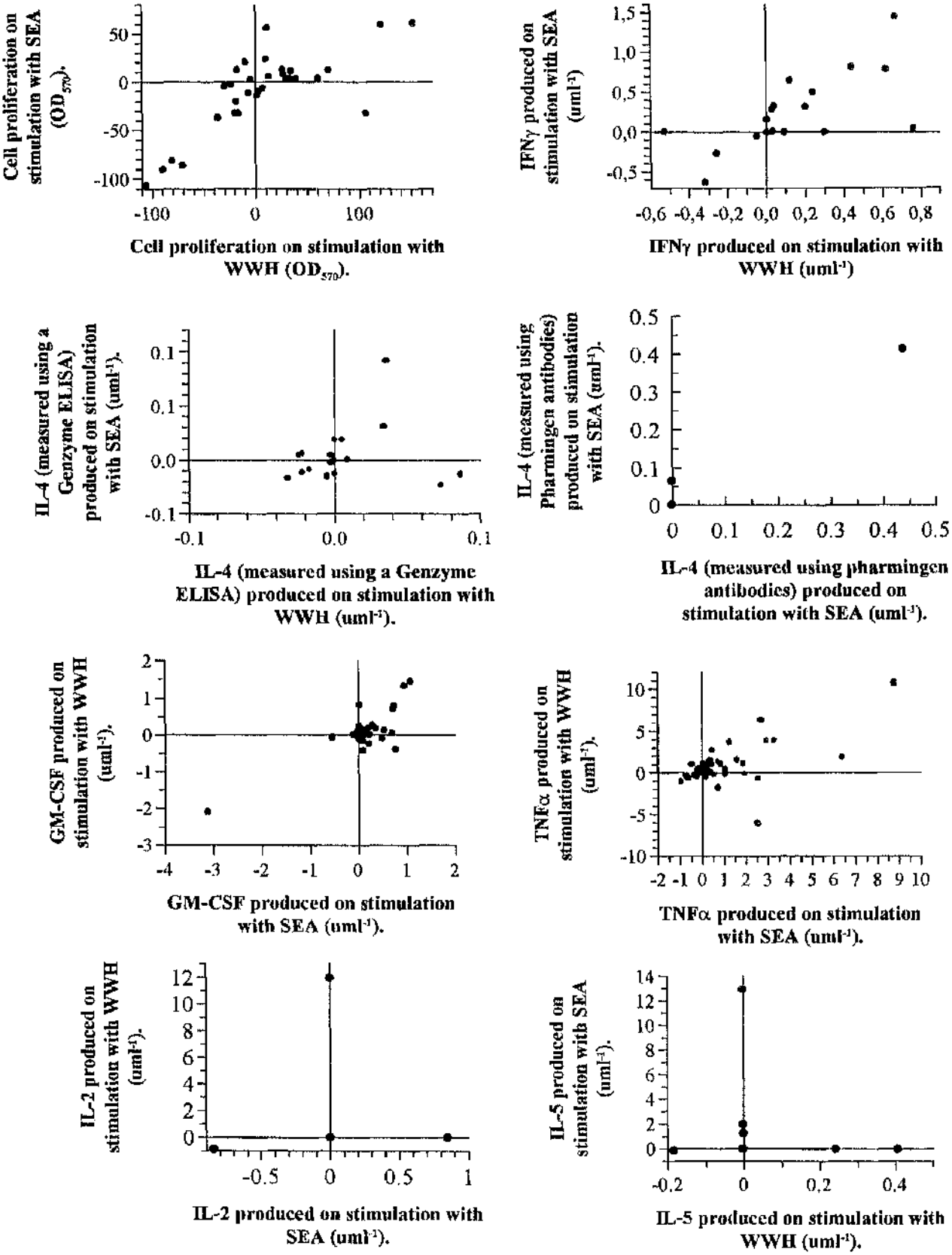


**Figure 3.8. An investigation into the potential effect of cross reactive antigen on cellular immune responses in Gambian individuals.**

Levels of net cell proliferation and net production of IL-4, GM-CSF, IFNγ, TNFα and IL-5 on incubation with 10μgml-1 SEA are plotted against the equivalent levels produced on incubation with 10μgml-1 WWH. These associations were positively correlated using a partial Kendall non-parametric correlation coefficient, controlling for cell proliferation and production of IL-4, GM-CSF, IFNγ and TNFα on incubation with 10μgml-1 PHA. Details of statistical results were: Cell proliferation: N=59, p<0.001, GM-CSF: N=59, p<0.001, IFNγ: N=45, p<0.001, IL-4 (Genzyme ELISA): N=45, p<0.001, TNFα: N=59, p<0.005, IL-2, IL-4 (Pharmingen ELISA) and IL-5 = the majority of samples contained undetectable levels of cytokine.

Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 1uml-1 and an accuracy of 0.01uml-1, IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturers instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line. PBMC were separated from blood taken before treatment and cultured at a concentration of 1 x 106 in 0.1 ml aliquots for 5 days the proliferation assays and 1ml aliquots for 48 hours for the production of cytokine with 10μgml-1 of either PHA, SEA or WWH. Net data are cell proliferation or the amount of cytokine produced by PBMC when cultured with either PHA, SEA or WWH minus cell proliferation or cytokine produced when cultured without stimulant.

**Figure 3.8. An investigation into the potential effect of cross reactive antigen on cellular immune responses in Gambian individuals.**



# Chapter 4.

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## Cytokine Production in response to *Schistosoma haematobium* infection in Zimbabwean children one year post treatment.

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**4.1 Introduction.**

This chapter describes the production of cytokines in whole blood, stimulated *in vitro* with mitogen or schistosome antigens taken from 137 Zimbabwean children who have been resident in one of two areas endemic for *S. haematobium* for one year following chemotherapy. Residents of one area had a high prevalence of infection and residents of the other a low prevalence of infection prior to treatment.

In a previous study in The Gambia (Chapter 3) a confounding difficulty was that, to a large extent, infected people were children and uninfected people were adults, such that the Th1/Th2 dichotomy observed could be explained either by age or infection status. The Gambian study could not therefore address the question of whether age or experience of infection was responsible for the observed changes in immune response.

An age-structured immigration-death model predicted that if protective immunity developed faster in areas of high transmission compared to low transmission (Woolhouse *et al.*, 1991) the age at which the peak of intensity of infection occurred would be younger in areas of high transmission than in areas of lower transmission, a phenomena known as peak shift. Peak shift was observed in a study of seventeen primary schools in the Zimbabwean highveld. This Zimbabwean study was designed to extrapolate from these ideas. It addresses the hypothesis that, given that protection against infection is associated with a Th2-type immune response, a protective type of immune response will develop faster in an area with a high transmission rate compared to an area with a low rate of transmission, if past experience of infection is associated with the acquisition of resistance to infection.

The Gambian study found that IL-4, GM-CSF, IFNγ and TNFα production was affected by cross-reactive antigen. This hypothesis has also been addressed in the Zimbabwean study.

Grogan and colleagues (1996) observed that IL-4 but not IL-5 was up-regulated as a result of treatment for *S.haematobium* infection with Praziquantel. They speculated that this was because IL-4 and IL-5, although both Th2-type cytokines, are separately regulated in schistosomiasis. This hypothesis is consistent with the results of intra-cellular cytokine staining experiments which have shown that IL-4 and IL-5 maybe produced by separate T cell populations in humans (Jung *et al.*, 1995; Sewell *et al.*, 1996). If IL-4 and IL-5 are produced by separate cell populations and only one of these populations is stimulated by schistosome antigen, then in blood from an individual, only one of the cytokines would be detected in a single supernatant. This hypothesis was also addressed.

**4.2 Methods**

One hundred and thirty seven children were recruited from two areas, approximately 10km apart in the Burma Valley in the Eastern Highlands of Zimbabwe in August 1994. All the children were permanent residents of the area. Their ages ranged from 6 to 15 years with age not reported in three cases. The main industry in the Burma Valley is fruit plantations. Families tend to live in communities of farm workers, associated with one of the several large commercial farms. *S.haematobium* is endemic throughout the valley, but communities differ in terms of the quality of sanitation provided and the accessibility of piped water. 70 children, 30 girls and 40 boys, attending Valhalla School, were resident in an area

with a high prevalence of *S.haematobium* infection. The residents of the high prevalence area used a river, which was not seasonal, for domestic purposes and had only limited access to piped water and Blair toilets. Blair toilets are pit latrines, commonly used in Zimbabwe, designed by The Blair Research Institute as low cost sanitary provision. In the high prevalence area, the water contact sites were heavily used by residents, compared with usage of water contact sites in the other area, as evidenced by analysis of returns of a questionnaire concerning water contact usage (Woolhouse, Hagan and colleagues, unpublished data).

Sixty-seven children attending Kaswa School, 32 girls and 35 boys were resident in an area with a low prevalence of *S.haematobium* infection. These children were resident in a village with better sanitary infrastructure when compared to the high prevalence area. Use of the river was limited and the river itself was seasonal. They had better access to piped water and Blair toilets compared to children resident in the high prevalence area.

In both areas *Ascaris spp.* eggs were observed in kato preparations from some stool samples, although prevalence of hookworm was low, probably due to the recent hookworm control program in the area. The area was selected because it had a low prevalence of *S.mansoni* infection. None of the children in the cohort were positive for *S.mansoni.* Prevalence of HIV infection in Zimbabwe is generally high, however HIV positive people tend to be either below school age or adult, so the children in the cohort were unlikely to be HIV positive.

Intensity of infection was determined for each child by counting *S.haematobium* eggs in 10ml of urine after filtration through 25mm diameter polycarbonate filters. Eggs were stained with Lugol's iodine (as described in section 3.2) to aid visibility. Prevalence of infection was measured in the cohort in November 1994, February, March, May and August 1995. Each child was successfully drug treated in November 1994 with 40mgkg$^{-1}$ Praziquantel and gave at least two urine samples in August 1995.

In November 1995 30ml blood samples were taken from each child. Ten millilitres was used for studies of cytokine production and 20ml for other studies. Blood was transported overnight at ambient temperature from the study site to Harare where it was diluted in 50% RPMI and incubated in a humidified environment at 37°C and 5% $CO_2$ in the presence of 10μgml$^{-1}$ of either the mitogen phytohaemagglutinin (PHA), antigen: schistosome egg antigen (SEA), whole adult worm homogenate (WWH), cercarial homogenate (CERC) or incubated without stimulant. 24, 48 or 72 hours post incubation, culture supernatants were harvested and stored at -20°C for future use.

Antigens were purchased in freeze dried form from the Schistosome Biological Supply Programme, Theodore Bilharz Research Institute, Giza, Egypt (section 2.5.2) IL-4, IL-5, IL-10, GM-CSF, IFNγ and TNFα were detected in culture supernatants using sandwich ELISAs (chapter 2). Because limited volumes of blood and antigen were available, not all samples provided supernatants at all three time points.

**4.3 Statistical treatment of results.**

Data were analysed using SPSS for Windows version 6. Net levels of cytokine have been used throughout. Net cytokine is cytokine produced as a result of culture in the presence of a stimulant such as antigen or mitogen minus cytokine produced with no stimulation. Six statistical tests have been used: Wilcoxon signed ranks test, logistic regression analyses, multiple linear regression, Mann Whitney U, $\chi^2$ and Kendall partial correlation.

Differences between cytokine produced at 24, 48 or 72 hours post incubation were tested using a Wilcoxon signed ranks test, because the data sets were not normally distributed (figure 4.1a) and could not be successfully transformed (figure 4.1b) (Siegel *et al.*, 1988b). There are more than two groups, so a Friedman two-way analysis of variance by ranks could be argued to be the more appropriate test (Siegel *et al.*, 1988a). However, the Friedman test could only be carried out on data that were available for all three time points from each individual. This reduced the sample size considerably. Instead the difference between 24 hours and 48 hours, 24 hours and 72 hours and 48 and 72 hours were tested separately. In each case the result was accepted if the total sample size was greater than 10. In order to correct for the multiple tests, the level of significance was set using a sequential Bonferroni technique (Rice, 1989).

A continuous dependent variable can be regressed linearly against a number of either continuous, categorical or ordinal variables provided that the residuals of the model are normally distributed. The dependent variable of a logistic regression must be binary, for example male and female, or cytokine producer compared to non-

cytokine producer, but independent variables can be continuous or categorical. For both tests, the sample size must be at least five times the number of independent variables.

Logistic regression analysis was used as the primary means of analysing cytokine data because models of most cytokine-dependent variables did not conform to the assumptions of linear regression. An individual was classified either as net cytokine producer (blood produces >0 $uml^{-1}$ of cytokine over background levels) or net cytokine non-producer (blood produces ≤0 $uml^{-1}$ of cytokine over background levels) (figure 4.2a). Multiple logistic and linear regression had the advantage over Mann-Whitney-U in that a number of factors could be considered at the same time. This allowed specific hypotheses to be addressed whilst controlling for potentially confounding factors. For example it was possible to address the hypothesis that area of residence explained a significant proportion of variation in cytokine production controlling for age, sex, pretreatment infection status and post-treatment infection status.

The logistic regression analysis has the disadvantage that any difference in the amount of cytokine produced was not taken into account, and thus the full information content of the data set is not exploited. There was also a particular problem in the case of PHA stimulated production of all cytokines, with the exception of IL-4, because blood from almost every child produced cytokines over the level in non-stimulated blood (figure 4.2b). However, it was possible in these instances to utilise a multiple linear regression because the residuals of the model were normally distributed.

For both linear or logistic regression the forward stepwise approach to model fitting was used to ascertain which independent variable or variables best explained the variation in cytokine data. Five categorical independent variables: area, sex, age, pretreatment infection status and infection status eight months after treatment were used to attempt to explain the variation in cytokine data. Age was split into ten one year intervals, although there was no difference to the conclusions of the analysis if two five year intervals were used instead (results not shown). A minimum sample size of 25 was considered appropriate because five independent variables were being considered. Cytokine data were first regressed against the independent variable which explained variation in cytokine data with the highest level of significance. If none of the independent variables explained cytokine variation to a significance level of $p<0.05$, none were entered into the model. Subsequent variables had to explain the remaining variation in the cytokine data to a significance level of $p<0.05$ to be included in the model.

The explanatory independent variable was then entered into the model after all other independent variables. If it could still significantly explain variation in cytokine data, it was accepted. For example, this method made it possible to test the hypothesis that children from one area produced more cytokine than children from the other area, controlling for the potentially confounding factors of sex, age, pretreatment infection status and infection status eight months after treatment.

To test the hypothesis that cytokine production is influenced by cross-reactive antigen, cytokine levels were compared in blood incubated for a defined duration with either of three antigens or with PHA. Cytokine levels produced on stimulation

with SEA were partially correlated against levels produced on stimulation with WWH, controlling for cytokine produced on stimulation with PHA. A Kendall partial correlation coefficient was used to analyse these data because the majority of cytokine data were not normally distributed (figure 4.1a). and could not be successfully be transformed (figure 4.2b). A minimum sample size of 10 was accepted for a valid comparison. Where a large proportion of the children produced no net cytokine in response to one or both antigens, a significant positive partial correlation between the two antigens could be the result of one or two outlying data points. For this reason, a correlation was only accepted as supporting the hypothesis if a simple majority samples produced detectable levels of cytokine over background for both antigens. It was not possible to control for age, sex, pretreatment or post treatment infection status using this type of analysis. However, for reasons explained in section 4.4, the major confounding factor was likely to be the area in which the child was resident. This method of analysis was used separately for data from each area.

For each of the above methods, non-statistically significant results have not been discussed because they do not prove that there is not a relationship between the variables considered, only that a relationship could not be proved with this data set and analysis, i.e. to avoid type II errors. For each combination of cytokine and antigen, there were data resulting from the three time points. This effectively allowed three opportunities to obtain a significant result, so the significance values were corrected to account for multiple tests using a sequential Bonferroni technique (Rice, 1989). Significance values have been quoted after correction for multiple tests.

A $\chi^2$ test was used to consider the hypothesis that children produced either IL-4 or IL-5 but not both, on stimulation with the same antigen or mitogen and incubated for the same length of time. A minimum sample size of 10 was considered adequate. Children were categorised as producing either IL-4 or IL-5 or both. Children who produced undetectable amounts of both cytokines were not included. This quantitative method treated the data as categorical, rather than as continuous variables and so had some imperfections: for example, a child whose blood produced a lot of IL-4 and very little IL-5 was categorised with a child whose blood produced a lot of IL-5 and very little IL-4, whereas it was evident from scatter graphs that they do not necessarily belong in the same category. This method could thus result in false negative conclusions on the basis of the $\chi^2$ test alone. A scatter graph has therefore been presented for each data set with a sample size of greater than ten whether the difference between the number of children whose blood produced both IL-4 and IL-5, compared to the number of children whose blood produced either one or the other cytokine was significant or non-significant according to the $\chi^2$ test, because a qualitative approach was considered a useful adjunct to the quantitative approach.

## 4.4 Results.

### 4.4.1 Prevalence of *S.haematobium* Infection.

Prevalence of infection was higher in children attending Valhalla School than Kaswa School, before treatment[1] and 8 month's post-treatment[2] after controlling for the effects of age and sex in a logistic regression analysis (figure 4.3 a and b).

[1]($p<0.0001$, B= -0.855, S.E.=0.2032, Wald=17.7005, R=-02936, Exp (B)=0.4253)

Prevalence of infection was not found to be significantly greater in young children (6-10 years) than in older children (11-15 years) before treatment or for three or four months following treatment. By the third follow up survey in May 1995, six months after treatment, prevalence of infection amongst younger children was significantly higher than in older children[3]. Nine months after treatment in August 1995, the younger children still had a higher prevalence and intensity of infection than older children[4] (figure 4.3a and b). These significant differences were measured by logistic regression analyses after controlling for the effects of area and sex. Intensity of infection followed a similar pattern to prevalence of infection (data not shown).

25.9% of children who were infected before treatment had been re-infected by eight months post treatment compared to 16.0% of the children who were uninfected before treatment. This difference was statistically significant[5] when tested with a $\chi^2$, but when area, sex and age had been taken into account by means of a logistic regression analysis, pretreatment infection was no longer a significant explanatory variable of post-treatment infection status.

### 4.4.2 Cytokine production over time.

Statistical analysis of cytokine production over time is summarised in tables 4.1 and 4.2. On stimulation with either SEA, WWH or CERC, there was no significant difference between IL-4 (figure 4.4a) or IL-10 (figure 4.4c) produced at any of the

[2] ($p<0.032$ B= -0.5038, S.E.=0.2351, Wald=4.5896, R= -0.1192, Exp (B)=0.6043)
[3] ($p<0.05$, B = 1.0689, S.E. = 0.5331, Wald = 4.0207, R = 0.1103, Exp (B) = 2.9121)
[4] ($p<0.023$, B = 0.6162 S.E. = 0.271, Wald = 5.1
697, R = 0.1336, Exp (B) = 1.8519)
[5] ($p<0.03$, $\chi 2 = 4.8$, d.f. = 1, N=137)

time points. Production of IL-5 on stimulation with antigen (figure 4.4b) appeared to increase gradually with time, although the differences were only significant when IL-5 produced 24 hours post incubation was compared with IL-5 produced after incubation for 72 hours. IFNγ production (figure 4.4d) did increase with time: more was produced after 72 hours compared to 48 hours, and more was produced at 48 hours compared to 24 hours. GM-CSF production peaked after 48 hours of incubation and TNFα production had peaked by 24 hours of incubation.

On incubation with PHA, there was no significant difference in production of IL-5 (figure 4.5b) or IL-10 (figure 4.5c ) between different time points, TNFα production (figure 4.5f) peaked after 24 hours of incubation, more GM-CSF was produced at 48 hours post incubation compared to the 24 hour time point (figure 4.5e), and IL-4 (figure 4.5a) and IFNγ (figure 4.5d) production peaked at 72 hours post-incubation.

**4.4.3 Cytokine produced in response to stimulation with mitogen or antigen.**

I have attempted to identify factors that could explain the difference observed between children in their levels of cytokine production when blood was stimulated with the mitogen, PHA, or any of the antigens: SEA, WWH or CERC. To do this, I analysed data for all six cytokines, at all the time points and for each stimulant using either multiple logistic or linear regression methods as appropriate. The criteria for the use of each method is discussed in section 4.3. In each analysis, the variables investigated were age, area (either low or high prevalence area), sex, pretreatment infection status and infection status eight months after treatment.

It was possible to use the more powerful method of analysis, multiple linear regression for only two data sets. These sets were for IFNγ and GM-CSF production, 24 hours after stimulation with PHA. As explained previously, to avoid type II errors, I will consider and discuss only those sets of data where a statistically significant explanatory variable (after correction for multiple tests) was identified. All significant results are summarised in Table 4.3.

**4.4.3.1 Cytokines produced on incubation with PHA.**

Area was a significant explanatory variable of IL-4 production on stimulation with PHA 24 hours post incubation after controlling for age, sex, pretreatment infection and infection eight months after treatment. Blood from 72% of children from the low prevalence area produced IL-4 after incubation for 24 hours with PHA, compared to blood from 13% of children from the high prevalence area (figure 4.6).

Blood from children resident in the low prevalence area produced more IFNγ on stimulation with PHA 24 hours post incubation than blood from children in the high prevalence area. Area explained 20% of the variation in IFNγ produced after incubation with PHA for 24 hours after controlling for age, sex pretreatment infection and infection eight months after treatment (figure 4.7). The five independent variables were regressed against IFNγ on data transformed using the equation [ln(data +25)] to normalise the residuals of the model.

Blood from children resident in the low prevalence area produced more GM-CSF produced on stimulation with PHA 24 hours post incubation than children in the high prevalence area. Area explained 26% of the variation in GM-CSF produced

after incubation with PHA for 24 hours after controlling for confounding variables (figure 4.8).

After controlling for age, sex pretreatment infection and infection eight months after treatment pre-treatment infection was a significant explanatory variable of IL-5 production on stimulation with PHA 24 hours post incubation (figure 4.9). Blood from 75% of children who were infected before treatment produced IL-5 on incubation with PHA for 24 hours compared to 40% of blood from children who were not infected.

**4.4.3.2 Cytokine produced in response to antigen.**

Pre-treatment infection was a significant explanatory variable of IL-4 production on stimulation with SEA, produced 24 hours post incubation after controlling for age, sex, pretreatment infection and infection eight months after treatment (figure 4.10). Blood from 42% of children who were uninfected before treatment produced IL-4 on incubation with SEA compared to 8.1% of blood from children who were infected before treatment.

After controlling for age, sex, pretreatment infection status and infection eight months after treatment, area was a significant explanatory variable of IL-10 production on stimulation with SEA produced after 48 hours of culture (figure 4.11). Blood from 36.3% of children resident in the low prevalence area produced IL-10 on stimulation with SEA compared to blood from 83.9% of children resident in the high prevalence area.

Area was a significant explanatory variable of GM-CSF production produced after incubation for 48 hours with SEA after controlling for age, sex, pretreatment infection status and infection eight months after treatment (figure 4.12). Blood from 25% of children in the low prevalence area produced GM-CSF when cultured with SEA, compared with blood from 69% of children from the high prevalence area.

Post treatment infection was an explanatory factor for IFNγ produced 48 hours post-incubation with WWH after controlling for age, sex pretreatment infection and infection eight months after treatment (figure 4.13). Blood from 57% of uninfected children produced IFNγ compared to blood from 23% of infected children.

**4.4.5 Is cytokine production influenced by cross reactive antigen?**

Evidence of cross reactive antigen between SEA and WWH was observed for IL-5 (figure 4.14) in the low prevalence area, and for IFNγ (figure 4.15), IL-5 (figure 4.16) and TNFα (figure 4.17) in the high prevalence area. Cytokine produced on stimulation with WWH by blood taken from children resident in the same prevalence area correlated positively with cytokine produced on stimulation with SEA, controlling for that produced on stimulation with PHA, for the following results: IL-5 produced at 72 hours post-incubation by blood from children resident in the low prevalence area, IFNγ produced at 48 hours post-incubation, IL-5 measured in supernatants after 48 hours of incubation, and TNFα produced at 24 and 48 hours of incubation by blood from children resident in the high prevalence area. These results are in agreement with results obtained in the Gambian study (section 3.4.5) and supports the hypothesis that cross reactive epitopes in the

antigen from the two life cycle stages have some effect on cytokine production. The statistical significance levels are summarised in table 4.4.

**4.4.6 Children either produce IL-4 or IL-5 but not both.**

Blood from more children produced either IL-4 or IL-5 than produced both cytokines on stimulation with SEA, WWH and PHA 24 hours post incubation (table 4.5, figure 4.18). Blood from more children produced either IL-4 or IL-5 but not both cytokines on stimulation with CERC, SEA and WWH 48 hours post incubation. There was no significant difference in the number of children whose blood produced either IL-4 or IL-5 compared to children whose blood produced both cytokines on stimulation with PHA 48 hours post incubation (table 4.6), although the scatter graph indicates that the majority of children produced predominantly one or the other cytokine (figure 4.19). There was no significant difference in the number of children whose blood produced either IL-4 or IL-5 compared to children whose blood produced both cytokines on stimulation with WWH and PHA 72 hours post incubation (figure 4.20, table 4.7).

## 4.5 Discussion.

### Prevalence of Infection.

The higher prevalence of infection amongst children attending Valhalla School compared to children attending Kaswa School was assumed to be due to difference in transmission dynamics in the two areas. However, with the absence of data regarding contact with infected water, it is also formally possible that it reflected in addition, differences in the level of immunity to *S.haematobium* between the two groups of children.

The observed differences in prevalence of re-infection between the younger (6-10 years) and older (11-15 years) children may be because the older children were either less exposed to infection, or have developed a partial immunity to infection. There are three possible hypotheses that could explain the effect that partial immunity might have had.

First, older children may have developed a level of immunity before treatment that allowed them to maintain a lower prevalence of infection than younger children. The worm burdens of older children before treatment would then reflect infections sustained earlier in life. A second possibility, is that partial immunity may have allowed older children to slow the build up of infection after treatment although eventually prevalence of infection would reach the same levels as their younger colleagues. This would explain why there was no difference between prevalence of infection in the two age groups before treatment because both groups had had enough time to gain maximal worm burdens. Thus the prevalence of infection before treatment would reflect maximum levels sustainable at each of the two sites

given their differing transmission dynamics. Alternatively, praziquantel used in treating the children or their subsequent exposure to antigen resulting from the death of the adult worms, may have stimulated the immune system of older children allowing them to successfully defend themselves against further infection in a way unavailable to the possibly less mature and/or less experienced immune systems of the younger children.

In this case, it might be expected that a combination of age and infection before treatment could explain a significant amount of post treatment infection. However, there is no evidence from this study to suggest that this is the case since pretreatment infection status does not explain any more of the post treatment infection than age, sex and area without a consideration of pretreatment infection.

**Cytokine production in response to antigen and mitogen.**

Blood from children resident in the low prevalence area produced more IL-4, IFNγ and GM-CSF than blood from children resident in the high prevalence area. These results, which are more significant than other comparisons, could imply that residence in the low prevalence area resulted in a stronger Th0-type response than residence in the high prevalence area, or that the more frequent challenge faced by children resident in the high prevalence area resulted in a reduction of their capacity to produce cytokines generally. Alternatively, since these differences in cytokine production were found in PHA stimulated samples, cytokine production may reflect something other than *S.haematobium* infection. For example, the immune systems of children resident in the low prevalence area may have a greater overall "fitness" than children resident in the high prevalence area, allowing their cytokine-producing

cells to have a larger capacity to produce any cytokine than their colleagues in the high intensity area.

IL-5 produced on stimulation with PHA was associated with being infected before treatment whereas, IL-4 produced on stimulation with SEA was associated with a lack of infection before treatment. This could imply that a capacity to produce IL-5 (a Th2 type cytokine) was associated with a susceptibility to infection and IL-4 (also a Th2 type cytokine) production, on stimulation with SEA was associated with an immunity to infection. Alternatively, treatment of an infection with praziquantel may effectively result in vaccination by means of the sudden release of antigen; children who were infected before treatment could have been 'vaccinated' whereas children who were uninfected would not have had their immune reactivity boosted in this way. The production of IL-5 by children who were infected before treatment, and the production of IL-4 by those uninfected before treatment might have been as a result of this 'vaccination'. The relevance of this IL-4 and IL-5 data will be discussed in more detail in the next section.

IL-10 and GM-CSF produced on stimulation with SEA was associated with residence in the high prevalence area. IL-10 and GM-CSF are both produced by macrophages (de Waal Malefyt *et al.*, 1991). IL-10 is classically considered to be a Th2-type cytokine although the cellular source of IL-10 is not as clear from experiments on human cells as it is for murine cell lines (Mosmann, 1994). GM-CSF is expressed by a number of cell types including Th1 and Th2 cells (Rasko *et al.*, 1994). Therefore macrophages or Th2 cells are likely to be responsible for more

of the cytokine production in the high prevalence area than they are in the low prevalence area.

IFNγ produced on stimulation with WWH was associated with a lack of infection after treatment. This might suggest that a Th1 type response was associated with immunity to infection after treatment. Araujo and colleagues (1994) suggested that IFNγ production was depressed in people exposed to *S.mansoni*. This result might be considered supporting evidence of this hypothesis, however this is the least statistically robust of the cytokine production results. The population of uninfected children, post treatment, could represent a much more heterogeneous group than uninfected children pre-treatment, in terms of the state of the development of their immune responses because very low rainfall as well as any degree of immunity, may have been a significant contributing factor to the low prevalence of infection eight months post treatment (see figure 4.12).

The cytokine profiles in both the high and low prevalence areas appear to be sending mixed signals, possibly as part of their regulatory processes. IL-10 is an inhibitor of macrophage cytokine synthesis, nitric oxide production and reduces their ability to present antigen to Th1 cells (Mosmann, 1994). This would appear to be antagonistic to the effects of macrophage colony stimulating factor. A mechanism could be envisaged whereby in the low prevalence area IL-4 enhances CD23, a low affinity receptor of IgE-$Fc\varepsilon R_{II}$, on B cells (Defrance *et al.*, 1987b; Hivroz *et al.*, 1989), whereas IFNγ blocks this IL-4 dependent increase of CD23 (Banchereau *et al.*, 1991). IgE has been shown to be a strong feature of an effective

immune response to schistosomiasis in humans (Hagan *et al.,* 1991; Rihet *et al.,* 1991; Dunne *et al.,* 1992).

Considering all of the cytokine results together, it would appear that area is the dominant factor in determining cytokine profile. I would postulate that the cytokine production is of a Th0 type in the low prevalence area, compared to the high prevalence area, whereas in the high prevalence area, a pattern that could be described as either macrophage or Th2 type predominates. This would explain the distribution of GM-CSF and IL-10 produced on stimulation with SEA and IL-4, GM-CSF and IFNγ produced on stimulation with PHA.

Studies in a community recently exposed to *S.mansoni* by Gryseels & colleagues (Gryseels, 1994a; Gryseels *et al.,* 1994b; van Dam *et al.,* 1996) have suggested that IgE-mediated immunity is an age-dependent, rather than an experience of infection-dependent phenomena. The current study would suggest that experience of infection does have an affect on the immune responses. In the absence of an adult immune cohort it was difficult to determine a cytokine profile that was definitively associated with resistance to infection. However previous studies would suggest that resistance to infection is mediated by Th2-type cytokines (Hagan *et al.,* 1985; Hagan *et al.,* 1991; Rihet *et al.,* 1991; Dunne *et al.,* 1992; Roberts *et al.,* 1993). If this is an accurate picture, I would like to speculate that the greater production of Th2-type cytokines by children resident in the high prevalence area suggests that their immune responses to schistosomiasis are developing towards a protective type at a faster rate than children from the low prevalence area, although IL-4 production is not significantly different. This does not preclude the possibility that

physiological changes that occur with age might also be of importance in the development of an effective immune response.

**A dichotomous relationship between IL-4 and IL-5**

Analysis of IL-4 and IL-5 production suggests a possible subdivision of the Th2 subset based on IL-4 and IL-5 production in both humans (Jung *et al.*, 1995) and mice (Bucy *et al.*, 1995). If Th2 cells are of two subtypes, one that produces IL-5 but not IL-4 and the other, a IL-4 producer, but not an IL-5 producer then this might imply that treatment of infected children with praziquantel resulted in the dominance of a IL-5+/IL-4- Th2 cell subtype whereas in children who were uninfected before treatment the IL-5-/IL-4+ Th2 cell subset was dominant.

Grogan and colleagues (1996) reported that IL-5 secretion by peripheral blood mononuclear cells taken from infected individuals, increased five weeks post treatment compared to pretreatment levels. They attributed this change to either the lifting of an adult worm-induced Th2 suppression or the stimulation of IL-4+ Th2 cells with antigen released by virtue of praziquantel treatment. Evidence from the current study would support the latter hypothesis. They did not report any changes in IL-4 levels after treatment, which they suggest indicates a lack of linkage between IL-4 and IL-5, implying that the two cytokines are produced by distinct cell sub-types. However, this conclusion could be considered a type II error, since it is based upon a result with a non-significant difference between two groups.

The dichotomy between IL-4 and IL-5 production, 24 and 48 hours post incubation, lends further support to the distinctive nature of IL-4 and IL-5

production. However at 72 hours post incubation blood began to produce both cytokines. Possibly there are two population of cells as suggested by the 24 hour and 48 hour data. First one or the other is stimulated, but by 72 hours the other population comes into play. Alternatively, maybe in an individual a single cell population starts by producing only IL-4 or only IL-5 and then later starts to produce the other cytokine. Jung and colleagues (1995) found that T-cell clones and freshly isolated human T-cells subjected to multiple stimulation and culture for fourteen days were capable of producing both IL-4 and IL-5, supporting the latter hypothesis.

**Cytokine production was influenced by cross reactive antigen.**

There was a correlation between cytokine produced on stimulation with SEA and WWH, controlling for PHA, for IL-5 in the low prevalence area, and in the high prevalence area, for IFNγ, IL-5, and TNFα. This suggests that cross reactive epitopes influence cytokine production in these cases as previously discussed in section 3.6.

**Figure 4.1a. An example of a non-normally distributed frequency distribution of cytokine production.**

A frequency distribution of net IL-4 produced by whole blood taken from Zimbabwean children 12 months after treatment and cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots, for 72 hours in the presence of $10\mu gml^{-1}$ PHA. IL-4 concentration was determined using a sandwich ELISA as described in section 2.3. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant.

**Figure 4.1b. An example of a frequency distribution of log +1 transformed data of cytokine production, which is also non-normally distributed.**

A frequency distribution of net IL-4 produced by whole blood taken from Zimbabwean children 12 months after treatment and cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots, for 72 hours in the presence of $10\mu gml^{-1}$ PHA. IL-4 concentration was determined using a sandwich ELISA as described in section 2.3. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. The data were transformed using the function log +1.

**Figure 4.1a. An example of a non-normally distributed frequency distribution of cytokine production.**

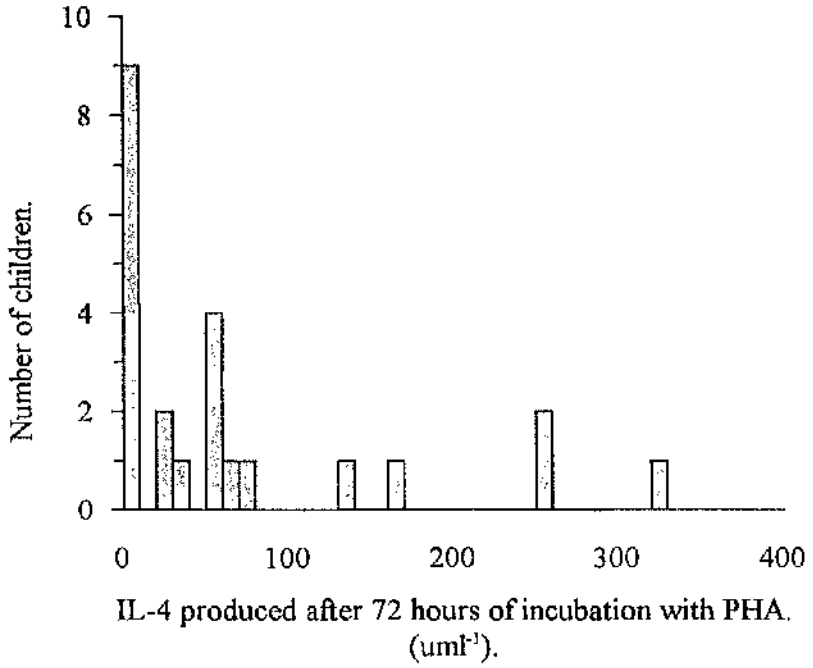



**Figure 4.1b. An example of a frequency distribution of log+1 transformed data of cytokine production, which is also non-normally distributed.**

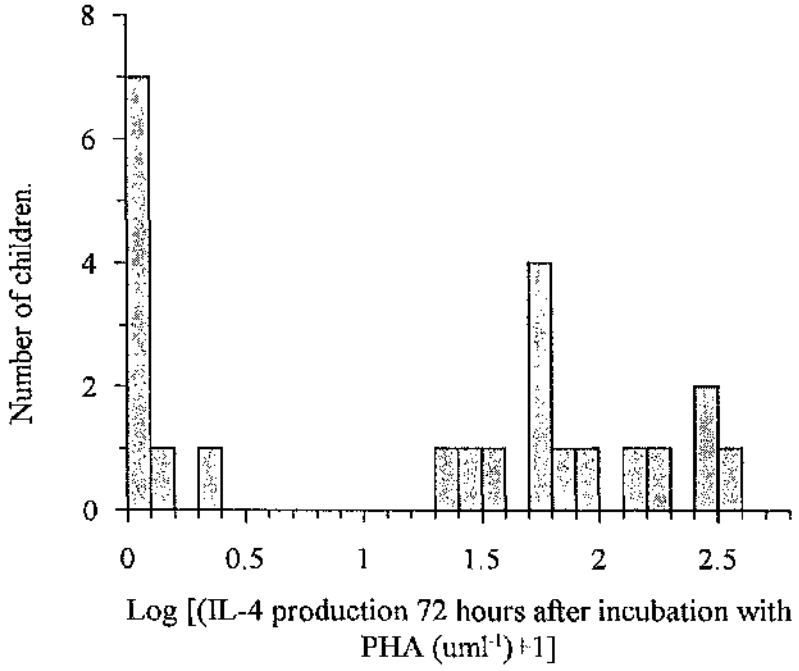


**Figure 4.2a. An example of a cytokine data set that was analysed using logistic regression analyses.**

A frequency distribution of net IL-4 produced by whole blood taken from Zimbabwean children 12 months after treatment and cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots, for 24 hours in the presence of $10\mu gml^{-1}$ PHA. IL-4 concentration was determined using a sandwich ELISA as described in section 2.3. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. The salient information for analysis by logistical regression was categorised as to whether blood from an individual produced cytokine or not.

**Figure 4.2a. An example of a cytokine data set that was analysed using multiple linear regression analyses.**

A frequency distribution of net IFNγ produced by whole blood taken from Zimbabwean children 12 months after treatment and cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots, for 24 hours in the presence of $10\mu gml^{-1}$ PHA. IFNγ concentration was determined using a sandwich ELISA as described in section 2.3. Net data are the amount of cytokine produced by blood when cultured with PHA minus levels produced by blood cultured without stimulant. Multiple linear regression analysis enabled the level of cytokine production to be considered as a continuous dependent variable.

**Figure 4.2a. An example of a cytokine data set that was analysed using logistic regression analyses.**

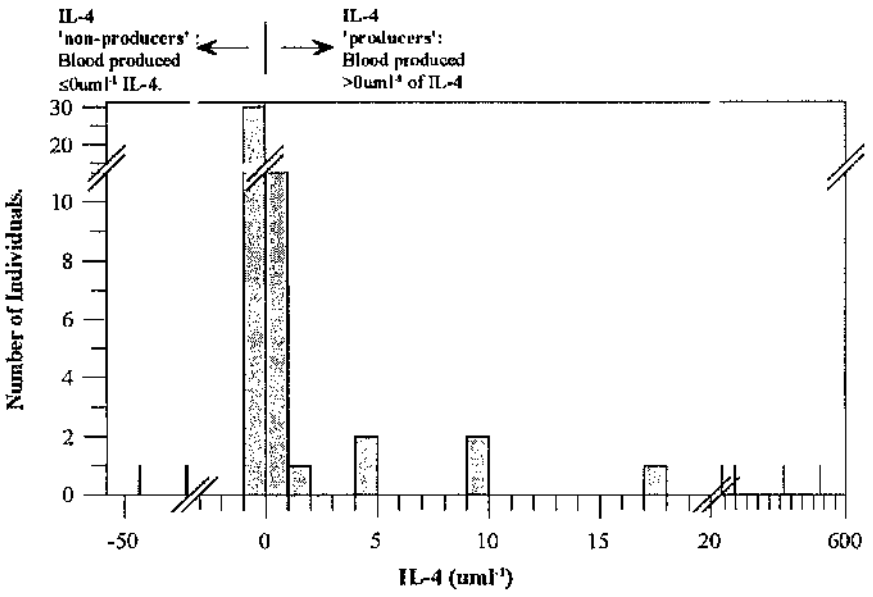



**Figure 4.2b. An example of a cytokine data set that was analysed using multiple linear regression analyses.**

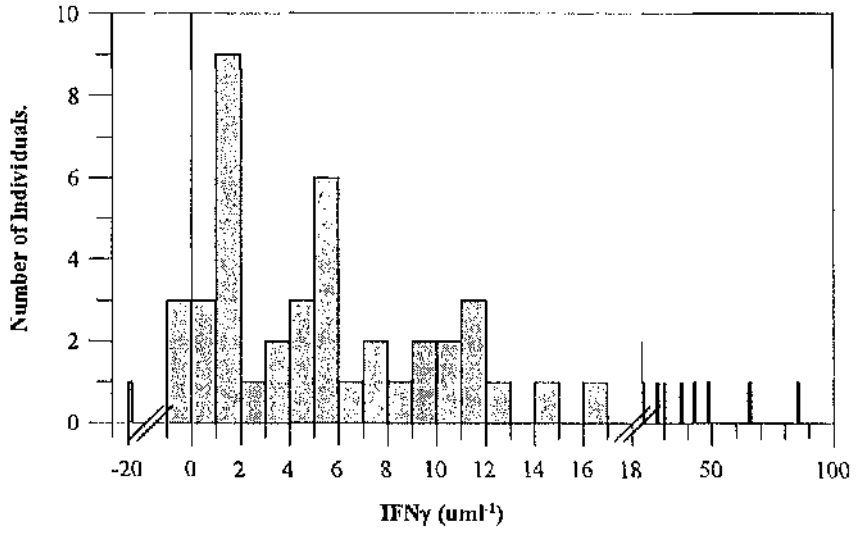


**Figure 4.3a. Prevalence of infection: Valhalla School.**

Prevalence of infection, as determined by *S.haematobium* eggs observed in urine samples from children attending Valhalla School aged 6 to 10 years compared with children aged 11 to 15 years before treatment and at 3,4,6 and 8 months post treatment.

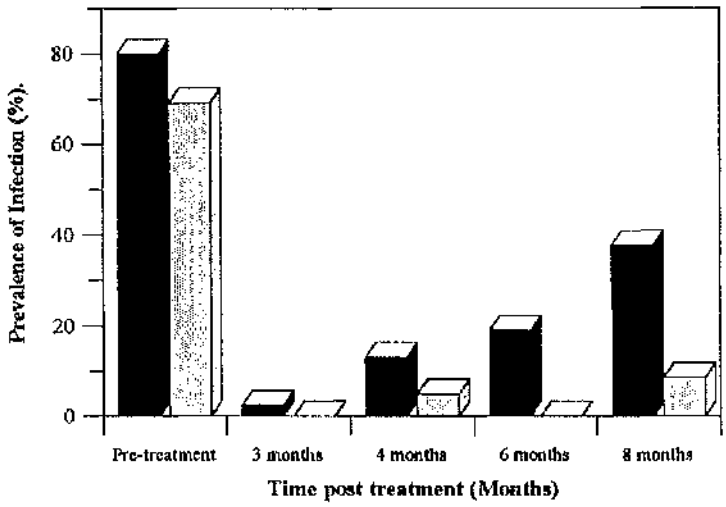



**Figure 4.3b. Prevalence of infection: Kaswa School.**

Prevalence of infection, as determined by *S.haematobium* eggs observed in urine samples from children attending Kaswa School aged 6 to 10 years compared with children ages 11 to 15 years before treatment and at 3,4,6 and 8 months post treatment

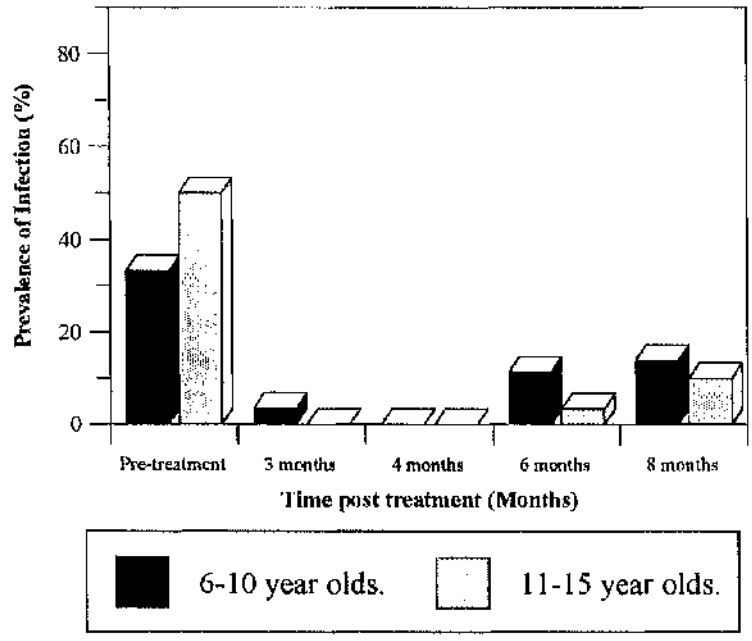


**Table 4.1. Summary of the analysis of differences in cytokine production with time on stimulation with either SEA, WWH or CERC.**

Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either SEA, WWH or CERC and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Differences in net cytokine produced at different time points were compared using Wilcoxon signed ranks test followed by a sequential Bonferroni correction for multiple tests. Net cytokine is cytokine produced on stimulation with SEA, WWH or CERC minus cytokine produced when cultured without stimulant.

**Table 4.2. Summary of the analysis of differences in cytokine production with time on stimulation with PHA.**

Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of PHA and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625 $uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Differences in cytokine produced at different time points were compared using Wilcoxon signed ranks test followed by a sequential Bonferroni correction for multiple tests. Net cytokine is cytokine produced on stimulation with PHA minus cytokine produced when cultured without stimulant.

**Table 4.1. Summary of the analysis of differences in cytokine production with time on stimulation with either SEA, WWH or CERC.**

| Cytokine | 24 verses 48 hours | | | 48 verses 72 hours | | | 24 verses 72 hours | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Z | p< | N | Z | p< | N | Z | p< |
| IL-4 | 117 | | ns | 43 | | ns | 46 | | ns |
| IL-5 | 111 | | ns | 40 | | ns | 46 | -1.225 | 0.01 |
| IL-10 | 77 | | ns | 39 | | ns | 40 | | ns |
| IFNg | 117 | -3.599 | 0.01 | 42 | -2.444 | 0.05 | 42 | -3.186 | 0.01 |
| GM-CSF | 74 | -2.857 | 0.02 | ~ | ~ | ~ | ~ | ~ | ~ |
| TNFα | 44 | -3.911 | 0.001 | ~ | ~ | ~ | ~ | ~ | ~ |

~ = no comparison could be made because of low sample size.

All p values have been corrected to take into account multiple tests.

ns = not significant.

**Table 4.2.Summary of the analysis of differences in cytokine production with time on stimulation with PHA.**

| Cytokine | 24 verses 48 hours | | | 48 verses 72 hours | | | 24 verses 72 hours | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Z | p< | N | Z | p< | N | Z | p< |
| IL-4 | 46 | | ns | 18 | | ns | 16 | -2.953 | 0.01 |
| IL-5 | 39 | | ns | 15 | | ns | 14 | | ns |
| IL-10 | 28 | | ns | 13 | | ns | 10 | | ns |
| IFNg | 46 | -4.441 | 0.01 | 18 | -3.332 | 0.01 | 15 | -3.237 | 0.01 |
| GM-CSF | 29 | -2.857 | 0.004 | ~ | ~ | ~ | ~ | ~ | ~ |
| TNFα | 17 | -2.223 | 0.026 | ~ | ~ | ~ | ~ | ~ | ~ |

~ = no comparison could be made because of low sample size.

All p values have been corrected to take into account multiple tests.

ns = not significant.

**Figure 4.4. A comparison of cytokine produced on stimulation with SEA, WWH or CERC at 24, 48 and 72 hours post incubation.**

Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either SEA, WWH or CERC and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Differences in net cytokine produced at different time points were compared using Wilcoxon signed ranks test followed by a sequential Bonferroni correction for multiple tests. The statistically significant differences between time points are summarised in table 4.1. Net cytokine is cytokine produced on stimulation with SEA, WWH or CERC minus cytokine produced when cultured without stimulant.

**Figure 4.4**

**A comparison of cytokine produced on stimulation with SEA, WWH or CERC at 24, 48 and 72 hours post incubation.**

**Figure 4.4a: IL-4**

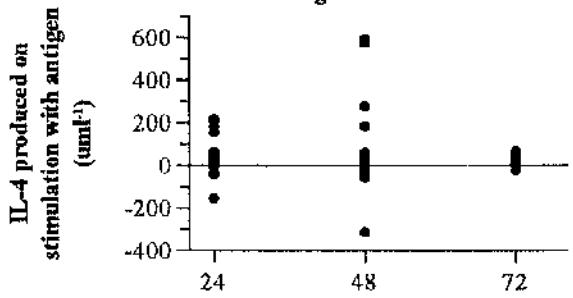



**Figure 4.4d: IFNγ**

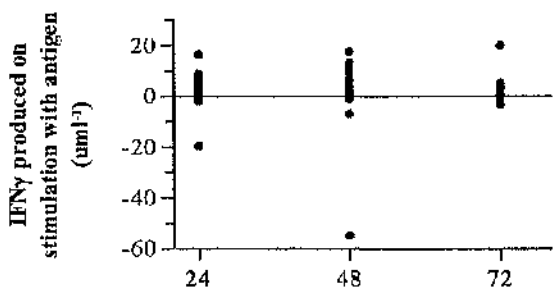



**Figure 4.4b: IL-5**

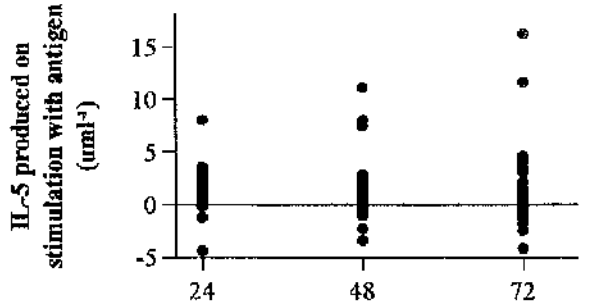



**Figure 4.4e: GM-CSF**

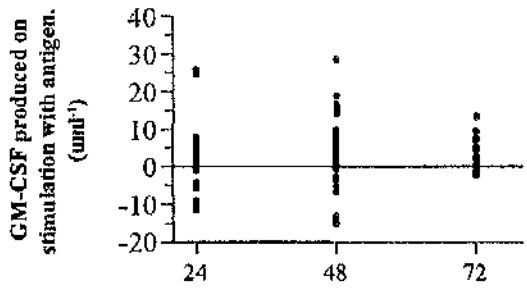



**Figure 4.4c: IL-10**

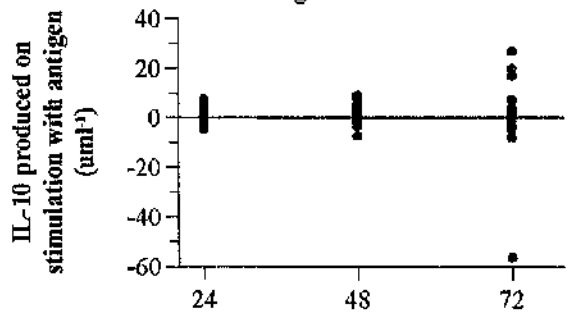



**Figure 4.4f: TNFα**

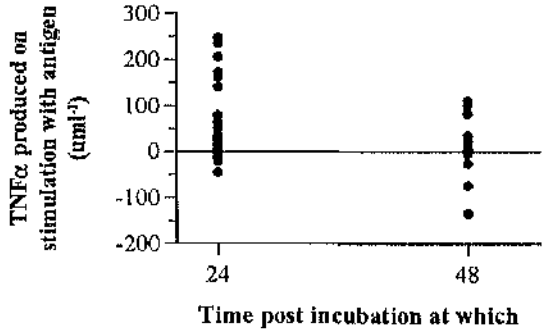


**Figure 4.5. A comparison of cytokine produced on stimulation with PHA at 24, 48 and 72 hours post incubation.**

Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of PHA and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625 $uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Differences in net cytokine produced at different time points were compared using Wilcoxon signed ranks test followed by a sequential Bonferroni correction for multiple tests. The statistically significant differences between time points are summarised in table 4.2. Net cytokine is cytokine produced on stimulation with PHA minus cytokine produced when cultured without stimulant.

**Figure 4.5**

**A comparison of cytokine produced on stimulation with PHA at 24, 48 and 72 hours post incubation.**

Figure 4.5a: IL-4

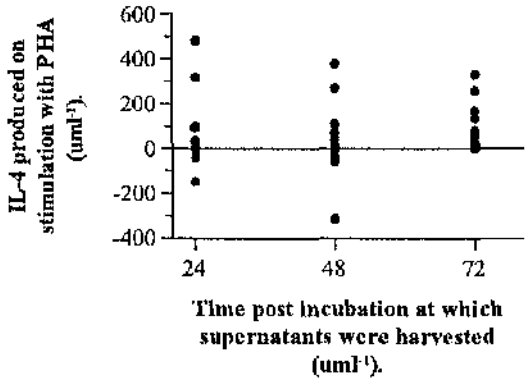



Figure 4.5d: IFNγ

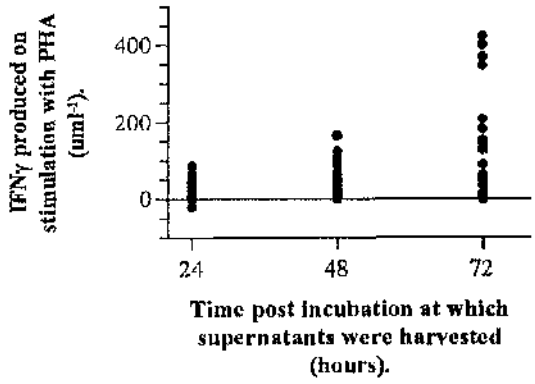



Figure 4.5b: IL-5

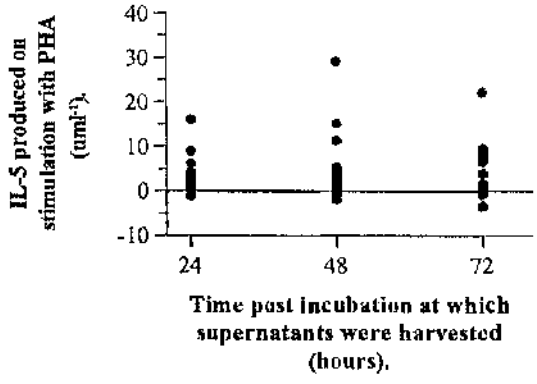



Figure 4.5e: GM-CSF

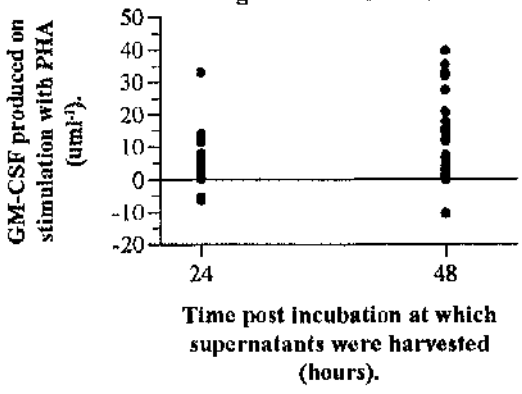



Figure 4.5c: IL-10

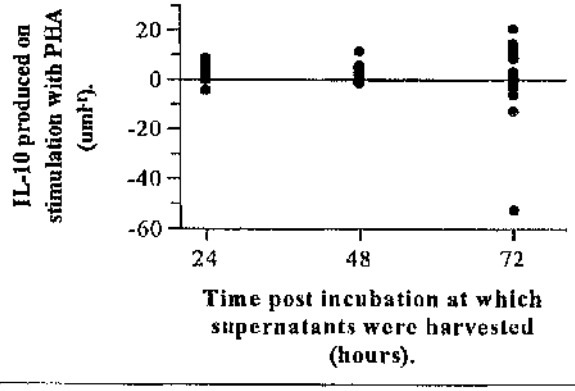



Figure 4.5f: TNFα

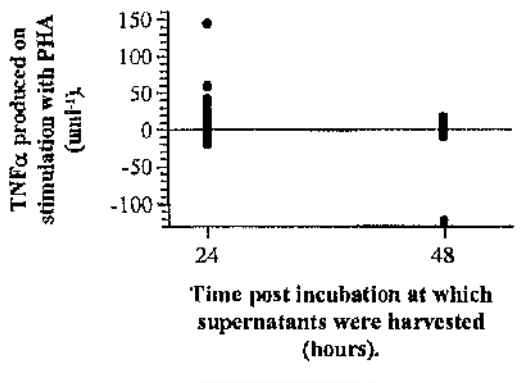


**Table 4.3 Summary of significant explanatory variables of cytokine production.**

Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either SEA, WWH or PHA and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625\ uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net data were used throughout. Net data are cytokine produced on culture with mitogen or antigen minus cytokine produced on culture without stimulus.

**Table 4.3. Summary of statistically significant explanatory variables of cytokine production.**

| Cytokine | Stimulant | Time point post incubation | Type of multiple regression. | Significant explanatory variable. | B | P< (N) | Explanation. |
|---|---|---|---|---|---|---|---|
| IL-4 | PHA | 24 | Logistic | Area | 1.7033 | 0.001 (55) | Production of IL-4 measured at 24 hours post incubation, on stimulation with PHA was associated with residence in the low prevalence area. |
| IFNγ | PHA | 24 | Linear | Area | -0.347 | 0.0005 (52) | IFNγ measured at 24 hours post incubation, on stimulation with PHA was associated with residence in the low prevalence area. |
| GM-CSF | PHA | 24 | Linear | Area | -6.12 | 0.0005 (42) | GM-CSF measured at 24 hours post incubation, on stimulation with PHA was associated with residence in the low prevalence area. |
| IL-10 | SEA | 48 | Logistic | Area | 2.367 | 0.05 (42) | Production of IL-10 measured at 48 hours post incubation, on stimulation with SEA was associated with residence in the high prevalence area. |
| GM-CSF | SEA | 48 | Logistic | Area | -1.2834 | 0.05 (42) | Production of GM-CSF measured at 48 hours post incubation, on stimulation with SEA was associated with residence in the high prevalence area. |
| IL-5 | PHA | 24 | Logistic | Pre-treatment infection status | -1.1466 | 0.05 (49) | Production of IL-5 measured at 24 hours post incubation, on stimulation with PHA was associated with infection before treatment. |
| IL-4 | SEA | 24 | Logistic | Pre-treatment infection status | -1.4095 | 0.05 (54) | Production of IL-4 measured at 24 hours post incubation, on stimulation with SEA was associated with a lack of infection before treatment. |
| IFNγ | WWH | 48 | Logistic | Post-treatment infection status | -1.3718 | 0.05 (67) | Production of IFNγ measured at 48 hours post incubation, on stimulation with WWH was associated with infection status eight months after treatment. |

**Figure 4.6. A comparison of the effects of residence in an area of low or high prevalence for *S.haematobium* on production of IL-4 by blood samples from Zimbabwean children when stimulated with PHA for 24 hours.**

Blood from children resident in the low prevalence area was more likely to produce net IL-4 than blood from children resident in the high prevalence area (multiple logistic regression analysis controlling for age, sex, pre- and post-treatment infection status: B=1.70, n=55, $p<0.001$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 24 hours with 10$\mu gml^{-1}$ PHA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625$uml^{-1}$ and an accuracy of 0.01$uml^{-1}$. Net IL-4 was comprised of IL-4 produced when blood was cultured with PHA minus IL-4 produced when blood was cultured without stimulant.

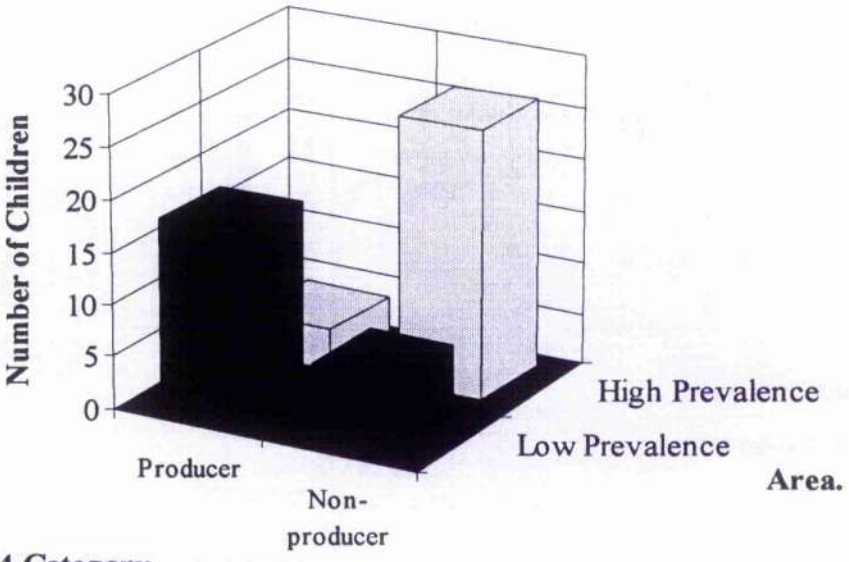


**Figure 4.7. A comparison of the effects of residence in an area of low or high prevalence for *S.haematobium* on production of IFNγ by blood samples from Zimbabwean children when stimulated with PHA for 24 hours.**

Blood from children resident in the low prevalence area produced more net IFNγ than blood from children resident in the high prevalence area (multiple linear regression analysis controlling for age, sex, pre- and post-treatment infection status B= -0.347, n=52, $p<0.0005$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 24 hours with 10μgml$^{-1}$ PHA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net IFNγ was comprised of IFNγ produced when blood was cultured with PHA minus IFNγ produced when blood was cultured without stimulant.

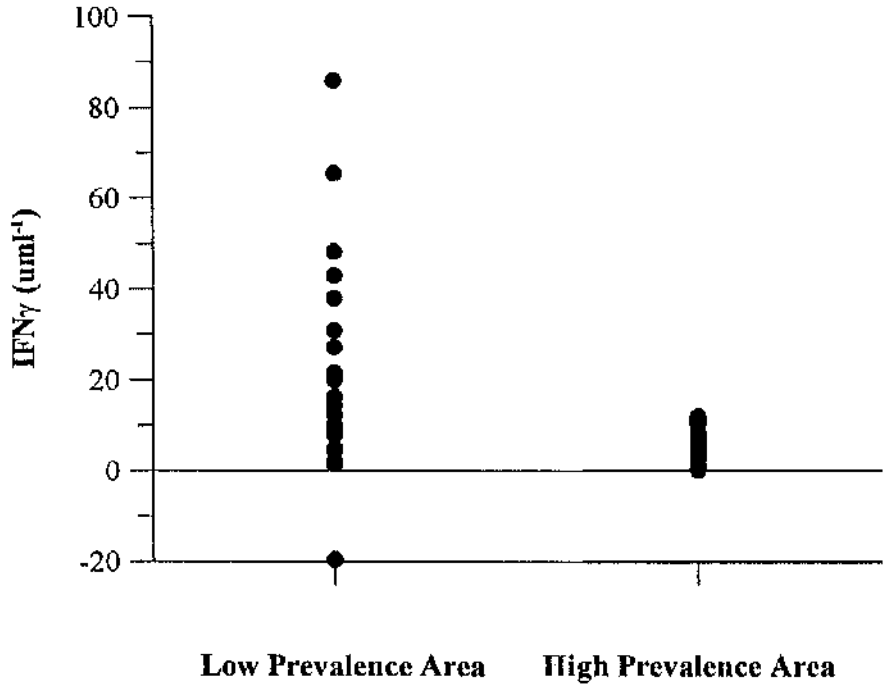


**Figure 4.8. A comparison of the effects of residence in an area of low or high prevalence for *S.haematobium* on production of GM-CSF by blood samples from Zimbabwean children when stimulated with PHA for 24 hours.**

Blood from children resident in the low prevalence area produced more net GM-CSF than blood from children resident in the high prevalence area (multiple linear regression analysis controlling for age, sex, pre- and post-treatment infection status B=-0.347, n=42, $p<0.0005$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 24 hours with 10μgml$^{-1}$ PHA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625 uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net GM-CSF was comprised of GM-CSF produced when blood was cultured with PHA minus GM-CSF produced when blood was cultured without stimulant.

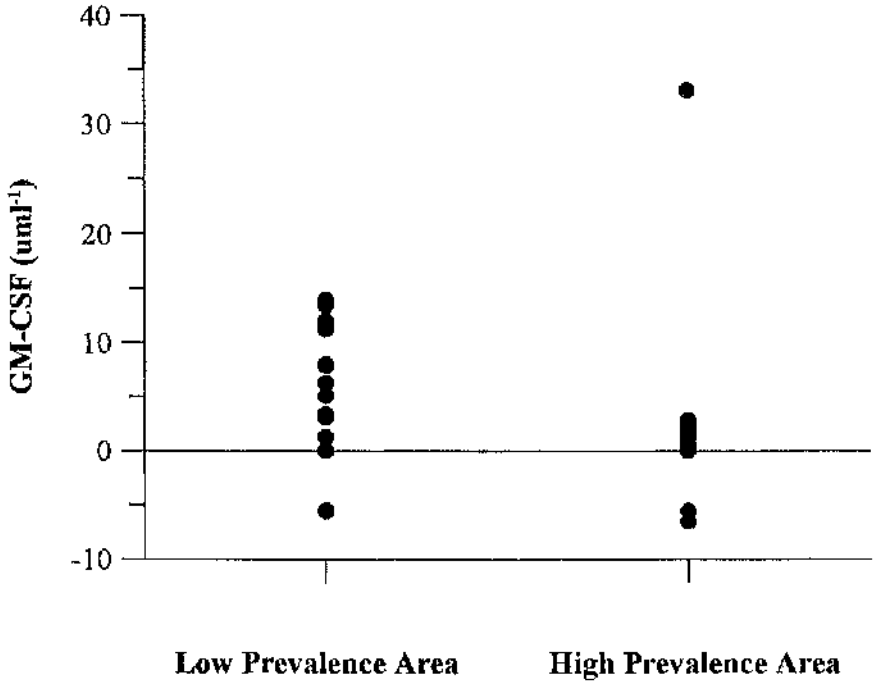


**Figure 4.9. A comparison of the effects pre-treatment infection status for *S.haematobium* on production of IL-5 by blood samples from Zimbabwean children when stimulated with PHA for 24 hours.**

Blood from children who were infected before treatment, as ascertained by *S.haematobium* eggs found in urine samples, were more likely to produce net IL-5 than blood from children who were not infected before treatment (multiple logistic regression analysis controlling for age, sex, post-treatment infection status and area: B=-1.147, n=49, $p<0.05$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 24 hours with 10μgml$^{-1}$ PHA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net IL-5 was comprised of IL-5 produced when blood was cultured with PHA minus IL-5 produced when blood was cultured without stimulant.

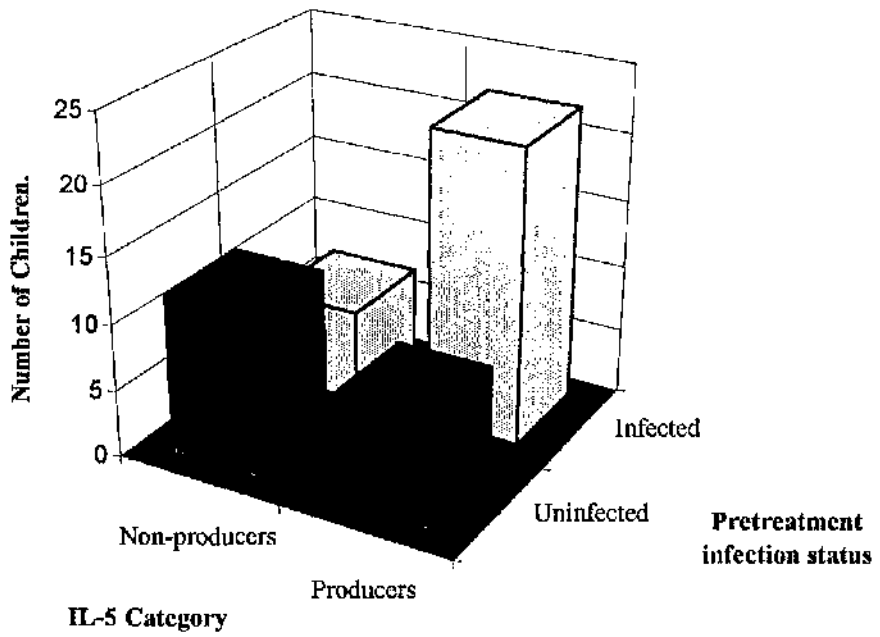


**Figure 4.10. A comparison of the effects of pre-treatment infection status for *S.haematobium* on production of IL-4 by blood samples from Zimbabwean children when stimulated with SEA for 24 hours.**

Blood from children who were not infected before treatment, as ascertained by *S.haematobium* eggs found in urine samples, were more likely to produce net IL-4 than blood from children who were infected before treatment (multiple logistic regression analysis controlling for age, sex, post-treatment infection status and area: B=-1.410, n=54, $p<0.05$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 24 hours with 10μgml$^{-1}$ PHA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net IL-4 was comprised of IL-4 produced when blood was cultured with PHA minus IL-4 produced when blood was cultured without stimulant.

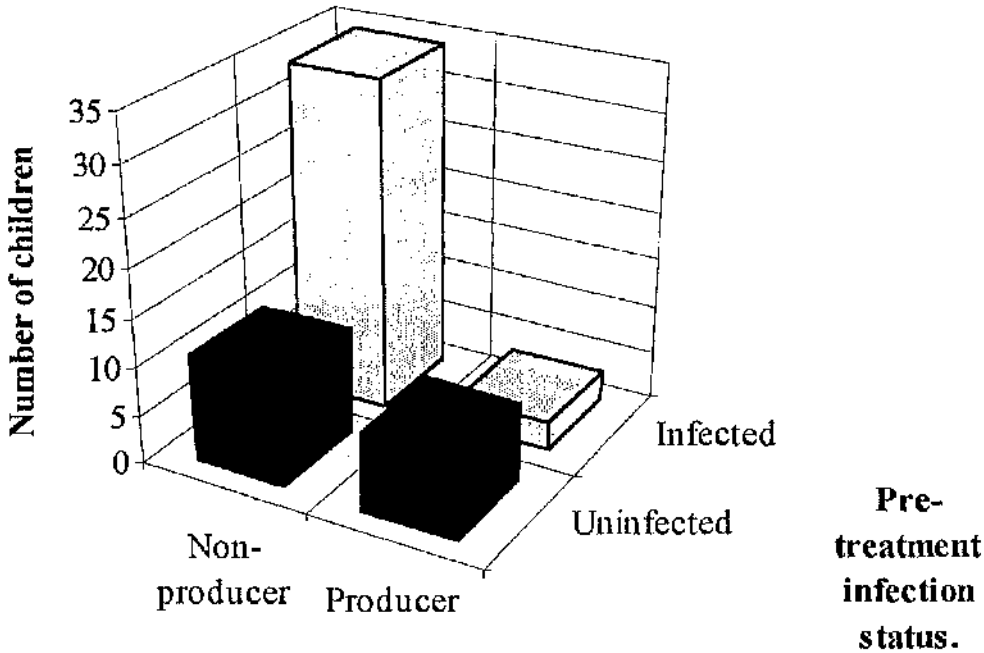


**Figure 4.11. A comparison of the effects of residence in an area of low or high prevalence for *S.haematobium* on production of IL-10 by blood samples from Zimbabwean children when stimulated with SEA for 48 hours.**

Blood from children resident in the high prevalence area was more likely to produce net IL-10 than blood from children resident in the low prevalence area (multiple logistic regression analysis controlling for age, sex, pre- and post-treatment infection status: B=2.37, n=42, $p<0.05$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 48 hours with 10$\mu gml^{-1}$ SEA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625$uml^{-1}$ and an accuracy of 0.01$uml^{-1}$. Net IL-10 was comprised of IL-10 produced when blood was cultured with SEA minus IL-10 produced when blood was cultured without stimulant.

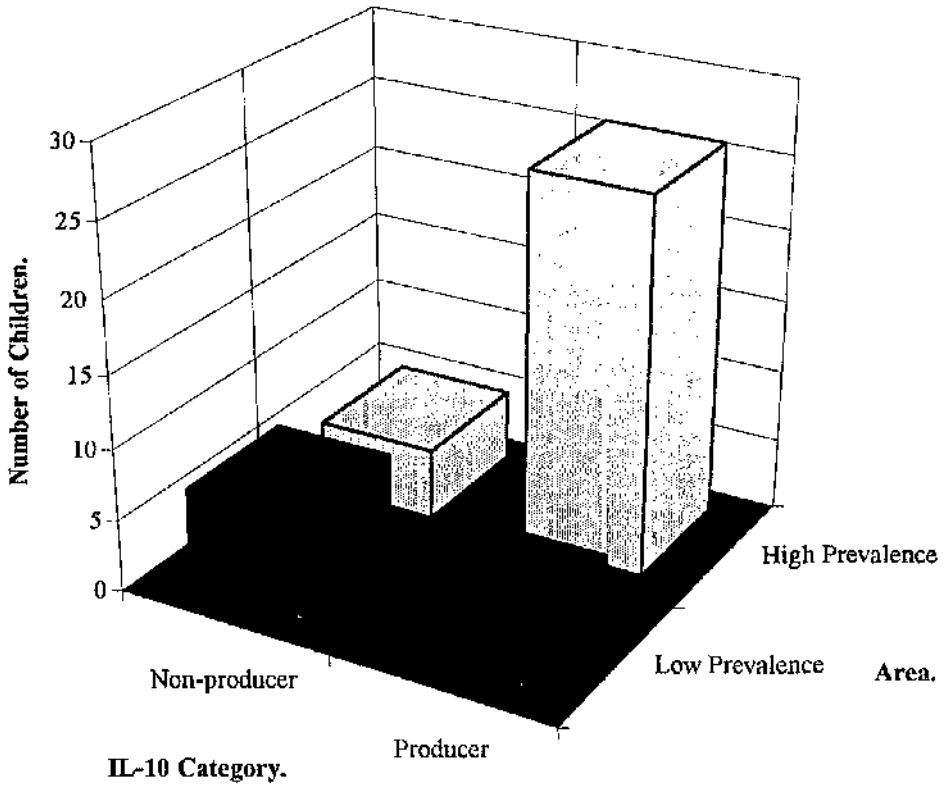


**Figure 4.12. A comparison of the effects of residence in an area of low or high prevalence for *S.haematobium* on production of GM-CSF by blood samples from Zimbabwean children when stimulated with SEA for 48 hours.**

Blood from children resident in the high prevalence area was more likely to produce net GM-CSF than blood from children resident in the low prevalence area (multiple logistic regression analysis controlling for age, sex, pre- and post-treatment infection status: B=-1.283, n=42, $p<0.05$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 48 hours with 10μgml$^{-1}$ SEA. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$. Net GM-CSF was comprised of GM-CSF produced when blood was cultured with SEA minus GM-CSF produced when blood was cultured without stimulant.

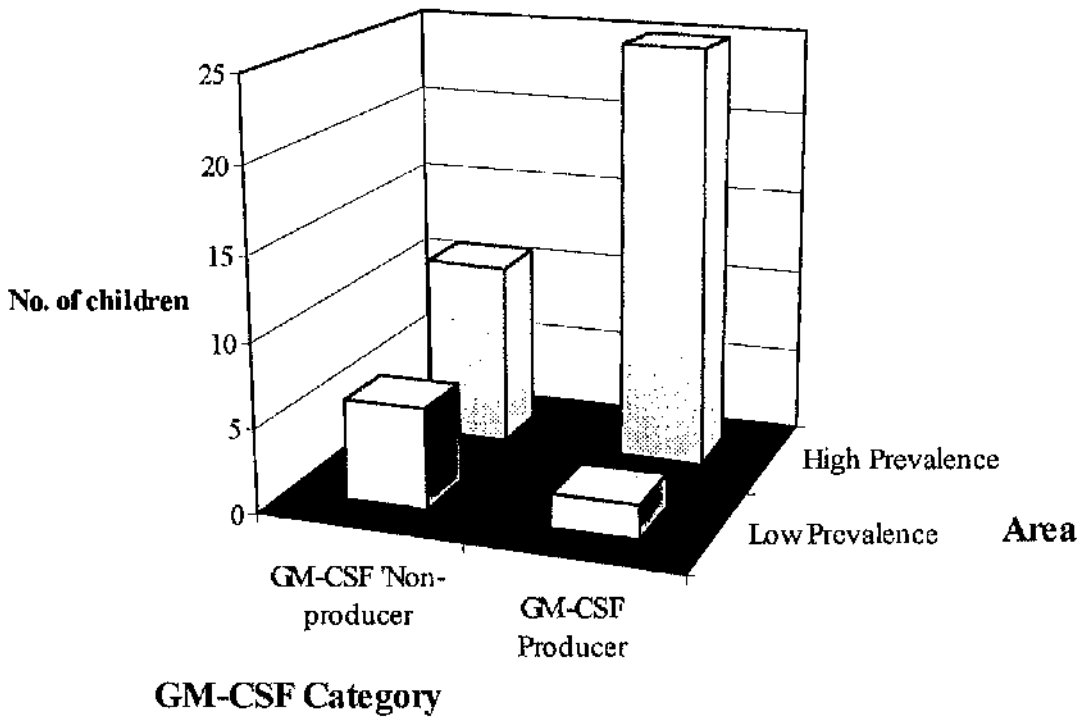


**Figure 4.13. A comparison of the effects of infection status for *S.haematobium* eight months after treatment on production of IFNγ by blood samples from Zimbabwean children when stimulated with WWH for 48 hours.**

Blood from children who were uninfected eight months after treatment for *S.haematobium* infection as ascertained by *S.haematobium* eggs found in urine samples were more likely to produce net IFNγ than blood from children who were uninfected eight months after treatment (Multiple logistic regression analysis controlling for age, sex, pre-treatment infection status and area: B=-1.3718, n=67, $p<0.05$). Blood was taken from Zimbabwean children 12 months post treatment for *S.haematobium* infection and cultured for 48 hours with 10μgml$^{-1}$ WWH. Blood was cultured at a dilution of 1 in 2 with RPMI in 1ml and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625uml$^{-1}$ and an accuracy of 0.01uml$^{-1}$.Net IFNγ was comprised of IFNγproduced when blood was cultured with SEA minus IFNγproduced when blood was cultured without stimulant.

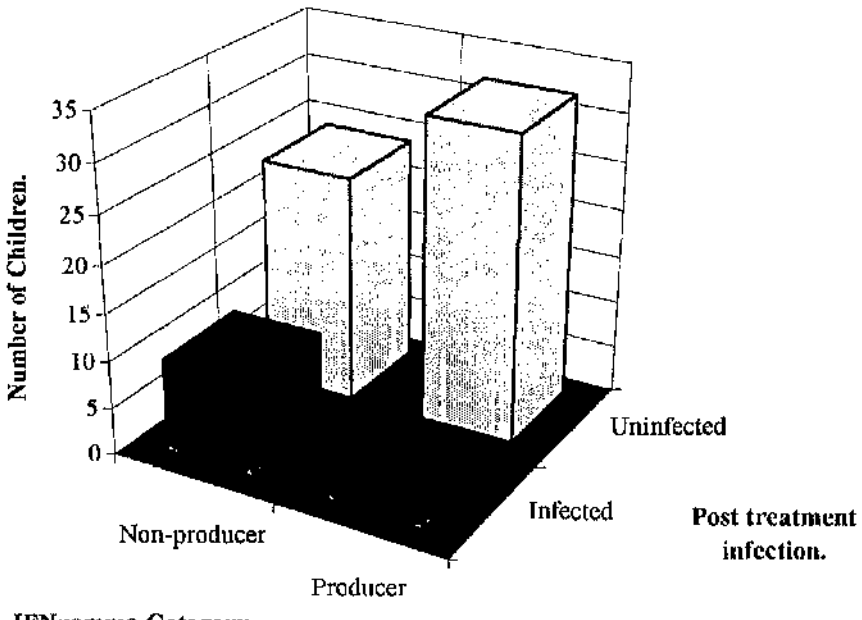


**Figure 4.14. A correlation analysis of levels of IL-5 produced from blood samples taken from Zimbabwean children when stimulated with either SEA or WWH.**

Net IL-5 produced by blood taken from children resident in the low prevalence area on stimulation with 10 $\mu gml^{-1}$ WWH correlated positively with net IL-5 on stimulation with 10 $\mu gml^{-1}$ SEA 72 hours post incubation controlling for production of net IL-5 produced on culture with 10 $\mu gml^{-1}$ PHA for 72 hours ($p<0.025$, $n=11$, $T_{xy.z} = 0.471$ using a Kendall partial correlation followed by a sequential Bonferroni correction for multiple tests). Blood was taken 12 months post treatment for *S.haematobium* and cultured at a dilution of 1 in 2 with RPMI in 1ml. IL-5 was measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net IL-5 was comprised of IL-5 produced when blood was cultured with stimulant minus IL-5 produced when blood was cultured without stimulant.

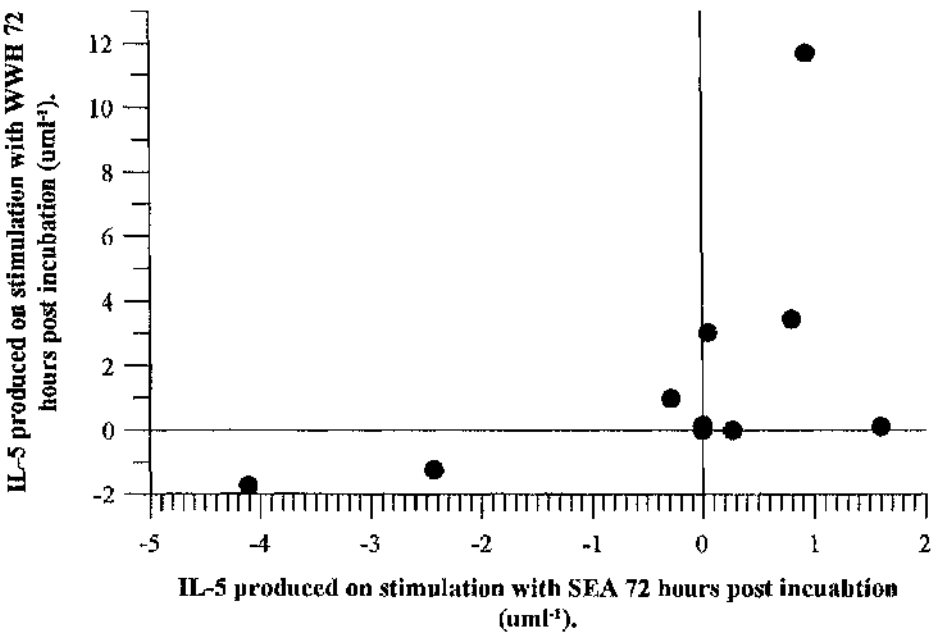


**Figure 4.15. A correlation analysis of levels of IFNγ produced from blood samples taken from Zimbabwean children when stimulated with either SEA or WWH.**

Net IFNγ produced by blood taken from children resident in the high prevalence area on stimulation with 10 $\mu gml^{-1}$ WWH correlated positively with net IFNγ on stimulation with 10 $\mu gml^{-1}$ SEA 48 hours post incubation controlling for production of net IL-5 produced on culture with 10 $\mu gml^{-1}$ PHA for 48 hours ($p<0.025$, $n=28$, $T_{xy.z} = 0.471$, using a Kendall partial correlation followed by a sequential Bonferroni correction for multiple tests). Blood was taken 12 months post treatment for *S.haematobium* and cultured at a dilution of 1 in 2 with RPMI in 1ml. IFNγ was measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net IFNγ was comprised of IFNγ produced when blood was cultured with stimulant minus IFNγ produced when blood was cultured without stimulant.

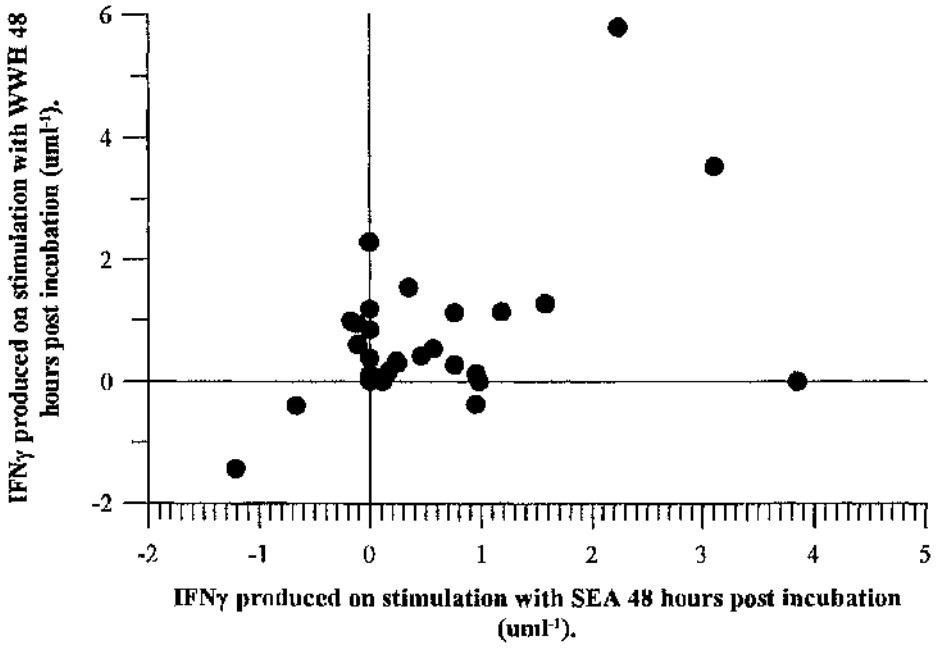


**Figure 4.16. A correlation analysis of levels of IL-5 produced from blood samples taken from Zimbabwean children when stimulated with either SEA or WWH.**

Net IL-5 produced by blood taken from children resident in the high prevalence area on stimulation with 10 $\mu gml^{-1}$ WWH correlated positively with net IL-5 on stimulation with 10 $\mu gml^{-1}$ SEA 48 hours post incubation controlling for production of net IL-5 produced on culture with 10 $\mu gml^{-1}$ PHA for 48 hours ($p<0.001$, $n=27$, $T_{xy.z} = 0.427$, using a Kendall partial correlation followed by a sequential Bonferroni correction for multiple tests). Blood was taken 12 months post treatment for *S.haematobium* and cultured at a dilution of 1 in 2 with RPMI in 1ml. IL-5 was measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of 0.0625$uml^{-1}$ and an accuracy of 0.01$uml^{-1}$. Net IL-5 was comprised of IL-5 produced when blood was cultured with stimulant minus IL-5 produced when blood was cultured without stimulant.

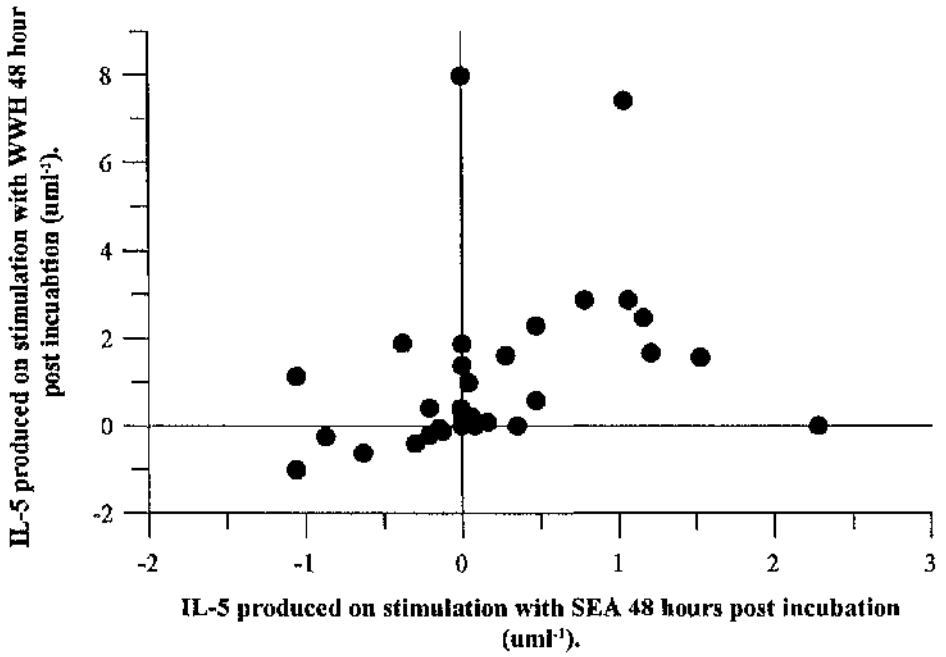
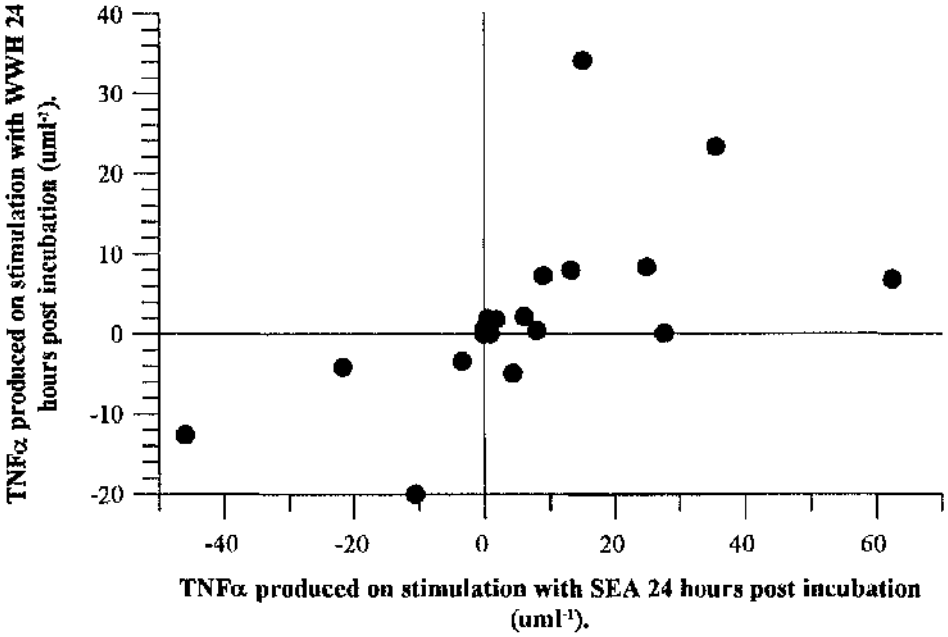


**Figure 4.17 . A correlation analysis of levels of TNFα produced from blood samples taken from Zimbabwean children when stimulated with either SEA or WWH.**

Net TNFα produced by blood taken from children resident in the high prevalence area on stimulation with 10 $\mu gml^{-1}$ WWH correlated positively with net TNFα on stimulation with 10 $\mu gml^{-1}$ SEA 24 or 48 hours post incubation controlling for production of net TNFα produced on culture with 10 $\mu gml^{-1}$ PHA for 24 or 48 hours respectively (24 hours: $p<0.002$, $n=19$, $T_{xy.z} = 0.608$, 48 hours: $p<0.025$, $n= 20$, $T_{xy.z} = 0.345$, in both cases, using a Kendall partial correlation followed by a sequential Bonferroni correction for multiple tests). Blood was taken 12 months post treatment for *S.haematobium* and cultured at a dilution of 1 in 2 with RPMI in 1ml. TNFα was measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net TNFα was comprised of TNFα produced when blood was cultured with stimulant minus TNFα produced when blood was cultured without stimulant.

**Figure 4.17. A correlation analysis of levels of TNFα produced from blood samples taken from Zimbabwean children when stimulated with either SEA or WWH.**

**TNFα produced at 24 hours post incubation.**




**TNFα produced at 48 hours post incubation.**

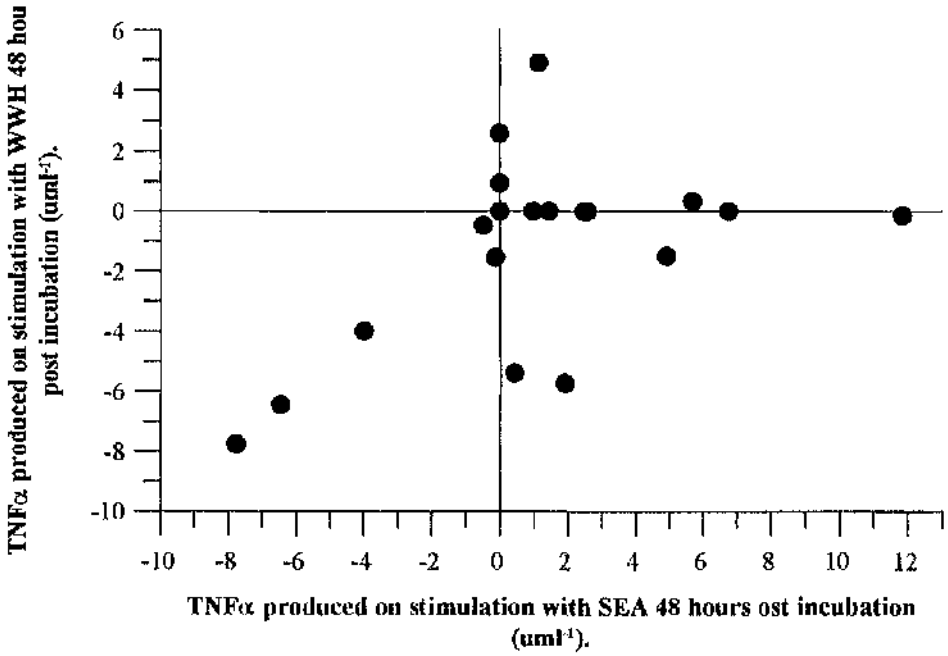


**Table 4.4.**

**A summary of the statistical analysis addressing the hypothesis that cytokine production is influenced by cross reactive antigens.**

A partial correlation analysis between levels of net cytokine production for each of cytokines on stimulation with 10 $\mu gml^{-1}$ of either SEA or WWH, controlling for levels produced on stimulation with 10 $\mu gml^{-1}$ of PHA. Kendall partial correlation analysis was conducted in each case and levels of significance determined after correction for multiple tests by a sequential Bonferroni technique. Statistical analysis using this methodology was inappropriate when $n<10$ or the majority of samples produced no net cytokines for one or both antigens. Cytokines were produced by whole blood from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml and measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net cytokine was comprised of cytokine produced when blood was cultured with a stimulant minus cytokine produced when blood was cultured without a stimulant.

**Table 4.4.**

**A summary of the statistical analysis addressing the hypothesis that cytokine production is influenced by cross reactive antigens.**

**Low Prevalence Area.**

| | Hour post incubation. | | |
|---|---|---|---|
| **Cytokine** | **24** | **48** | **72** |
| **GM-CSF** | - | - | - |
| **IFNγ** | ns | ~ | ns |
| **IL-10** | - | - | - |
| **IL-4** | ns | ns | ns |
| **IL-5** | ns | ~ | 0.025 |
| **TNFα** | - | - | - |

**High Prevalence Area.**

| | Hour post incubation. | | |
|---|---|---|---|
| **Cytokine** | **24** | **48** | **72** |
| **GM-CSF** | ns | ns | - |
| **IFNγ** | ~ | 0.025 | - |
| **IL-10** | ns | ns | - |
| **IL-4** | ns | ~ | - |
| **IL-5** | ~ | 0.001 | - |
| **TNFα** | 0.002 | 0.025 | - |

ns = Not significant

- = sample size of less than 10.

~ = a majority of data points were $0uml^{-1}$ for one or both antigens.

**Table 4.5, 4.6 and 4.7. A comparison of the number of children whose blood produced either IL-4 or IL-5 compared to those whose blood produced both IL-4 and IL-5.**

Blood samples were taken from Zimbabwean children 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of one of four antigens SEA, WWH, CERC or PHA for either 24, 48 or 72 hours. Levels of net IL-4 and IL-5 were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Statistical tests were made by $\chi^2$ test. Net cytokine was comprised of cytokine produced when blood was cultured with a stimulant minus cytokine produced when blood was cultured without a stimulant.

**Table 4.5. IL-4 and IL-5 produced 24 hours post incubation.**

| Stimulant. | Number of children whose blood produced either IL-4 or IL-5 | Number of children whose blood produced both IL-4 and IL-5. | (p<) | $\chi^2$ | Degrees of freedom |
|---|---|---|---|---|---|
| **CERC** | ~ | ~ | | | |
| **SEA** | 14 | 1 | 0.0008 | 11.27 | 1 |
| **WWH** | 28 | 5 | 0.0001 | 16.03 | 1 |
| **PHA** | 31 | 10 | 0.001 | 10.8 | 1 |

~ = sample size of less than 10.

**Table 4.6. IL-4 and IL-5 produced 48 hours post incubation.**

| Stimulant. | Number of children whose blood produced either IL-4 or IL-5 | Number of children whose blood produced both IL-4 and IL-5. | (p<) | $\chi^2$ | Degrees of freedom |
|---|---|---|---|---|---|
| **CERC** | 9 | 1 | 0.01 | 6.40 | 1 |
| **SEA** | 24 | 4 | 0.0002 | 14.29 | 1 |
| **WWH** | 27 | 13 | 0.03 | 4.9 | 1 |
| **PHA** | 23 | 19 | ns | 0.38 | 1 |

~ = sample size of less than 10. ns = not significant.

**Table 4.7 IL-4 and IL-5 produced 72 hours post incubation.**

| Stimulant. | Number of children whose blood produced either IL-4 or IL-5 | Number of children whose blood produced both IL-4 and IL-5. | (p<) | $\chi^2$ | Degrees of freedom |
|---|---|---|---|---|---|
| **CERC** | ~ | ~ | | | |
| **SEA** | ~ | ~ | | | |
| **WWH** | 10 | 8 | ns | 0.11 | 1 |
| **PHA** | 10 | 10 | ns | 0.00 | 1 |

~ = sample size of less than 10. ns = not significant.

**Figure 4.18. A comparison of the levels of production of IL-4 and IL-5 after culturing for 24 hours in the presence of one of three stimulants: SEA, WWH or PHA.**

Cytokines were produced by whole blood samples from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either SEA, WWH or PHA. Cytokines were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net levels of cytokine were compared. Net cytokine was comprised of cytokine produced when blood was cultured with a stimulant minus cytokine produced when blood was cultured without a stimulant.

**Figure 4.18.**

**A comparison of the levels of production of IL-4 and IL-5 after culturing for 24 hours in the presence of one of three stimulants: SEA, WWH or PHA.**

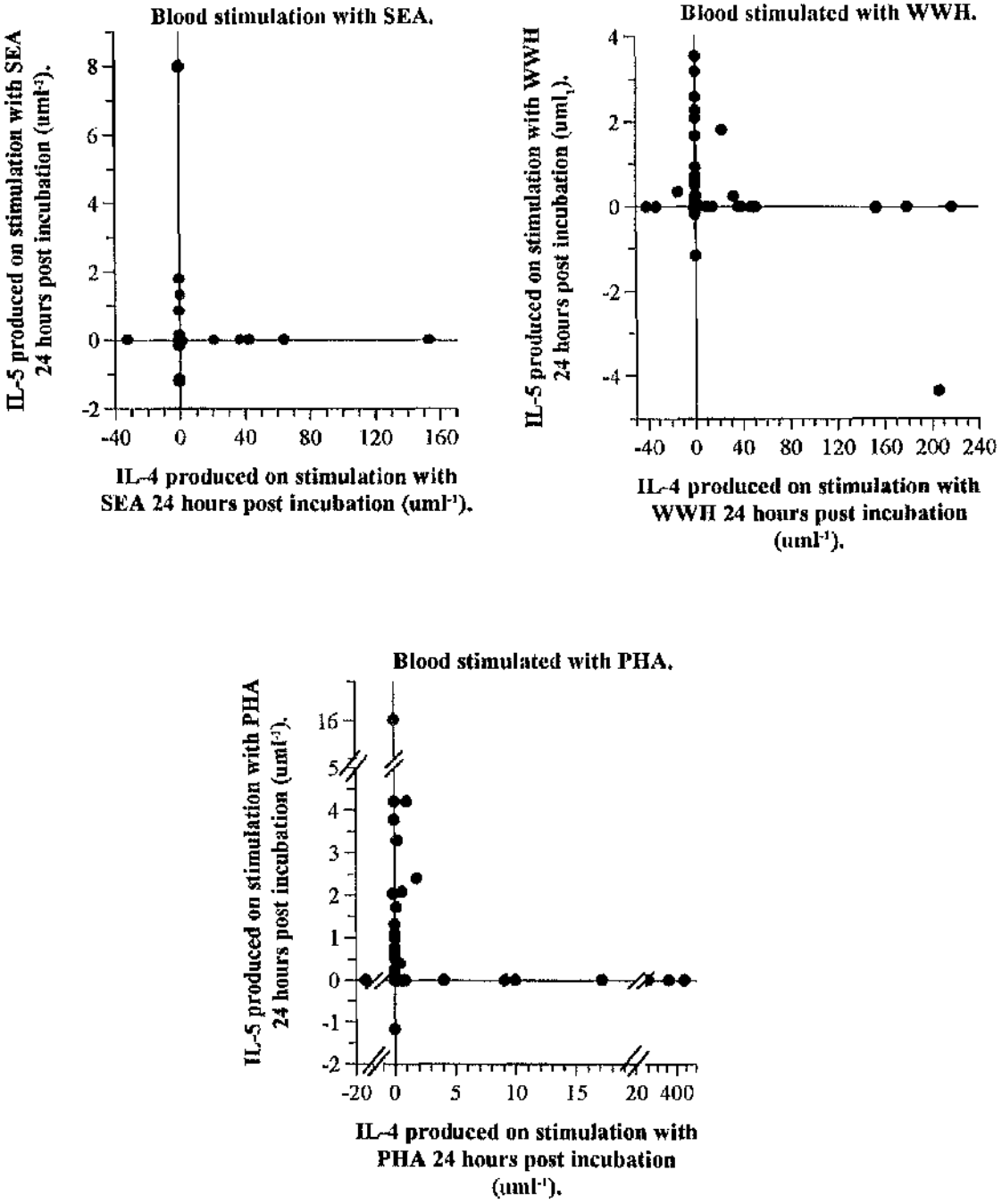


**Figure 4.19. A comparison of the levels of production of IL-4 and IL-5 after culturing for 48 hours with presence of one of four stimulants: CERC, SEA, WWH or PHA.**

Cytokines were produced by whole blood samples from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either SEA, WWH, CERC or PHA. Cytokines were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net levels of cytokine were compared. Net cytokine was comprised of cytokine produced when blood was cultured with a stimulant minus cytokine produced when blood was cultured without a stimulant.

**Figure 4.19.**

**A comparison of the levels of production of IL-4 and IL-5 after culturing for 48 hours in the presence of one of four stimulants: CERC, SEA, WWH or PHA.**

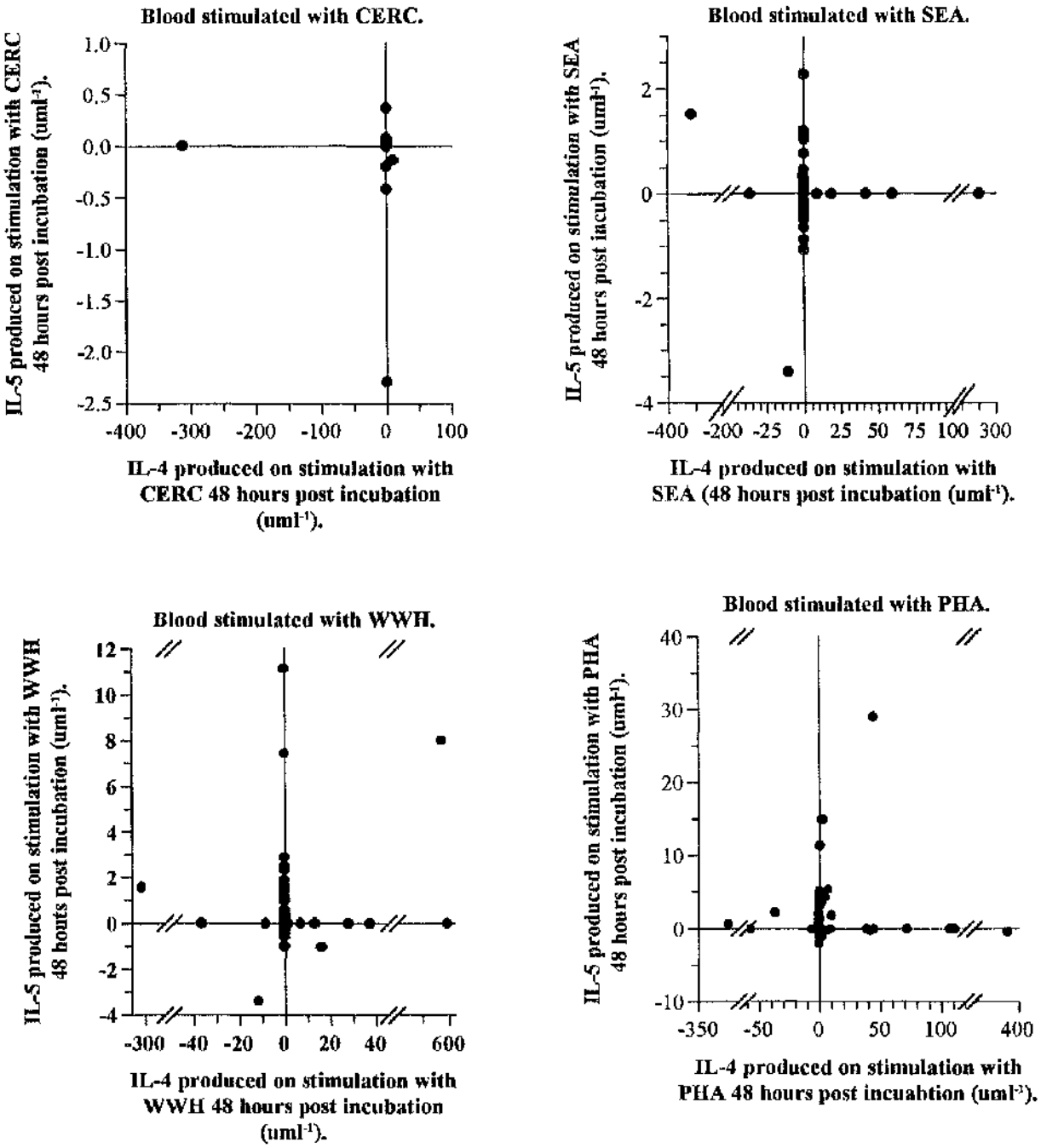
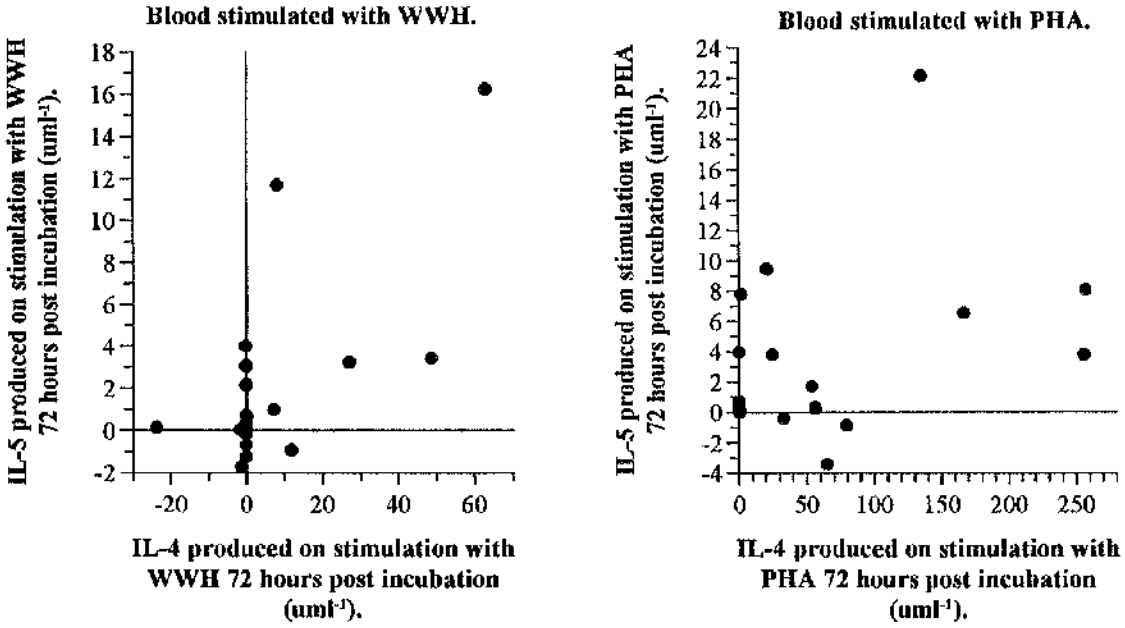


**Figure 4.20. A comparison of the levels of production of IL-4 and IL-5 after culturing for 72 hours with presence of one of two stimulants: WWH or PHA.**

Cytokines were produced by whole blood samples from Zimbabwean children taken 12 months after treatment for *S.haematobium* infection, cultured at a dilution of 1 in 2 with RPMI in 1ml aliquots in the presence of $10\mu gml^{-1}$ of either WWH or PHA . Cytokines were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $0.0625uml^{-1}$ and an accuracy of $0.01uml^{-1}$. Net levels of cytokine were compared. Net cytokine was comprised of cytokine produced when blood was cultured with a stimulant minus cytokine produced when blood was cultured without a stimulant.

**Figure 4.20 .**

**A comparison of the levels of production of IL-4 and IL-5 after culturing for 72 hours in the presence of one of two stimulants: WWH or PHA.**



# Chapter 5.

# General Discussion

**5.1 PBMC from children produce more IFNγ on stimulation with antigen than PBMC from adults.**

In the cross-sectional study in The Gambia, PBMC from children or infected individuals produced more net IFNγ on stimulation with WWH and SEA compared to PBMC from adults or uninfected individuals. I would therefore like to propose that the overriding immune response exhibited by children and infected individuals to schistosome antigens could be considered comparable to a murine Th1-type response.

Resistance to *S.mansoni* infection in the mouse can be mediated either by a Th1-type response when mice are vaccinated a single time with attenuated cercariae, or a Th2-type response in mice vaccinated three times with the same vaccine (Caulada-Benedetti *et al.,* 1991; Wynn *et al.,* 1996). If it is assumed that adults have a response that is more closely related to a Th-2-type, due to their lack of a Th1-type response, the multiple-vaccination model could be considered to mimic the type of immune response observed in adult humans, who would have gained their exposure as a result of trickle infections over a period of years.

In contrast with responses to antigen stimulation, PBMC stimulated with the mitogen PHA, from adults or uninfected individuals tended to produce more cytokine and proliferate than PBMC from children or infected individuals. Since these observations were due to mitogen, rather than antigen stimulation, they could represent some difference other than schistosome infection, the most likely being age since in the Gambian study uninfected individuals roughly corresponded with adults and infected individuals with children. It is reasonable to assume that

the adults in this study had past and continuing exposure to schistosomiasis because the people had a similar lifestyle to residents of the village of Madina, in the same area, where detailed water contact observations have been carried out (Wilkins *et al.* 1987, Hagan *et al.*, 1991). Given this assumption, the adults in this study were likely to have been exposed to infection for many more years than the children and have gained some degree of protection against infection since they had no detectable worm burden. The differences in immune response between children and adults could therefore be due to age related factors or reflect number of years of experience of *S.haematobium*.

Some evidence, however, suggests that Th2-type responses are also associated with pathology in mice (Sher *et al.*, 1991; Wynn *et al.*, 1995) and humans (Williams *et al.*, 1994). Extrapolating from the murine model, although natural immunity may be Th2-mediated in humans, it may be possible for a future vaccine to stimulate an artificial, but effective Th1-type of protection, which might avoid inducing pathology.

**5.2 Evidence that a protective profile of cytokine production may be due to the absence of IFNγ rather than increased IL-4 production.**

If the immune response switches from a Th1-type in infected individuals to a Th2-type in uninfected individuals, it might be expected that a person with high levels of IFNγ would also have low levels of IL-4 and *vice versa*. However no such dichotomous relationship was observed. This would suggest that the underlying mechanism of acquiring an appropriate protective immunity is not simply a switch from the dominance of one T-cell subtype to another. Instead, I would suggest that

the cytokine profile observed in adults in The Gambia, was not that of a positive Th2-type response, but was due rather, to the lack of a strong Th1-type response.

This hypothesis is supported by the observations that net IFNγ production was significantly different between infected and uninfected individuals on stimulation with both WWH and SEA, whereas the difference in net IL-4 production was significant only as a result of stimulation with the non-specific mitogen PHA. If this hypothesis is correct, then the induction of an artificially induced strong Th1-type response, although protective in mice, may have precisely the opposite effect in humans.

**5.3 Is resistance to *S. haematobium* infection acquired as a result of age or experience of infection?**

Evidence from new foci of schistosomiasis has indicated that the drop in intensity of infection observed with adulthood, is due to resistance acquired as a result of age, rather than as a result of years of experience of infection (Gryseels *et al.*, 1989; Gryseels, 1991a; Gryseels *et al.*, 1991b; Gryseels *et al.*, 1994b). Other studies, however, have indicated that experience of infection is the dominant factor in the development of resistance (Kloetzel *et al.*, 1967; Woolhouse, 1994) as previously discussed in section 1.2.

The study design in The Gambia did not differentiate between older people and those with more experience of infection, so it was not possible to address the question of age verses experience with that study. However, the Zimbabwean study did afford an opportunity to observe differences in the immune responses of

children who had experienced *S.haematobium* for the same length of time, but in areas of differing transmission dynamics.

The place of residence of a child was the dominant factor in determining cytokine profile. More children resident in the low prevalence area produced IL-4 and they produced more IFNγ and GM-CSF than children from the high prevalence area. These results implied that children from the low prevalence area had a stronger Th0-type immune response than children from the high prevalence area. More children resident in the high prevalence area, however, produced GM-CSF and IL-10 (a Th2- or macrophage-type profile) on stimulation with SEA than children from the low prevalence area.

If it is assumed that a protective cytokine profile is Th2-like, a possible interpretation of these results is that children resident in the high prevalence area, were developing faster towards a protective type of immune response than children resident in the low prevalence area, and therefore that experience of infection was a dominant factor in altering the type of immune response that the children exhibited to *S.haematobium* infection.

Although age was not a significant explanatory variable of cytokine production in Zimbabwe, in The Gambian study, there was a distinct difference in immune response between children and adults. Possibly, the immune response of children gradually changes as they gain more experience of infection, as seen in the Zimbabwe study from a Th0-type response, to a Th2-type response (as defined by production of IL-10). IL-10, possibly derived from macrophages rather than T-

cells, down-regulates the Th1-type responses, allowing what appears to be a predominant Th2 response to develop in adulthood, due to the relative lack of Th1-type cytokines such as IFNγ, as observed in The Gambia. There could either be a sudden switch to Th2 (or from Th1), as might be expected given the sudden drop in age-related intensity of infection observed in many endemic areas, or a gradual shift from Th0 to macrophage-induced suppression of Th1, resulting in a net Th2-type cytokine profile.

**5.4 IL-4 and IL-5 are produced by separate cell populations.**

IL-4 and IL-5 (both Th2-type cytokines) were rarely co-expressed in blood from the same child, when it was cultured for either 24 or 48 hours (section 4.4.6). One interpretation of these results is that IL-4 and IL-5 were expressed by separate cell populations. This hypothesis is supported by intra-cellular cytokine staining experiments (Jung *et al.,* 1995). Jung and colleagues (1995) observed that IL-4 and IL-5 were predominantly produced by different human peripheral T-cells, in blood from healthy volunteers and in patients with hyper-IgE syndrome. IFNγ and IL-2 (Th-1 type cytokines) were produced by the same cells.

Sewell and Mu (1996) have suggested that cytokines in micro-environments may mediate the development of Th0 cells to one of four T-cell sub-types, rather than the conventional two. They discuss evidence that IL-12 and IFNγ together, could mediate production of Th1 cells, IL-4 alone may mediate production of Th2 cells that can produce both IL-4 and IL-5, IL-12 and IL-4 in the absence of IFNγ could result in the development of IL-4 producing cells that do not produce IL-5, and

that IL-2, with some other unknown co-factor may modulate the development of IL-5 producing cells that do not also produce IL-4.

After culture for 72 hours, blood from a significant proportion of Zimbabwean children produced both IL-4 and IL-5 on stimulation with WWH and PHA. This could be interpreted in two ways. Either, after 72 hours cells co-expressed both cytokines, or another cell population began to produce cytokine at this time. The experimental design was not capable of distinguishing between these two scenarios. However, intra-cellular cytokine staining has revealed that, unlike freshly sampled T-cells, T-cell clones are capable of co-expressing both IL-4 and IL-5 and further, that it is possible to induce co-expression in fresh human T-cells by multiple stimulation and culture for fourteen days (Jung *et al.*, 1995). One possible interpretation of the Zimbabwean results therefore could be that in blood from an individual, cells started by producing only IL-4 or only IL-5 and then later started to produce the other cytokine as a result of multiple stimulation of the cells *in vitro*.

IL-4 and IL-5 are independently regulated in several disease systems (Sewell *et al.*, 1996) including atopic dermatitis (Kagi *et al.*, 1994), asthma (Walker *et al.*, 1992) and psoriasis (Vollmer *et al.*, 1994). IL-5 is generally associated with eosinophilia, whereas IL-4 is associated with IgE production. Where one of these cytokines was produced in the absence of the other, this was reflected in the resulting pathology (Sewell *et al.*, 1996). It is possible that immunity to schistosomiasis could be mediated by either IL-4 or IL-5.

It is interesting that almost no samples contained detectable IL-5 produced on stimulation with SEA or WWH in the Gambian study, and yet high levels of eosinophils were observed in children. IL-5, however, was detected in antigen-stimulated samples from the Zimbabwean study using the same ELISA protocol. A major difference between the two studies was the culture method. PBMC were utilised in The Gambia, whereas a whole blood stimulation method was used in Zimbabwe. It is possible that this difference between the two studies could be explained by eosinophils which, in The Gambia, because of their density, would have been pelleted with the red blood cells in the ficol-gradient centrifugation method of separating PBMC from whole blood.

Other studies, however, have reported detectable IL-5 produced on stimulation of PBMC with schistosome antigens (Roberts *et al.*, 1993; Grogan *et al.*, 1996), suggesting that the other cell types may also produce IL-5 under some circumstances. These results not with standing, it would be interesting to compare levels of IL-5 produced by PBMC and whole blood taken from the same cohort of people from a schistosomiasis endemic area, and measured using the same protocol. A potentially more powerful method is intra-cellular cytokine staining. If the intra-cellular cytokine staining method could be developed further, such that cytokines produced by cell types other than T-cells could be investigated, it may be possible to better elucidate the actual, rather than simply the potential, source of many cytokines.

Grogan and colleagues (1996) observed that IL-4 secretion by PBMC from people (both children and adults) infected with *S.haematobium* increased five weeks after

treatment, but there was no change in levels of IL-5. As previously discussed (sections 1.3.3 and 4.5), this could be due either to down-modulation of IL-4 production by the live adult worm, or stimulation of IL-4 producing cells as a result of antigen release on treatment with praziquantel. Irrespective of which hypothesis is correct, these observations indicate that IL-4 and IL-5 are modulated independently in *S.haematobium* infection.

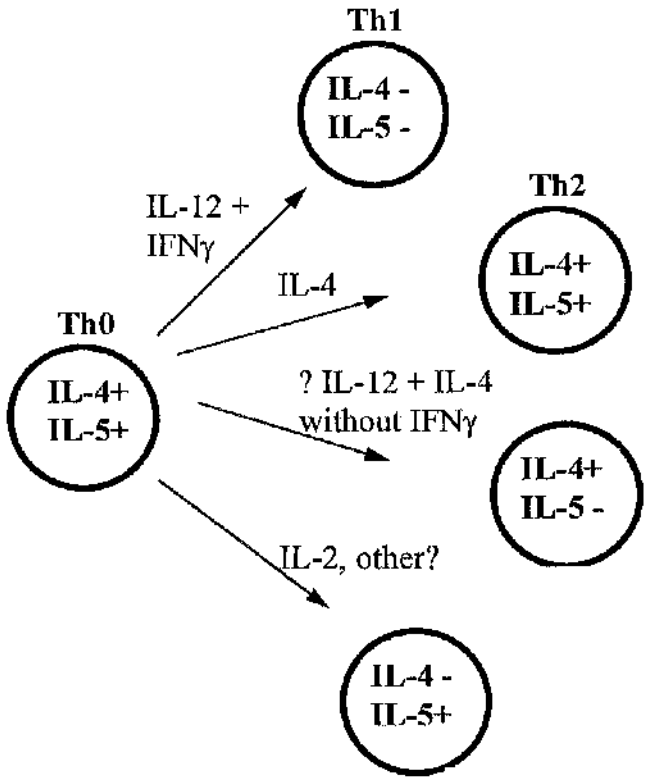
IL-5 produced on stimulation with PHA in Zimbabwe, one year post-treatment, was associated with pre-treatment infection status whereas, IL-4 produced on stimulation with SEA was associated with a lack of infection before treatment. These results could imply that antigen released on treatment resulted in an increased capacity to produce IL-5, but a decreased IL-4 response to stimulation with SEA (previously discussed in section 4.5).

**5.5 Summary of Conclusions**

In summary, the major conclusions of this thesis are as follows. PBMC from children or infected individuals produced more net IFNγ on stimulation with WWH and SEA compared to PBMC from adults or uninfected individuals. I would like to propose therefore, that the overriding immune response exhibited by children and infected individuals to schistosome antigens could be considered comparable to a murine Th1-type response. In children, experience is one factor in determining the type of immune response to *S.haematobium,* but this does not preclude the possibility that age is also an important factor. IL-4 and IL-5 are rarely co-expressed in blood from children resident in an area endemic for *S.haematobium* supporting the hypothesis that they are expressed by separate cell populations.

**Figure 5.1. Modulation of T-cell subtype development.**

Sewell and Mu (1996), suggested that different micro-environments, could result in Th0 cells developing into one of four different Th-subtypes, instead of the conventional two.




Sewell & Mu 1996

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# Appendices

**Appendix 1a.**

**A comparison of Gambian children and adults: gross data**

The population was split into children (<16 years) and adults (>25 years). Differences between the groups were tested with a Mann-Whitney U Rank comparison and accepted as statistically significant differences if $p<0.05$. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. Cell proliferation is expressed as $OD_{570nm}$. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $1uml^{-1}$ and an accuracy of $0.01uml^{-1}$. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line. Concentrations of IL-2, IL-4 (measured as described in section 2.3), IL-5, TNFα, GM-CSF are expressed as $uml^{-1}$. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturer's instructions. Results from Genzyme ELISAs are reported in $pgml^{-1}$. PBMC were separated from blood taken before treatment and cultured at a concentration of $1 \times 10^6$ in 0.1 ml aliquots for 5 days for the proliferation assays and 1ml aliquots for 48 hours for the production of cytokine with $10\mu gml^{-1}$ of either PHA, SEA or WWH.

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| GM-CSF | No stimulant | 0.06 $uml^{-1}$ | 1.33 $uml^{-1}$ | 3.02 $uml^{-1}$ | 27.00 | 27 | 0.79 $uml^{-1}$ | 1.86 $uml^{-1}$ | 2.46 $uml^{-1}$ | 32.53 | 32 | 0.218 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured without stimulation. |
| GM-CSF | PHA | 27.13 $uml^{-1}$ | 36.61 $uml^{-1}$ | 45.79 $uml^{-1}$ | 28.59 | 27 | 28.03 $uml^{-1}$ | 37.22 $uml^{-1}$ | 55.05 $uml^{-1}$ | 31.19 | 32 | 0.563 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with PHA. |
| GM-CSF | SEA | 0.15 $uml^{-1}$ | 2.25 $uml^{-1}$ | 9.40 $uml^{-1}$ | 29.57 | 27 | 0.97 $uml^{-1}$ | 1.89 $uml^{-1}$ | 3.06 $uml^{-1}$ | 30.36 | 32 | 0.861 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with SEA. |
| GM-CSF | WWH | 0.22 $uml^{-1}$ | 1.95 $uml^{-1}$ | 6.05 $uml^{-1}$ | 31.09 | 27 | 0.76 $uml^{-1}$ | 1.87 $uml^{-1}$ | 2.74 $uml^{-1}$ | 29.08 | 32 | 0.654 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with WWH. |
| IFNγ | No stimulant | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 21.96 | 24 | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 24.19 | 21 | 0.416 | There was no significant difference between production of IFNγ by PBMC from children or adults cultured without stimulation. |
| IFNγ | PHA | 371.20 $pgml^{-1}$ | 629.00 $pgml^{-1}$ | 876.75 $pgml^{-1}$ | 26.58 | 24 | 229.00 $pgml^{-1}$ | 394.00 $pgml^{-1}$ | 644.50 $pgml^{-1}$ | 18.90 | 21 | 0.050 | When cultured with PHA, PBMC from adults produced more IFNγ compared to PBMC from children. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IFNγ | SEA | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 1.75 $pgml^{-1}$ | 18.38 | 24 | 0.00 $pgml^{-1}$ | 12.00 $pgml^{-1}$ | 50.50 $pgml^{-1}$ | 28.29 | 21 | 0.006 | When cultured with SEA, PBMC from children produced more IFNγ compared to PBMC from adults. |
| IFNγ | WWH | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 1.75 $pgml^{-1}$ | 17.98 | 24 | 0.00 $pgml^{-1}$ | 7.00 $pgml^{-1}$ | 38.50 $pgml^{-1}$ | 28.74 | 21 | 0.003 | When cultured with WWH, PBMC from children produced more IFNγ compared to PBMC from adults. |
| IL-2 | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 21.05 | 20 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.95 | 20 | 0.515 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured without stimulation. |
| IL-2 | PHA | 6.88 $uml^{-1}$ | 11.75 $uml^{-1}$ | 20.00 $uml^{-1}$ | 24.63 | 19 | 0.69 $uml^{-1}$ | 4.94 $uml^{-1}$ | 9.31 $uml^{-1}$ | 25.60 | 20 | 0.013 | When cultured with PHA, PBMC from adults produced more IL-2 compared to PBMC from children. |
| IL-2 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 24.40 | 20 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.60 | 20 | 0.433 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured without stimulation. |
| IL-2 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 21.86 | 21 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.10 | 20 | 0.362 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured with WWH. |
| IL-4 (Genzyme Kit) | No stimulant | 1.50 $pgml^{-1}$ | 11.50 $pgml^{-1}$ | 23.25 $pgml^{-1}$ | 24.15 | 24 | 0.50 $pgml^{-1}$ | 11.00 $pgml^{-1}$ | 18.50 $pgml^{-1}$ | 21.69 | 21 | 0.529 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured without stimulation. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-4 (Genzyme Kit) | PHA | 673.00 $pgml^{-1}$ | 1109 .00 $pgml^{-1}$ | 1518.50 $pgml^{-1}$ | 29.92 | 24 | 265.50 $pgml^{-1}$ | 441.00 $pgml^{-1}$ | 613.00 $pgml^{-1}$ | 15.10 | 21 | 0.001 | PBMC from adults produced more IL-4 on stimulation with PHA than PBMC from children. |
| IL-4 (Genzyme Kit) | WWH | 5.75 $pgml^{-1}$ | 14.5 $pgml^{-1}$ | 23.00 $pgml^{-1}$ | 24.63 | 24 | 0.00 $pgml^{-1}$ | 2.00 $pgml^{-1}$ | 22.50 $pgml^{-1}$ | 21.14 | 21 | 0.367 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with WWH. |
| IL-4 (Genzyme kit) | SEA | 0.75 $pgml^{-1}$ | 13.50 $pgml^{-1}$ | 24.50 $pgml^{-1}$ | 24.40 | 24 | 0.00 $pgml^{-1}$ | 8.00 $pgml^{-1}$ | 22.00 $pgml^{-1}$ | 21.40 | 21 | 0.440 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with SEA. |
| IL-4 (ELISA as in section 2.3) | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.00 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.00 | 32 | 1.000 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured without stimulation. |
| IL-4 (ELISA as in section 2.3) | PHA | 0.41 $uml^{-1}$ | 4.34 $uml^{-1}$ | 5.89 $uml^{-1}$ | 36.74 | 27 | 0.11 $uml^{-1}$ | 0.88 $uml^{-1}$ | 2.00 $uml^{-1}$ | 24.31 | 32 | 0.005 | PBMC from adults produced more IL-4 on stimulation with PHA than PBMC from children.. |
| IL-4 (ELISA as in section 2.3) | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.50 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.27 | 32 | 0.106 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with WWH. |
| IL-4 (ELISA as in section 2.3) | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-2}$ | 27.07 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 32.47 | 32 | 0.043 | PBMC from children produced more IL-4 on stimulation with SEA than PBMC from adults. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-5 | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.50 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.27 | 32 | 0.106 | There was no significant difference between production of IL-5 by PBMC from children or adults cultured without stimulation. |
| IL-5 | PHA | 1.50 $uml^{-1}$ | 8.10 $uml^{-1}$ | 11.86 $uml^{-1}$ | 30.91 | 27 | 2.27 $uml^{-1}$ | 7.20 $uml^{-1}$ | 10.47 $uml^{-1}$ | 29.23 | 32 | 0.709 | There was no significant difference between production of IL-5 by PBMC from children or adults cultured with PHA. |
| IL-5 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.56 | 26 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.27 | 32 | 0.383 | There was no significant difference between production of IL-5 by PBMC from children or adults cultured with SEA. |
| IL-5 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.94 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.24 | 31 | 0.250 | There was no significant difference between production of IL-5 by PBMC from children or adults cultured with WWH. |
| TNFα | No stimulant | 0.17 $uml^{-1}$ | 0.62 $uml^{-1}$ | 2.12 $uml^{-1}$ | 31.96 | 27 | 0.01 $uml^{-1}$ | 0.33 $uml^{-1}$ | 1.70 $uml^{-1}$ | 28.34 | 32 | 0.418 | There was no significant difference between production of TNFα by PBMC from children or adults cultured without stimulant. |
| TNFα | PHA | 17.25 $uml^{-1}$ | 27.80 $uml^{-1}$ | 41.69 $uml^{-1}$ | 30.00 | 27 | 21.56 $uml^{-1}$ | 30.82 $uml^{-1}$ | 38.31 $uml^{-1}$ | 29.97 | 32 | 0.988 | There was no significant difference between production of TNFα by PBMC from children or adults cultured with PHA |
| TNFα | SEA | 0.35 $uml^{-1}$ | 0.642 $uml^{-1}$ | 3.143 $uml^{-1}$ | 31.57 | 27 | 0.10 $uml^{-1}$ | 0.55 $uml^{-1}$ | 2.77 $uml^{-1}$ | 28.67 | 32 | 0.518 | There was no significant difference between production of TNFα by PBMC from children or adults cultured with SEA. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| TNFα | WWH | 0.29 $uml^{-1}$ | 0.76 $uml^{-1}$ | 3.32 $uml^{-1}$ | 33.09 | 27 | 0.02 $uml^{-1}$ | 0.38 $uml^{-1}$ | 2.67 $uml^{-1}$ | 27.39 | 32 | 0.203 | There was no significant difference between production of TNFα by PBMC from children or adults cultured withWWH. |
| Proliferative response | No stimulant | 0.42 $OD_{570nm}$ | 0.65 $OD_{570nm}$ | 0.68 $OD_{570nm}$ | 36.94 | 27 | 0.27 $OD_{570nm}$ | 0.40 $OD_{570nm}$ | 0.49 $OD_{570nm}$ | 24.14 | 32 | 0.004 | PBMC from adults proliferate more than PBMC from children when cultured without stimulant. |
| Proliferative response | PHA | 0.46 $OD_{570nm}$ | 0.62 $OD_{570nm}$ | 0.81 $OD_{570nm}$ | 39.11 | 27 | 0.38 $OD_{570nm}$ | 0.42 $OD_{570nm}$ | 0.48 $OD_{570nm}$ | 22.31 | 32 | 0.001 | PBMC from adults proliferate more than PBMC from children when cultured with PHA. |
| Proliferative response | SEA | 0.36 $OD_{570nm}$ | 0.60 $OD_{570nm}$ | 0.67 $OD_{570nm}$ | 37.13 | 27 | 0.26 $OD_{570nm}$ | 0.40 $OD_{570nm}$ | 0.51 $OD_{570nm}$ | 23.98 | 32 | 0.003 | PBMC from adults proliferate more than PBMC from children when cultured with SEA. |
| Proliferative response | WWH | 0.42 $OD_{570nm}$ | 0.63 $OD_{570nm}$ | 0.70 $OD_{570nm}$ | 37.89 | 27 | 0.27 $OD_{570nm}$ | 0.39 $OD_{570nm}$ | 0.50 $OD_{570nm}$ | 23.34 | 32 | 0.001 | PBMC from adults proliferate more than PBMC from children when cultured with WWH. |

**Appendix 1b.**

**A comparison of children and adults: Net data.**

The population was split into children (<16 years) and adults (>25 years). Differences between the groups were tested with a Mann-Whitney U Rank comparison and accepted as statistically significant differences if $p<0.05$. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. Cell proliferation is expressed as $OD_{570nm}$. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $1uml^{-1}$ and an accuracy of $0.01uml^{-1}$. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturer's instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line. Concentrations of IL-2, IL-4, IL-5, TNFα, GM-CSF and IFNγ are expressed as $uml^{-1}$. PBMC were separated from blood taken before treatment and cultured at a concentration of $1 \times 10^6$ in 0.1 ml aliquots for 5 days for the proliferation assays and 1ml aliquots for 48 hours for the production of cytokine with $10\mu gml^{-1}$ of either PHA, SEA or WWH. All comparisons were made using net data. Net data are the amount of cytokine or cell proliferation produced by PBMC when cultured with stimulant minus levels produced by PBMC cultured without stimulant.

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| GM-CSF | PHA | 21.99 $uml^{-1}$ | 33.66 $uml^{-1}$ | 38.63 $uml^{-1}$ | 28.63 | 27 | 20.95 $uml^{-1}$ | 31.31 $uml^{-1}$ | 53.88 $uml^{-1}$ | 31.16 | 32 | 0.574 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with PHA. |
| GM-CSF | SEA | -0.20 $uml^{-1}$ | 0.02 $uml^{-1}$ | 0.43 $uml^{-1}$ | 26.85 | 27 | 0.02 $uml^{-1}$ | 0.10 $uml^{-1}$ | 0.63 $uml^{-1}$ | 32.66 | 32 | 0.196 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with SEA. |
| GM-CSF | WWH | -0.09 $uml^{-1}$ | 0.08 $uml^{-1}$ | 0.36 $uml^{-1}$ | 32.22 | 27 | -0.10 $uml^{-1}$ | 0.02 $uml^{-1}$ | 0.23 $uml^{-1}$ | 28.13 | 32 | 0.361 | There was no significant difference between production of GM-CSF by PBMC from children or adults cultured with WWH. |
| IFNγ | PHA | 9.67 $uml^{-1}$ | 28.26 $uml^{-1}$ | 69.38 $uml^{-1}$ | 26.63 | 24 | 4.51 $uml^{-1}$ | 10.65 $uml^{-1}$ | 29.84 $uml^{-1}$ | 18.86 | 21 | 0.048 | PBMC from adults produced more IFNγ on culture with PHA than PBMC from children. |
| IFNγ | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.42 | 24 | 0.00 $uml^{-1}$ | 0.05 $uml^{-1}$ | 0.58 $uml^{-1}$ | 27.10 | 21 | 0.039 | PBMC from children produced more IFNγ on culture with SEA than PBMC from adults. |
| IFNγ | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 19.42 | 24 | 0.00 $uml^{-1}$ | 0.04 $uml^{-1}$ | 0.37 $uml^{-1}$ | 27.10 | 21 | 0.042 | PBMC from children produced more IFNγ on culture with WWH than PBMC from adults. |
| IL-2 | PHA | 3.33 $uml^{-1}$ | 8.10 $uml^{-1}$ | 10.26 $uml^{-1}$ | 18.68 | 11 | 0.00 $uml^{-1}$ | 4.33 $uml^{-1}$ | 7.33 $uml^{-1}$ | 13.66 | 19 | 0.128 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured with PHA. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-2 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.39 | 19 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.63 | 20 | 0.753 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured with PHA. |
| IL-2 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.89 | 19 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.15 | 20 | 0.447 | There was no significant difference between production of IL-2 by PBMC from children or adults cultured with WWH. |
| IL-4 (Genzyme Kit) | PHA | 0.78 $uml^{-1}$ | 1.79 $uml^{-1}$ | 3.61 $uml^{-1}$ | 29.96 | 24 | 0.27 $uml^{-1}$ | 0.48 $uml^{-1}$ | 0.69 $uml^{-1}$ | 15.05 | 21 | 0.001 | PBMC from adults produced more IL-4 on culture with PHA than PBMC from children. |
| IL-4 (Genzyme Kit) | SEA | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 23.17 | 24 | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 22.81 | 21 | 0.927 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with SEA. |
| IL-4 (Genzyme Kit) | WWH | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 23.83 | 24 | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 22.05 | 21 | 0.648 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with WWH. |
| IL-4 (ELISA as in section 2.3) | PHA | 0.18 $uml^{-1}$ | 0.89 $uml^{-1}$ | 4.34 $uml^{-1}$ | 28.19 | 27 | 0.02 $uml^{-1}$ | 1.47 $uml^{-1}$ | 4.80 $uml^{-1}$ | 31.53 | 32 | 0.454 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with PHA. |
| IL-4 (ELISA as in section 2.3) | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.96 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.34 | 32 | 0.151 | There was no significant difference between production of IL-4 by PBMC from children or adults cultured with SEA. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-4 (ELISA as in section 2.3) | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.11 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 29.91 | 32 | 0.884 | There was no significant difference between IL-4 produced on culture with WWH by PBMC from adults and PBMC from children. |
| IL-5 | PHA | 1.49 $uml^{-1}$ | 8.10 $uml^{-1}$ | 11.86 $uml^{-1}$ | 30.91 | 27 | 2.27 $uml^{-1}$ | 7.20 $uml^{-1}$ | 10.74 $uml^{-1}$ | 29.23 | 32 | 0.709 | There was no significant difference between IL-5 produced on culture with PHA by PBMC from adults and PBMC from children. |
| IL-5 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.00 | 26 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 29.09 | 32 | 0.719 | There was no significant difference between IL-5 produced on culture with SEA by PBMC from adults and PBMC from children. |
| IL-5 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.80 | 27 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 27.50 | 31 | 0.107 | There was no significant difference between IL-5 produced on culture with WWH by PBMC from adults and PBMC from children. |
| TNFα | PHA | 17.25 $uml^{-1}$ | 27.10 $uml^{-1}$ | 41.52 $uml^{-1}$ | 30.44 | 27 | 18.57 $uml^{-1}$ | 29.66 $uml^{-1}$ | 38.04 $uml^{-1}$ | 29.63 | 32 | 0.855 | There was no significant difference between TNFα produced on culture with PHA by PBMC from adults and PBMC from children. |
| TNFα | SEA | -0.17 $uml^{-1}$ | 0.17 $uml^{-1}$ | 0.88 $uml^{-1}$ | 29.30 | 27 | 0.00 $uml^{-1}$ | 0.18 $uml^{-1}$ | 1.02 $uml^{-1}$ | 30.59 | 32 | 0.772 | There was no significant difference between TNFα produced on culture with SEA by PBMC from adults and PBMC from children. |
| TNFα | WWH | -0.27 $uml^{-1}$ | 0.04 $uml^{-1}$ | 1.10 $uml^{-1}$ | 29.20 | 27 | -0.15 $uml^{-1}$ | 0.09 $uml^{-1}$ | 1.03 $uml^{-1}$ | 30.67 | 32 | 0.743 | There was no significant difference between TNFα produced on culture with WWH by PBMC from adults and PBMC from children. |

| | | Adults | | | | | Children | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| Proliferative response | PHA | -0.13 $OD_{570nm}$ | 0.10 $OD_{570nm}$ | 0.28 $OD_{570nm}$ | 31.93 | 27 | -0.12 $OD_{570nm}$ | -0.02 $OD_{570nm}$ | 0.20 $OD_{570nm}$ | 28.38 | 32 | 0.429 | There was no significant difference between proliferation on culture with PHA by PBMC from adults and PBMC from children. |
| Proliferative response | SEA | -0.06 $OD_{570nm}$ | -0.01 $OD_{570nm}$ | 0.01 $OD_{570nm}$ | 28.11 | 27 | -0.03 $OD_{570nm}$ | 0.00 $OD_{570nm}$ | 0.01 $OD_{570nm}$ | 31.59 | 32 | 0.438 | There was no significant difference between proliferation on culture with SEA by PBMC from adults and PBMC from children. |
| Proliferative response | WWH | -0.06 $OD_{570nm}$ | 0.02 $OD_{570nm}$ | 0.04 $OD_{570nm}$ | 31.46 | 27 | -0.02 $OD_{570nm}$ | 0.00 $OD_{570nm}$ | 0.03 $OD_{570nm}$ | 28.77 | 32 | 0.548 | There was no significant difference between proliferation on culture with WWH by PBMC from adults and PBMC from children. |

**Appendix 1c.**

**A comparison of infected and uninfected individuals from The Gambia: Gross data**

The population was split into individuals either infected or uninfected with *S.haematobium* from The Gambia. Infection status was determined by the presence of *S.haematobium* eggs in urine. Differences between the groups were tested with a Mann-Whitney U Rank comparison and accepted as statistically significant differences if $p<0.05$. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. Cell proliferation is expressed as $OD_{570nm}$. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $1uml^{-1}$ and an accuracy of $0.01uml^{-1}$. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line. Concentrations of IL-2, IL-4 (measured as described in section 2.3), IL-5, TNFα, GM-CSF are expressed as $uml^{-1}$. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturer's instructions. Results from Genzyme ELISAs are reported in $pgml^{-1}$. PBMC were separated from blood taken before treatment and cultured at a concentration of $1 \times 10^6$ in 0.1 ml aliquots for 5 days for the proliferation assays and 1ml aliquots for 48 hours for the production of cytokine with $10\mu gml^{-1}$ of either PHA, SEA or WWH.

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| GM-CSF | No stimulant | 0.12 $uml^{-1}$ | 1.43 $uml^{-1}$ | 2.48 $uml^{-1}$ | 26.07 | 29 | 0.73 $uml^{-1}$ | 1.98 $uml^{-1}$ | 3.02 $uml^{-1}$ | 33.80 | 30 | 0.084 | There was no significant difference between GM-CSF produced by PBMC from infected or uninfected individuals cultured without stimulation. |
| GM-CSF | PHA | 25.81 $uml^{-1}$ | 36.56 $uml^{-1}$ | 44.14 $uml^{-1}$ | 26.86 | 29 | 30.38 $uml^{-1}$ | 39.96 $uml^{-1}$ | 56.26 $uml^{-1}$ | 33.03 | 30 | 0.168 | There was no significant difference between GM-CSF produced by PBMC from infected or uninfected individuals cultured with PHA. |
| GM-CSF | SEA | 0.29 $uml^{-1}$ | 1.91 $uml^{-1}$ | 3.41 $uml^{-1}$ | 28.88 | 29 | 0.87 $uml^{-1}$ | 2.00 $uml^{-1}$ | 3.77 $uml^{-1}$ | 31.08 | 30 | 0.622 | There was no significant difference between GM-CSF produced by PBMC from infected or uninfected individuals cultured with SEA. |
| GM-CSF | WWH | 0.33 $uml^{-1}$ | 1.81 $uml^{-1}$ | 3.30 $uml^{-1}$ | 30.22 | 29 | 0.74 $uml^{-1}$ | 1.89 $uml^{-1}$ | 2.89 $uml^{-1}$ | 29.78 | 30 | 0.921 | There was no significant difference between GM-CSF produced by PBMC from infected or uninfected individuals cultured with WWH. |
| IFNγ | No stimulant | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 22.11 | 23 | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 1.00 $pgml^{-1}$ | 23.93 | 22 | 0.505 | There was no significant difference between IFNγ produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| IFNγ | PHA | 359.00 $pgml^{-1}$ | 599.00 $pgml^{-1}$ | 888.00 $pgml^{-1}$ | 26.39 | 23 | 231.00 $pgml^{-1}$ | 397.00 $pgml^{-1}$ | 685.75 $pgml^{-1}$ | 19.45 | 22 | 0.077 | There was no significant difference between IFNγ produced by PBMC from infected or uninfected individuals cultured with PHA. |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IFNγ | SEA | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 1.00 $pgml^{-1}$ | 17.65 | 23 | 0.00 $pgml^{-1}$ | 16.50 $pgml^{-1}$ | 48.25 $pgml^{-1}$ | 28.59 | 22 | 0.002 | PBMC from infected individuals produced more IFNγ more when cultured with SEA than PBMC from uninfected individuals. |
| IFNγ | WWH | 0.00 $pgml^{-1}$ | 0.00 $pgml^{-1}$ | 1.00 $pgml^{-1}$ | 17.30 | 23 | 0.00 $pgml^{-1}$ | 8.00 $pgml^{-1}$ | 35.75 $pgml^{-1}$ | 28.95 | 22 | 0.001 | PBMC from infected individuals produced more IFNγ more when cultured with WWH than PBMC from uninfected individuals. |
| IL-2 | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.78 | 23 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.12 | 17 | 0.697 | There was no significant difference between IL-2 produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| IL-2 | PHA | 6.26 $uml^{-1}$ | 10.76 $uml^{-1}$ | 18.88 $uml^{-1}$ | 23.64 | 22 | 0.00 $uml^{-1}$ | 4.33 $uml^{-1}$ | 11.17 $uml^{-1}$ | 15.29 | 17 | 0.023 | PBMC from uninfected individuals produced more IL-2 more when cultured with PHA than PBMC from infected individuals. |
| IL-2 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.89 | 23 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.97 | 17 | 0.692 | There was no significant difference between IL-2 produced by PBMC from infected or uninfected individuals cultured with SEA. |
| IL-2 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 21.50 | 24 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.29 | 17 | 0.537 | There was no significant difference between IL-2 produced by PBMC from infected or uninfected individuals cultured with WWH. |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-4 (Genzyme Kit) | No stimulant | 1.00 $pgml^{-1}$ | 14.00 $pgml^{-1}$ | 24.00 $pgml^{-1}$ | 24.33 | 23 | 0.75 $pgml^{-1}$ | 10.50 $pgml^{-1}$ | 18.25 $pgml^{-1}$ | 21.61 | 22 | 0.486 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| IL-4 (Genzyme Kit) | PHA | 666.00 $pgml^{-1}$ | 1080.00 $pgml^{-1}$ | 1511.0 $pgml^{-1}$ | 29.48 | 23 | 272.75 $pgml^{-1}$ | 455.00 $pgml^{-1}$ | 626.75 $pgml^{-1}$ | 16.23 | 22 | 0.001 | PBMC from uninfected individuals produced more IL-4 more when cultured with PHA than PBMC from infected individuals. |
| IL-4 (Genzyme Kit) | SEA | 0.00 $pgml^{-1}$ | 14.00 $pgml^{-1}$ | 25.00 $pgml^{-1}$ | 24.67 | 23 | 0.00 $pgml^{-1}$ | 8.00 $pgml^{-1}$ | 19.00 $pgml^{-1}$ | 21.25 | 22 | 0.376 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured with SEA. |
| IL-4 (Genzyme Kit) | WWH | 5.00 $pgml^{-1}$ | 14.00 $pgml^{-1}$ | 23.00 $pgml^{-1}$ | 23.87 | 23 | 0.00 $pgml^{-1}$ | 9.50 $pgml^{-1}$ | 26.75 $pgml^{-1}$ | 22.09 | 22 | 0.645 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured with WWH. |
| IL-4 (ELISA as described in section 2.3) | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.00 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.00 | 30 | 1.000 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| IL-4 (ELISA as described in section 2.3) | PHA | 0.49 $uml^{-1}$ | 3.63 $uml^{-1}$ | 5.618 $uml^{-1}$ | 36.19 | 29 | 0.02 $uml^{-1}$ | 0.88 $uml^{-1}$ | 2.25 $uml^{-1}$ | 24.02 | 30 | 0.006 | PBMC from uninfected individuals produced more IL-4 when cultured with PHA than PBMC from infected individuals. |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-4 (ELISA as described in section 2.3) | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.50 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.45 | 30 | 0.083 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured with WWH. |
| IL-4 (ELISA as described in section 2.3) | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 27.97 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 31.97 | 30 | 0.133 | There was no significant difference between IL-4 produced by PBMC from infected or uninfected individuals cultured with SEA. |
| IL-5 | No stimulant | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 29.55 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.43 | 30 | 0.605 | There was no significant difference between IL-5 produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| IL-5 | PHA | 1.45 $uml^{-1}$ | 7.30 $uml^{-1}$ | 11.58 $uml^{-1}$ | 29.16 | 29 | 2.58 $uml^{-1}$ | 7.62 $uml^{-1}$ | 10.83 $uml^{-1}$ | 30.82 | 30 | 0.710 | There was no significant difference between IL-5 produced by PBMC from infected or uninfected individuals cultured with PHA. |
| IL-5 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.48 | 28 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.45 | 30 | 0.313 | There was no significant difference between IL-5 produced by PBMC from infected or uninfected individuals cultured with SEA. |
| IL-5 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.64 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.36 | 29 | 0.332 | There was no significant difference between IL-5 produced by PBMC from infected or uninfected individuals cultured with WWH. |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| TNFα | No stimulant | 0.16 $uml^{-1}$ | 0.62 $uml^{-1}$ | 1.77 $uml^{-1}$ | 31.72 | 29 | 0.00 $uml^{-1}$ | 0.35 $uml^{-1}$ | 1.81 $uml^{-1}$ | 28.33 | 30 | 0.446 | There was no significant difference between TNFα produced by PBMC from infected or uninfected individuals cultured without stimulant. |
| TNFα | PHA | 19.05 $uml^{-1}$ | 33.38 $uml^{-1}$ | 41.27 $uml^{-1}$ | 30.78 | 29 | 21.24 $uml^{-1}$ | 29.83 $uml^{-1}$ | 36.28 $uml^{-1}$ | 29.25 | 30 | 0.733 | There was no significant difference between TNFα produced by PBMC from infected or uninfected individuals cultured with PHA. |
| TNFα | SEA | 0.35 $uml^{-1}$ | 0.96 $uml^{-1}$ | 3.29 $uml^{-1}$ | 32.53 | 29 | 0.10 $uml^{-1}$ | 0.45 $uml^{-1}$ | 2.52 $uml^{-1}$ | 27.55 | 30 | 0.265 | There was no significant difference between TNFα produced by PBMC from infected or uninfected individuals cultured with SEA. |
| TNFα | WWH | 0.28 $uml^{-1}$ | 1.27 $uml^{-1}$ | 3.34 $uml^{-1}$ | 30.05 | 29 | 0.01 $uml^{-1}$ | 0.36 $uml^{-1}$ | 2.09 $uml^{-1}$ | 26.08 | 3* | 0.074 | There was no significant difference between TNFα produced by PBMC from infected or uninfected individuals cultured with WWH. |
| Proliferative response | No stimulant | 0.34 $OD_{570nm}$ | 0.62 $OD_{570nm}$ | 0.68 $OD_{570nm}$ | 33.64 | 29 | 0.32 $OD_{570nm}$ | 0.42 $D_{570nm}$ | 0.50 $OD_{570nm}$ | 26.48 | 30 | 0.110 | There was no significant difference between proliferative responses of PBMC from infected or uninfected individuals cultured without stimulation. |
| Proliferative response | PHA | 0.42 $OD_{570nm}$ | 0.60 $OD_{570nm}$ | 0.77 $OD_{570nm}$ | 37.59 | 29 | 0.37 $OD_{570nm}$ | 0.42 $OD_{570nm}$ | 0.48 $OD_{570nm}$ | 22.67 | 30 | 0.001 | PBMC from uninfected individuals proliferated more when cultured with PHA than PBMC from infected individuals. |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Gross cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| Proliferative response | SEA | 0.33 $OD_{570nm}$ | 0.56 $OD_{570nm}$ | 0.67 $OD_{570nm}$ | 33.95 | 29 | 0.30 $OD_{570nm}$ | 0.41 $OD_{570nm}$ | 0.55 $OD_{570nm}$ | 26.18 | 30 | 0.083 | There was no significant difference between proliferative responses of PBMC from infected or uninfected individuals cultured with SEA. |
| Proliferative response | WWH | 0.34 $OD_{570nm}$ | 0.56 $OD_{570nm}$ | 0.69 $OD_{570nm}$ | 34.72 | 29 | 0.32 $OD_{570nm}$ | 0.41 $OD_{570nm}$ | 0.63 $OD_{570nm}$ | 25.43 | 30 | 0.038 | PBMC from uninfected individuals proliferated more when cultured with WWH than PBMC from infected individuals. |

**Appendix 1d.**

**A comparison of infected and uninfected individuals from The Gambia:**

**Net data.**

The population was split into individuals either infected or uninfected with *S.haematobium* from The Gambia. Infection status was determined by the presence of *S.haematobium* eggs in urine. Differences between the groups were tested with a Mann-Whitney U Rank comparison and accepted as statistically significant differences if $p<0.05$. Cell proliferation was measured using a colorimetric assay (Promega), according to the manufacturer's instructions. Cell proliferation is expressed as $OD_{570nm}$. IL-4, IL-5, TNFα and GM-CSF, were measured using sandwich ELISAs as described in section 2.3, which have a sensitivity of $1uml^{-1}$ and an accuracy of $0.01uml^{-1}$. IL-4 and IFNγ were measured using Genzyme sandwich ELISA kits according to the manufacturer's instructions. IL-2 was measured using a bioassay based on the proliferation of a CTLL cell line. Concentrations of IL-2, IL-4, IL-5, TNFα, GM-CSF and IFNγ are expressed as $uml^{-1}$. PBMC were separated from blood taken before treatment and cultured at a concentration of $1 \times 10^6$ in 0.1 ml aliquots for 5 days for the proliferation assays and 1ml aliquots for 48 hours for the production of cytokine with $10\mu gml^{-1}$ of either PHA, SEA or WWH. All comparisons were made using net data. Net data are the amount of cytokine or cell proliferation produced by PBMC when cultured with stimulant minus levels produced by PBMC cultured without stimulant.

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| GM-CSF | PHA | 19.83 $uml^{-1}$ | 32.35 $uml^{-1}$ | 37.86 $uml^{-1}$ | 27.78 | 29 | 21.90 $uml^{-1}$ | 32.58 $uml^{-1}$ | 55.38 $uml^{-1}$ | 32.17 | 30 | 0.324 | There was no significant difference between production of GM-CSF by PBMC from infected or uninfected individuals cultured with PHA |
| GM-CSF | SEA | -0.12 $uml^{-1}$ | 0.07 $uml^{-1}$ | 0.69 $uml^{-1}$ | 28.34 | 29 | 0.02 $uml^{-1}$ | 0.10 $uml^{-1}$ | 0.55 $uml^{-1}$ | 31.60 | 30 | 0.467 | There was no significant difference between production of GM-CSF by PBMC from infected or uninfected individuals cultured with SEA. |
| GM-CSF | WWH | -0.06 $uml^{-1}$ | 0.07 $uml^{-1}$ | 0.48 $uml^{-1}$ | 32.72 | 29 | -0.14 $uml^{-1}$ | 0.02 $uml^{-1}$ | 0.22 $uml^{-1}$ | 27.37 | 30 | 0.231 | There was no significant difference between production of GM-CSF by PBMC from infected or uninfected individuals cultured with WWH |
| IFNγ | PHA | 9.09 $uml^{-1}$ | 25.03 $uml^{-1}$ | 72.09 $uml^{-1}$ | 26.43 | 23 | 4.58 $uml^{-1}$ | 10.83 $uml^{-1}$ | 34.91 $uml^{-1}$ | 19.41 | 22 | 0.073 | There was no significant difference between production of IFNγ by PBMC from infected or uninfected individuals cultured with PHA |
| IFNγ | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 18.65 | 23 | 0.00 $uml^{-1}$ | 0.11 $uml^{-1}$ | 0.54 $uml^{-1}$ | 27.55 | 22 | 0.017 | PBMC from infected individuals produced more IFNγ on stimulation with SEA than PBMC from uninfected individuals. |
| IFNγ | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 18.72 | 23 | 0.00 $uml^{-1}$ | 0.07 $uml^{-1}$ | 0.34 $uml^{-1}$ | 27.48 | 22 | 0.020 | PBMC from infected individuals produced more IFNγ on stimulation with WWH than PBMC from uninfected individuals. |

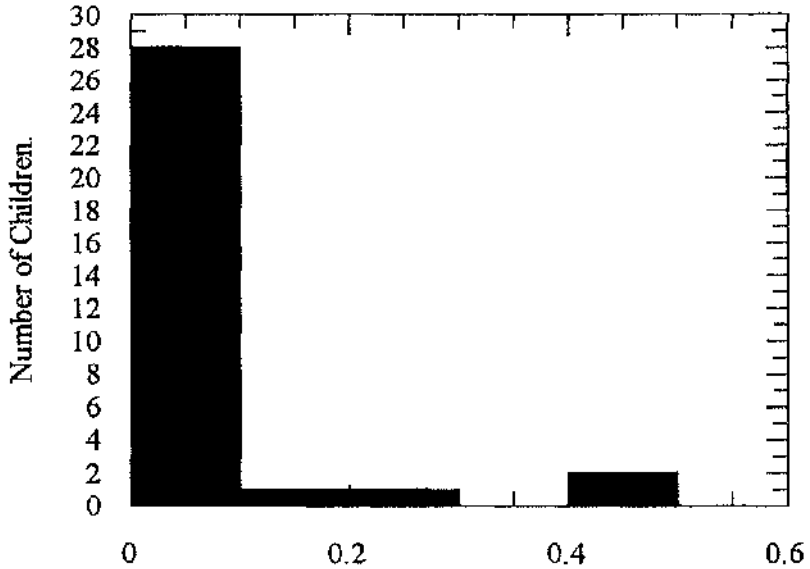
| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-2 | PHA | 4.11 $uml^{-1}$ | 7.67 $uml^{-1}$ | 9.52 $uml^{-1}$ | 18.61 | 14 | 0.00 $uml^{-1}$ | 3.04 $uml^{-1}$ | 6.91 $uml^{-1}$ | 12.78 | 16 | 0.068 | There was no significant difference between production of IL-2 by PBMC from infected or uninfected individuals cultured with PHA |
| IL-2 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.14 | 22 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.82 | 17 | 0.899 | There was no significant difference between production of IL-2 by PBMC from infected or uninfected individuals cultured with SEA |
| IL-2 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 20.64 | 22 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 19.18 | 17 | 0.528 | There was no significant difference between production of IL-2 by PBMC from infected or uninfected individuals cultured with WWH |
| IL-4 (Genzyme Kit) | PHA | 0.78 $uml^{-1}$ | 1.70 $uml^{-1}$ | 3.55 $uml^{-1}$ | 29.52 | 23 | 0.28 $uml^{-1}$ | 0.48 $uml^{-1}$ | 0.70 $uml^{-1}$ | 16.18 | 22 | 0.001 | PBMC from uninfected individuals produced more IL-4 on stimulation with PHA than PBMC from infected individuals. |
| IL-4 (Genzyme Kit) | SEA | -0.02 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 23.39 | 23 | -0.12 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 22.59 | 22 | 0.838 | There was no significant difference between production of IL-4 by PBMC from infected or uninfected individuals cultured with SEA |
| IL-4 (Genzyme Kit) | WWH | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.01 $uml^{-1}$ | 23.04 | 23 | -0.01 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.02 $uml^{-1}$ | 22.95 | 22 | 0.982 | There was no significant difference between production of IL-4 by PBMC from infected or uninfected individuals cultured with WWH |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| IL-4 (ELISA as in section 2.3) | PHA | 0.10 $uml^{-1}$ | 0.89 $uml^{-1}$ | 3.98 $uml^{-1}$ | 27.59 | 29 | 0.07 $uml^{-1}$ | 1.81 $uml^{-1}$ | 4.96 $uml^{-1}$ | 32.33 | 30 | 0.296 | There was no significant difference between production of IL-4 by PBMC from infected or uninfected individuals cultured with PHA |
| IL-4 (ELISA as in section 2.3) | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 32.53 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 27.55 | 30 | 0.047 | PBMC from uninfected individuals produced more IL-4 on stimulation with SEA than PBMC from infected individuals. |
| IL-4 (ELISA as in section 2.3) | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.03 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 29.97 | 30 | 0.961 | There was no significant difference between production of IL-4 by PBMC from infected or uninfected individuals cultured with WWH |
| IL-5 | PHA | 1.45 $uml^{-1}$ | 7.30 $uml^{-1}$ | 11.59 $uml^{-1}$ | 29.16 | 29 | 2.58 $uml^{-1}$ | 7.62 $uml^{-1}$ | 10.83 $uml^{-1}$ | 30.82 | 30 | 0.710 | There was no significant difference between production of IL-5 by PBMC from infected or uninfected individuals cultured with PHA |
| IL-5 | SEA | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.93 | 28 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.03 | 30 | 0.660 | There was no significant difference between production of IL-5 by PBMC from infected or uninfected individuals cultured with SEA |
| IL-5 | WWH | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 30.59 | 29 | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 0.00 $uml^{-1}$ | 28.41 | 29 | 0.414 | There was no significant difference between production of IL-5 by PBMC from infected or uninfected individuals cultured with WWH |

| | | Uninfected Individuals | | | | | Infected Individuals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentiles | | | | | Percentiles | | | | | | |
| Net cytokine/ proliferative response | Stimulant | 25% | 50% median | 75% | Mean rank | N | 25% | 50% median | 75% | Mean rank | N | 2 tailed p< (MWU) | Interpretation. |
| TNFα | PHA | 18.56 $uml^{-1}$ | 31.53 $uml^{-1}$ | 41.19 $uml^{-1}$ | 31.17 | 29 | 17.48 $uml^{-1}$ | 28.20 $uml^{-1}$ | 36.05 $uml^{-1}$ | 28.87 | 30 | 0.606 | There was no significant difference between production of TNFα by PBMC from infected or uninfected individuals cultured with PHA |
| TNFα | SEA | -0.09 $uml^{-1}$ | 0.23 $uml^{-1}$ | 1.43 $uml^{-1}$ | 31.10 | 29 | 0.00 $uml^{-1}$ | 0.12 $uml^{-1}$ | 0.85 $uml^{-1}$ | 28.93 | 30 | 0.627 | There was no significant difference between production of TNFα by PBMC from infected or uninfected individuals cultured with SEA |
| TNFα | WWH | -0.25 $uml^{-1}$ | 0.11 $uml^{-1}$ | 1.32 $uml^{-1}$ | 30.78 | 29 | -0.22 $uml^{-1}$ | 0.07 $uml^{-1}$ | 0.96 $uml^{-1}$ | 29.25 | 30 | 0.733 | There was no significant difference between production of TNFα by PBMC from infected or uninfected individuals cultured with WWH |
| Proliferative response | PHA | -0.13 $OD_{570nm}$ | 0.13 $OD_{570nm}$ | 0.31 $OD_{570nm}$ | 32.69 | 29 | -0.13 $OD_{570nm}$ | -0.03 $OD_{570nm}$ | 0.22 $OD_{570nm}$ | 27.40 | 30 | 0.237 | There was no significant difference between the proliferative response of PBMC from infected or uninfected individuals cultured with PHA. |
| Proliferative response | SEA | -0.04 $OD_{570nm}$ | 0.00 $OD_{570nm}$ | 0.01 $OD_{570nm}$ | 29.84 | 29 | -0.03 $OD_{570nm}$ | -0.01 $OD_{570nm}$ | 0.01 $OD_{570nm}$ | 30.15 | 30 | 0.946 | There was no significant difference between the proliferative response of PBMC from infected or uninfected individuals cultured with SEA |
| Proliferative response | WWH | -0.04 $OD_{570nm}$ | 0.02 $OD_{570nm}$ | 0.04 $OD_{570nm}$ | 32.03 | 29 | -0.03 $OD_{570nm}$ | 0.00 $OD_{570nm}$ | 0.03 $OD_{570nm}$ | 28.03 | 30 | 0.371 | There was no significant difference between the proliferative response of PBMC from infected or uninfected individuals cultured with WWH |

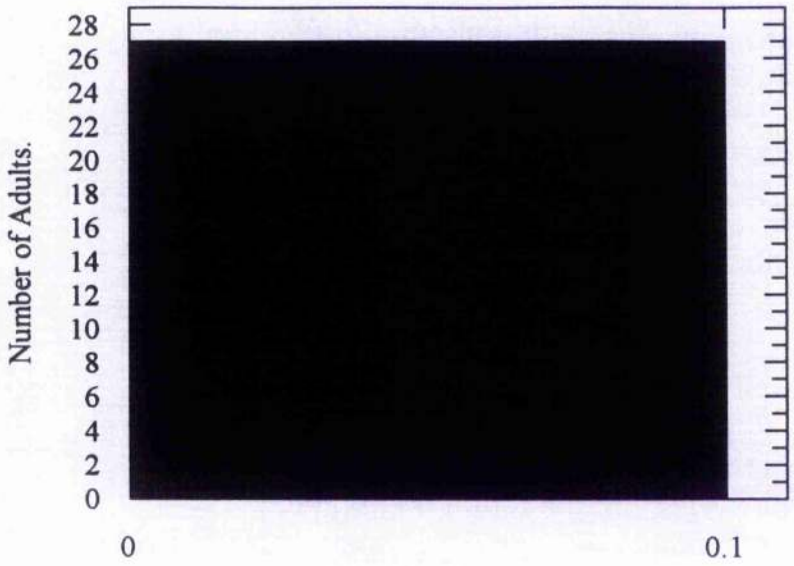
**Appendix 2a.**

A frequency distribution of gross IL-4 produced by PBMC from Gambian children when cultured at a concentration of 1 x $10^6$ cells$ml^{-1}$ in 1ml aliquots, for 48 hours in the presence of 10$\mu gml^{-1}$ of SEA. IL-4 concentration was determined using a sandwich ELISA as described in section 2.3 which had a sensitivity of 1$uml^{-1}$ and an accuracy of 0.01 $uml^{-1}$.



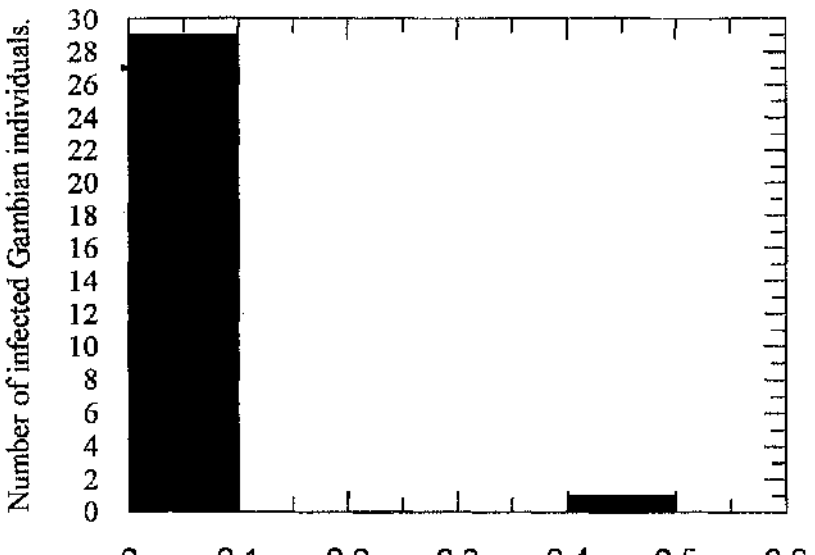
**Appendix 2b.**

A frequency distribution of gross IL-4 produced by PBMC from Gambian adults when cultured at a concentration of 1 x $10^6$ cells$ml^{-1}$ in 1ml aliquots, for 48 hours in the presence of 10$\mu g ml^{-1}$ of SEA. IL-4 concentration was determined using a using a sandwich ELISA as described in section 2.3 which had a sensitivity of 1$uml^{-1}$ and an accuracy of 0.01 $uml^{-1}$.



**Appendix 2c.**

A frequency distribution of net IL-4 produced by PBMC from infected Gambian individuals when cultured at a concentration of 1 x $10^6$ cells$ml^{-1}$ in 1ml aliquots, for 48 hours in the presence of 10$\mu g ml^{-1}$ of SEA. IL-4 concentration was determined using a using a sandwich ELISA as described in section 2.3 which had a sensitivity of 1$uml^{-1}$ and an accuracy of 0.01 $uml^{-1}$. Net IL-4 is IL-4 produced by PBMC when cultured with SEA minus IL-4 produced by PBMC when cultured without stimulant.



**Appendix 2d.**

A frequency distribution of net IL-4 produced by PBMC from uninfected Gambian individuals when cultured at a concentration of 1 x $10^6$ cells$ml^{-1}$ in 1ml aliquots, for 48 hours in the presence of 10$\mu g ml^{-1}$ of SEA. IL-4 concentration was determined using a using a sandwich ELISA as described in section 2.3 which had a sensitivity of 1$uml^{-1}$ and an accuracy of 0.01 $uml^{-1}$. Net IL-4 is IL-4 produced by PBMC when cultured with SEA minus IL-4 produced by PBMC when cultured without stimulant.