REPEATED INFECTION OF INBRED MICE WITH *HELIGMOSOMOIDES POLYGYRUS*

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by

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ABSTRACT

To approximate naturally occurring conditions of continued exposure to infection, two strains of mice (NIH and CBA) were given repeated low-level doses of *Heligmosomoides polygyrus*. The dynamics of the parasite and the host's immunological responses were investigated throughout the infection.

The host's cellular response was assayed using the *in vitro* lympho-proliferative assay of Corradin *et al.*, (1977). The assay was found to show the variability inherent on sampling an exponentially growing population.

The antigens of the parasite can be mitogenic. The level of mitogenicity depended on their method of preparation and the source and condition of the cells they were cultured with. All the antigens caused proliferation with the cells of infected mice although proliferation to certain antigens appeared to be organ specific. The level of proliferation induced by each antigen was different. Cross-reactivity was shown between some of the antigens.

On repeated low-level infection, differences between the two mouse strains used in the experimental system were noted. These differences were evident in the worm burdens of the mice and the fecundities of the worms they harboured. These parameters were lower for NIH mice than CBA mice and the parasite showed different dynamics in the two types of host. These differences were reflected in the ability of the spleen cells of the two strains to proliferate to the antigens of *H. polygyrus* The cells of the NIH mice proliferated to the antigens to a greater degree than the cells of the CBA mice. In addition the dynamics of the cellular responses were different in the two strains. However, the humoral responses were somewhat similar indicating that immunosuppression has little effect on the humoral response.

On anthelminitc interruption of a repeated infection and subsequent re-exposure to the parasite it was found that antibody levels appeared to be dependent on the input of larvae. The increase in anti-adult antibody occurred in the absence of adult worms. It was concluded that there was a large degree of cross-reactivity between the homogenates of the two stages (D6L4 and adult). Investigation of the dynamics of the parasite indicated that the CBA strain showed some resistance after anthelmintic abbreviation of an infection. This "anti-worm" effect could be distinguished from an "anti-fecundity" effect. Analysis of the cellular responses was made difficult as the mice were found to be concurrently infected with mouse hepatitis virus.

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1.1 *H. polygyrus* life cycle

The parasite *Heligmosomoides polygyrus* (Dujardin 1845. Nematoda; Heilgmosomoidae) has a simple, direct life cycle. In a primary infection the eggs are passed out in the faeces of the host mouse and hatch, the development of the L3, the infective free-living stage, takes 5-6 days at 26°C (1). The mouse is infected when it ingests the L3 (although subcutaneous infection is possible (but not percutaneous, Maema, (1987)). It then penetrates the intestinal mucosa and reaches the subserosa 24-36hr later .The subsequent tissue phase of development, during which the parasite reaches the L4 stage, takes approximately 6-8 days after which it emerges as a "preadult". Maturation to the adult takes 2-3 days after which eggs are produced by female worms. The infection lasts 8-10 months after a single infection (Keymer and Hiorns, 1986 .Bryant, 1973).

1.2 Uses of the *H. polygyrus*/mouse model

This murine trichostrongylid nematode, like many medically important parasites, gives rise to a chronic primary infection. The system would, therefore, appear to be a suitable model to allow an analysis of the immunological and epidemiological aspects of chronic intestinal parasitism. The majority of the research performed so far has concentrated, understandably, on the analysis of mechanisms of immunity during a primary or repeated/multiple infection. However, a feature of this present study is that the mice are given repeated doses of the parasite in an attempt to approximate more closely naturally occurring conditions of continued exposure to infection. Bearing this in mind, it maybe that the mechanisms of immunity described for a primary or repeated/multiple infection may not apply to the repeated infections.

The survival of this parasite in a host which has the ability to remove other intestinal nematodes such as *Trichuris muris* (Behnke *et al.*, 1984) suggests that it possesses a mechanism which allows it to survive in the face of an otherwise effective immune response. Thus, in any discussion of immune responses against the nematode account must be taken of this. Furthermore, although "immunity" will be discussed, the assaying of an effective response may be hampered by the homologous and heterologous suppression that occurs.

For the purposes of this report published research will be considered under the following categories:-

1.3 Host responses to *H. polygyrus*.

1.4 Evasion/suppression of the response by the parasite.

1.5 Host genetics and the immune response.

1.6 T-cell involvement.

A number of important points should be made before any mechanisms are discussed. The mechanisms of immunity to *H. polygyrus* in mice are not well understood and various components of the immune system have been implicated in the protective response to the larvae. The response to the parasite is a dynamic one and it is likely that many (compensating) events which are both immunological and physiological are involved (Dawkins, Windon and Eagleson, 1989). It is also probable that these effector mechanisms operate in combination (Mitchell and Munoz, 1983). Furthermore, although a host may generate an immune response it is not necessarily protective, although its relevance may be poorly understood. The protective response could be correlated with a response not yet observed. Similarly any observed suppression may have no bearing on the parasite's survival.

1.3 Host responses to the parasite.

Host protective immunity is thought to manifest itself in 5 distinct ways (Behnke and Parish, 1981):-

(a). Reduction in the number of larvae surviving tissue development. In an infection in resistant LAF1 mice only 2.5% of larvae were observed as encysted, the rest had either not penetrated the mucosa, had been killed at an earlier stage of penetration or were unidentifiable (Cypess *et al.*, 1988).

(b). Arrested development of a proportion of worms in the intestinal tissue.

(c).Delay in development of worms and a reduction in their size. It was shown by Cypess *et al.*, (1988) that in resistant mice only 25.5% of the encysted L3 developed and the adults took up to thirteen days to emerge.

(d). Reduction in fecundity of the worms.

(e). Expulsion of adult worms from the intestine.

This pattern of expression of immunity could offer an explanation of the observed convex pattern of worm burden seen in high responder mice during a trickle infection demonstrated by Maema (1987). Thus there is an initial increase in numbers when no protective host response is expressed but there is a decrease in establishment when anti-larval immunity occurs. The worm burden is then reduced as an anti-adult response develops. However this pattern is not seen in low responder mice exposed to a trickle regime, during which the parasite numbers increase and then reach a plateau.

It would appear from the literature that immunity is directed mainly against the larval stages of the parasite and acts to reduce establishment (Pritchard & Behnke 1985; Jacobson, Brooks and Cypess, 1982). However, Wakelin (1986) suggests that in immune hosts hatching or exsheathing is unaffected, allowing larvae to penetrate the mucosa. It is after this that the larvae are trapped and killed. If they are the sole targets of an effective response then this would offer an

explanation for chronic primary infection with the parasite. The observation that transplanted adult worms can survive in mice totally immune to larval challenge (Behnke, Hannah and Pritchard, 1983) would appear to support the idea of stage specific immune responses. However other observations suggest that protective anti-adult immune reactions can occur. For example, there is a loss of adult worms developing from a larval infection in S.J.L/J female mice, the infection dynamics resembling those of self cure (Mitchell and Prowse, 1979). Furthermore, there is a loss of adult worms in some strains of immunised mice (Behnke and Wakelin, 1977; Sayles and Jacobson, 1983).

Thus, although larvae may induce and be the targets of an effective immune response, they are not the sole targets of a response (Behnke, Hannah and Pritchard, 1983; Mitchell and Munoz, 1983; Hurley, Day and Mitchell, 1980). Although the effector mechanisms of the response to the two stages are probably qualitatively distinct due to the differences in their anatomical position. However, some responses may be common to both. The responses can be considered for each of the stages, even though the control may be due to the same cell, that is, T-lymphocytes (more especially T-helper cells (Prowse, 1982)). These responses are not understood well. The situation is complicated by the fact that there is little immunity shown in many (inbred) mice even after prolonged and repeated exposure to infection (Sayles and Jacobson, 1983). Even when immunity is shown (often after repeated/anthelmintic abbreviated regimes or treatment with irradiated larvae), it is complex in its effects and involves both humoral and cell mediated components.

1.3.1 Antibody responses in infected mice

1.3.1.1 Changes in immunoglobulin levels

As mentioned above, a single infection gives rise to a chronic infection during which the most striking observation is an increase in the serum immunoglobulin

levels, up to twice that seen in controls (Chapman, Knopf, Hicks and Mitchell, 1979). The immunoglobulin which shows the greatest increase is an isotype of IgG, IgG1. This phenomenon of hypergammaglobulinaemia is a common feature of parasitic infection and a number of mechanisms as to its cause have been postulated (Chapman *et al.*, 1979). It should be mentioned that high levels of one isotype may depress levels of another, IgG1 may depress levels of IgG2a, IgG2b and IgG3 (Dobson *et al.*, 1985) and thus the increase in total serum immunoglobulins may not be as great as expected.

The observed increase in immunoglobulin levels would require an additional $2x10^8$ B-cells per mouse (Chapman *et al.*, 1979). There is no single tissue in which immunoglobulin synthesis by these cells occurs. However, it would appear that the antigen trapping organ in line from the source of antigen, the mesenteric lymph node, is the major source of IgG1-B cells (Chapman *et al.*, 1979). This would appear to be supported by the observation by Ali and Behnke (1985) that there is enlargement of the mesenteric lymph node on a primary infection with *H. polygyrus*.

It has been shown by many workers that serum from a primary infection is not protective. This was demonstrated by Williams and Behnke (1983) who transferred up to 6 ml of primary infection serum with mesenteric lymph nodes (IMLNC) from immune mice and observed no protection on infection of the recipients. This does not mean that no protective humoral response occurs as the transfer of 0.25 ml of immune serum with IMLNC confers protection (Williams & Behnke, 1983). The difference between the two types of sera may be in the titre of parasite-specific antibody or it could be in the "quality" of the sera. For example, Monroy, East, Dobson and Adams (1989) noted that primary infection serum did not recognise a 60 kilodalton *H. polygyrus* antigen whereas immune serum did.

As hypergammaglobulinaemia occurs during a chronic infection, Prowse (1982) suggests that the rise in antibody causes a blocking of potentially protective host

responses. Work by Pritchard *et al* (1984b) shows that it is unlikely to be the case as IgG1 from primary infection sera purified by affinity chromatography is host protective and fails to block immunity transferred by IMLNC. Thus, any failure to transfer protection in other experiments may be due to the low specific antibody titre. However, it could also be the case that the antibody when combined with antigen forms immune complexes which block protection. Precipitation of these complexes in polyethylene glycol and their subsequent incubation with IMLNC prior to the cells' transfer fails to increase susceptibility (Pritchard, Behnke and Williams, 1984).

An interesting observation is that anti-parasite activity of immune serum can mostly be attributed to the IgG1 fraction as judged by immunofluoresence activity, immunoprecipitation and ELISA (Molinari, Ebersole and Cypess, 1978; Crandall, Crandall and Franco, 1974; Prowse, Mitchell, Ey and Jenkin, 1978; Pritchard, Williams, Behnke and Lee, 1983; Price and Turner, 1986). However, in primary infection serum activity is also found in the Ig2a, IgG3 and IgG2b sub classes (Dobson *et al.*, 1985). Consequently, other workers have found that the levels of these isotypes may be depressed or unaffected by *H. polygyrus* (Pritchard, Williams and Behnke, 1983; Crandall, Crandall and Franco, 1974). It has been suggested that it is these isotypes which block cell adherence (Pritchard, Behnke and Williams, 1984).

1.3.1.2 The role of antibody during an infection.

Just under half the IgG1 from immune serum is specific to adult homogenate, the remaining serum expresses immunofluorescence against larval stages (Williams and Behnke, 1983). Transfer experiments show that this is the only isotype to have a significant effect on worm numbers with or without IMLNC. It shows its effects by stunting worms as well as increasing destruction in the mucosa (Behnke and Parish, 1979; Dobson, 1982). The sera is shown to exhibit protective activity during the 3rd and 4th weeks of a multiple immunizing regime

reaching a peak level at about 6 weeks, beyond which there is no further increase in protective activity (Williams and Behnke, 1983).

The IgG1 fraction is the only one which shows a corresponding increase and this can also be compared with an increase in the number of eosinophils which reach their peak levels at 6 weeks (Pritchard *et al.*, 1983). The rise in immunoglobulins is often, but not always, different between high and low responder strains of mice (Dobson, 1982). Williams and Behnke (1983) suggest that the difference between these mice is in the efficiency of the cellular, bone marrow derived components of protective immunity.

Antibody may have a direct effect on larval establishment (Dobson, 1982) and on worm survival (Dobson, Brindley and Sitepu, 1982). It may reduce larval establishment by binding to larvae through the cuticle and thus interfere with tegument function or they may react with secreted enzymes which are necessary for development (Phillip, Parkhouse and Ogilvie, 1980). Antibody has been detected in sites where immune attrition may occur, in immune mice the intestinal mucosa stains brightly with the fluorescent antibody specific to IgG1 (Crandall, Crandall and Franco, 1974). It has also been observed that on incubation with the fluorescent antibody both the gut and body wall of *H. polygyrus* stain positively (Crandall, Crandall and Franco, 1974). The surface of exsheathed larvae have been shown to bind antibody (Ninneman and Leuker, 1974) and immunoprecipitates have been observed round the pores of *H. polygyrus* (Panter, 1969b).

Although it is likely that larvae are killed in the granulomas there is also thought to be a reduction in the number of larvae that penetrate and develop in the intestinal tissue, secretory IgA being a requirement in the mechanism (Bienenstock and Befus, 1983). Molinari, Ebersole and Cypess (1978) have demonstrated parasite specific IgA. However, Crandall, Crandall and Franco (1974) could not detect specific IgA after initial and challenge infection and found no change in the luminal immunoglobulin concentration. They showed that the

intestinal IgG1 concentration doubles and this may or may not reflect changes in secretory IgG1 levels. There is likely to be transmucosal stimulation of secretory IgA production by adult antigen but it is difficult to draw any firm conclusions regarding the relevance of the reduction of larval penetration as a host protective mechanism (Crandall, Crandall and Franco, 1974).

In a primary infection adult nematodes can survive for up to 8 months and their ultimate loss is probably as a result of their own senescence rather than a host response (Williams & Behnke, 1983). However, in a mouse expressing a significant degree of immunity, it is likely that a parasite's fecundity is reduced and the duration of residence of any one worm within the intestine is lessened. Worm expulsion may be similar to that for other intestinal helminths. Supporting evidence comes from work investigating the effects of immune serum on worm expulsion. It was found that mice given immune serum expelled most of their worm burden about 4 weeks post infection and 2 to 3 weeks after the last injection of immune serum. The serum was only effective at evoking expulsion if given one day before or up to four days after the infection (Behnke & Parish 1979).

Behnke and Parish (1979) report that the half-life of injected IgG in normal mice is about 4 to 8 days and in mice infected with *H. polygyrus* it can be as short as 2.6 to 3.5 days. Therefore it is unlikely that immune serum alone effected expulsion, although as stunting is observed it may have had some effect. It is more probable that transferred serum plays a vital initial role damaging worms and rendering them more susceptible to other components (Behnke & Parish 1979). The time course of serum transfer required for expulsion is of interest as transfer is only effective before the adults emerge. As discussed at a later stage (section 1.4), adult worms are thought to be the mediators of suppression.

The worms are stunted early on in development, but IgG could also have a direct effect on the worms in the lumen (Dobson, 1982). It has been reported that the luminal concentration of IgG increases substantially 3-7 days post infection

with *H. polygrus* probably as a result of a leakage of serum proteins into the gut (Molinari *et al.*, 1977). Protective activity for such immunoglobulins has been shown for other parasite systems (Molinari *et al.*, 1977) However, there is no evidence that the cytopathological changes seen in other systems such as *Nippostrongylus brasiliensis* are necessary for expulsion. These changes can be induced by sub-optimal *in vitro* conditions (Lee, 1969)

1.3.2 Cellular responses to the parasite

In a primary infection there is a pronounced leucocytosis which is probably in part due to the mobilisation of cells and their migration to the intestinal wall (Cypess, 1972). However, this in itself does not constitute a host protective response although its maintenance does require the presence of the adult parasite. It would appear that the leucocytosis is stimulated by the larvae which on day 2 post infection give rise to haemorrhages in the intestinal mucosa which then become inflamed and infiltrated by neutrophils. Subsequent larval development gives rise to necrosis of adjacent tissues and damage to the capillaries (Liu, Cypess and Van Zandt, 1974). On emergence of the worms there is extensive leucocytotic infiltration of their "cysts" (which are in fact granuloma) in the sub-mucosa. There is still migration into these cysts after the parasites develop into adults, probably in response to remaining larval excretory or secretory antigen (ES) and the cuticles shed at the L3 and L4 moults.

In contrast to the sequence of events in a host given a single infection, in a host immunised by a number of infections there is a different pattern of cellular production and distribution. It may be that these changes correlate with the development of immunity.

1.3.2.1 The role of macrophages in resistance to *H. polygyrus*.

It is thought that L3 (and later larval stages) may play a major role in the stimulation of resistance to reinfection (Chaicumpa, Jenkins and Fischer, 1977).

Macrophages have been shown to kill L3 *in vitro* (Hagan *et al.*, 1981) and a role for them in killing the larvae as they penetrate the gut wall has been proposed. These cells have also been reported to be active against the larvae of other nematodes as well (Ninneman and Leuker, 1974). They are thought to be responsible for the partial immunity shown in a primary infection. Prowse *et al* (1979) suggest that the larvae are killed 24-36 hours post-infection whilst still in the third stage. However, Pentilla *et al* (1985) show that peritoneal exudate (which contains many macrophages) exhibits no larvicidal activity 5 days post-infection, activity being shown 10 and 24 days post infection. This implies that the macrophages are not important host protective cells. The cells which did show activity at 5 days were neutrophils which suggests these maybe the effector cells.

1.3.2.2 The role of neutrophils in resistance.

Once in the gut wall the larvae are inhibited and slowly killed in the granulomata which form around their site of development in the muscularis mucosa (Cypess *et al.*, 1988). Within the granulomas there are neutrophils, lymphocytes and eosinophils as well as macrophages (Liu, 1965; Prowse, Ey and Jenkin, 1979).

The effector cells may be neutrophils as well as eosinophils (Hurley and Vadas, 1983). Both have been studied *in vitro* and both exhibit larvicidal (anti-L3) activity in the presence of immune mouse serum. Neutrophils can damage some parasites. With *Schistosoma mansoni* they can cause lesions of the tegument and they have also been observed penetrating the cuticle of *Ascaris suum* and extending pseudopodia into the underlying tissues (Thompson *et al.*, 1977). Neutrophils can also damage some parasites. Neutrophils have also been observed causing *in vitro* damage to *Dipetalonema vitae* and *Onchocerca volvulus* (Pentilla *et al.*, 1985).

Neutrophils from infected mice appear to be, cell for cell, more larvicidal than eosinophils from infected mice (Pentilla, Ey and Jenkin, 1985). However the neutrophils were only slightly more active in immune mouse serum than normal

serum suggesting that complement is more important and that eosinophils were only active in the presence of complement and specific antibody (Pentilla *et al.*, 1985). This correlates with the observation that *H. polygyrus* can activate complement by the alternative pathway (Prowse *et al.*, 1977) and that neutrophils have been shown to be capable of killing schistosomulae *in vitro* in the presence of complement (Prowse *et al.*, 1985). It may also be that the neutrophils play a greater role than eosinophils in the early stages of an infection or during a primary infection (Pentilla, Ey, Lopez and Jenkin, 1985). However, in the latter case little effective resistance is observed.

The ability of the cells to interact with complement may aid resistance. The relevance of this is uncertain as the level of complement in the interstitial fluid surrounding the larvae in the muscularis mucosa is probably not as high as that in the serum/*in vivo* (Butterworth, 1984) It is likely that antibody which is at a higher level is more important, at least in the later stages of an infection.

1.3.2.3 The role of eosinophils in resistance

Pritchard *et al.* (1983) and Pentilla *et al.* (1985) suggest that IgG1 dependent eosinophilic killing is the main form of anti-helminth immunity in immune animals. It is the eosinophils which rise most in the peripheral blood and peritoneal exudate on a challenge (Jones and Rubin, 1974). It is also the eosinophils that are responsible for larval destruction in the granulomata (Hagan *et al.*, 1981). Furthermore, there is also a correlation between the capacity of different inbred strains to produce an eosinophil response and their resistance to *H. polygyrus* (Wakelin and Donachie, 1983). This correlation is also seen in other host parasite systems (e.g. for *Trichostrongylus colubriformis*: Hadlinger and Rothwell, 1981; Dawkins, Windon and Eagleson, 1989. For *Trichinella spiralis*: Wakelin and Donachie, 1983)). Eosinophils have been implicated in immunity to *Nippostrongylus brasiliensis*, *T. spiralis* and *Schistosoma mansoni* (Pentilla *et al.*, 1985).

The exact role of eosinophils remains uncertain and the precise mechanisms of larval killing remain uncertain. They have been observed in intimate contact with the parasite *in vitro* (Prowse, Ey and Jenkin, 1978) and *in vivo* (Hurley and Vadas, 1983). However, direct killing of the parasite has not been observed (Hurley and Vadas, 1983). Gansmuller *et al.*, (1987) have noted that eosinophils adhere to the cuticle of *T. spiralis* via the contents of their granules (unlike in *S. mansoni* no fusion of the membranes was observed). When the cells had degranulated and detached the granule contents and cell debris remained and lifting of the parasite's epicuticle was generally seen. In an *in vitro* system major basic protein and eosinophil cationic protein are active. The role of the oxidative processes, which involve partially reduced products of oxygen and eosinophil peroxidase, is controversial (Butterworth, 1984).

It is interesting to note that neutrophils and eosinophils from normal mice are unable to damage the L3 *in vitro*, but neutrophils and eosinophils from immune mice can (Pentilla *et al.*, 1985). This suggests that neutrophils must be "activated" in some way (at least *in vitro*) for killing to take place. The "activation", which lasts for six weeks after the initial stimulation, may be a stimulatory or maturation event and may parallel the activation of macrophages which require accessory cells and have also been implicated in larval killing (Chaicumpa and Jenkin, 1978).

1.3.2.4 The time course of cellular changes during an immune response.

There is some disagreement over the actual time course of loss and killing. Work by Jones and Rubin (1974) suggests that larvae in a challenge are rapidly expelled as opposed to being gradually eliminated by host sequestration due to the disappearance of inflammation, few granulomas and an exponential decline in parasite numbers. This is not a view shared by Behnke and Parish (1981) who suggest that granuloma formation plays an important part in mediating resistance, delayed type hypersensitivity playing a more important role than

immediate hypersensitivity as little loss occurs within the first 4 days of a second infection. They propose that larvae are arrested in an early stage of development and larval destruction in the intestinal wall is a slow process.

The changes in cell numbers and function have been summarised by Pentilla *et al.*, (1985). They propose the following changes in the immune system in infected mice:- (1): within 4 days of an infection a (sub)population of neutrophils becomes altered such that the cells are able to damage exsheathed L3; (2): by day 10 post-infection both activated macrophages and neutrophils are present. In the presence of specific antibody both cell types can mediate partial protection; (3): within 1 week after a secondary infection eosinophils appear in the circulation in increased numbers. These probably function as effector cells, and in concert with neutrophils and macrophages damage and kill larvae developing in the muscularis mucosa. However deletion experiments show that neutrophils are not essential for expression of full resistance but, like macrophages, appear to play a part in partial protection.

1.3.3 Cell and antibody interactions

One of the functions of antibody appears to be to cooperate with cells such as eosinophils to bring about immunity to *H. polygyrus* (Pentilla, Ey, Lopez and Jenkin, 1985; Pritchard, Williams, Behnke and Lee, 1983).

Evidence for *in vivo* antibody mediated helmintho-toxic effect of eosinophils comes from the observed impairment of immunity on the treatment of mice with heterologous anti-eosinophil serum (Hurley and Vadas, 1983). It is unlikely that antibody's main effect is the entrapment and destruction of worms in the intestinal lumen, although expulsion of adults does occur. Cell mediated immunity against larvae in the mucosa is thought to be important (Behnke and Parish, 1979).

The IgG1 isotype has been studied in a variety of experiments and its ability to promote cell adherence has been studied. This isotype has little effect on IMLNC

adherence to L3 (Behnke and Parish, 1981) but this supports the observation that the L3 are not very immunogenic. Spontaneous adherence may occur, Ninneman & Leuker (1974) have observed attachment of macrophages to the head and tail region of exsheathed larvae. For L4 and adults the adherence of peritoneal exudate cells and IMLNC is markedly enhanced by the presence of heat inactivated serum and therefore complement is not an essential requirement. However, Prowse *et al.* (1979) have shown that *H. polygyrus* can activate complement by the alternative pathway and early in an infection, complement dependent activity may be an important method of reducing parasite establishment.

No larvae are lost during a primary infection and if the eosinophil is the major cell involved in killing trapped larvae then they are ineffective in a primary infection. Their ineffectiveness may be explained if the eosinophil mediated killing of the larvae is IgG1 dependent. The low titre of anti-*H. polygrus* antibody in primary infection serum (Williams and Behnke, 1983) may mean that little killing can take place. An alternative explanation is that the larvae escape the host reaction before it is completed. That this may be the case is supported by the observation that larvae in the cysts can survive the immune attrition. These larvae are reactivated when the worms are treated with cortisone. However, the later the treatment is initiated, the lower the worm recovery, suggesting that the arrested larvae are slowly being rejected by the host. Corticosteroids, of which cortisone is one, act against B cells before exposure to antigen and committed cells are relatively resistant. Consequently existing immunoglobulin levels should remain unchanged. This gives an indication of the importance of the dynamics of antibody production in the rejection of the worm.

1.3.4 Stage specific immunity

Immunity to the parasite would appear to be stage specific and this may be related to the presence of stage specific antigens.

Stage specific antigens (or stage specific expression) have been shown for many parasites (e.g. for *T. spiralis*: Parkhouse and Ortega-Pierres, 1984; for *Haemonchus contortus*: Adams *et al*, 1989).

Pritchard (pers. comm.) has shown, using immunoprecipitation, that ova and L3 are not very immunogenic but that the L4 is more so, with the adult showing a greater number of more complex antigens but similar immunogenicity. Pritchard *et al.* (1984c) also reports that there is a stage specific antigen on the D6L4 of 16kd which is not recognised by sera from mice infected only once. It may be that this molecule is immunogenic and triggers the host protective response against the L4 and is then lost on maturation to the adult stage. There is also an antigen of 65kd which is different to that on the adult and this may have some bearing on the fact that only 48% of immune serum reacted with adult antigen; of the remaining serum a large proportion reacted with larval antigen.

The immunity may also be sex-specific. Adams, East, Monroy and Dobson (1989) have detected sex-specific antigens in the secretions and on the surface of *H. polygyrus*. They suggest that if the molecules found on the male are highly immunogenic then this may draw the host's response away from the females allowing egg production to continue.

1.4 Suppression of the host's immune response by *H. polygyrus*

Heligmosomoides polygyrus can, and does, survive in the face of host defence mechanisms. It also impairs the ability of the host to expel other parasites such as *Trichuris muris* (Behnke, Ali and Jenkins, 1984). This long term survival has as yet to be satisfactorily explained. It may be that as the immune mechanisms are stage specific the responses which are effective against the larvae are ineffective

against the adult, or by virtue of antigen masking the adult is not recognised by the host. Cayzer and Dobson (1983) suggest this is achieved by using non-protective antibody although they offer no supporting evidence. Jacobson *et al.* (1982) have shown using LAF1/J mice, that intestinal implantation results in no resistance to homologous infection but worms implanted intraperitoneally effect a significant reduction in subsequent intraluminal infection resulting from a *per os* challenge. This suggests that adult antigens can be recognised if presented to the host in a particular way.

There are other explanations for its survival, including the proposal that the worms occupy an immunologically privileged site that they create and are not reached by the effector mechanisms. However, the fact that the adults can be expelled from the jird which has similar gut physiology to the mouse (Behnke and Hannah, 1982) and from mice which have received immune serum and IMLNC suggests that this is not the case (Behnke and Parish, 1977). It has also been suggested that the worms are more resistant than other worms, but the events which take place during their expulsion would suggest that this is not the case although Smith and Boyd (1986) give evidence of resistance mechanisms. Another possibility is that the worms reduce the level of specific anti-parasite antibody (Cayzer & Dobson, 1983). This does not take account of the increase in specific anti-*H. polygyrus* antibody observed during an infection (Crandall, Crandall and Franco, 1974).

An interesting observation is that the worms surviving in a chronic infection are not evenly distributed along the intestine but occur in pockets and may therefore be creating an environment of relative safety (Behnke, Hannah and Pritchard, 1983).

Thus an alternative explanation is that the parasite itself affects the host's ability to generate and express an effective anti-parasite response. This is shown by the transfer of adults by laparotomy and the subsequent development of an

infection in previously immune mice (Behnke, Hannah and Pritchard, 1985). Thus the parasite in some way depresses the host's response to it.

It is recognised that during an infection there occurs two types of immunodepression during an infection: non-specific immunodepression and specific immunodepression (NSID and SID respectively). NSID occurs during heavy infections (more than 400 adults) acting against responses to heterologous and homologous infections whereas SID to homologous antigens occurs during relatively light infections. Both can be shown to be cell mediated by cell transfer experiments (Behnke & Parish, 1981).

Despite this immunodepression, immunity can be induced by many regimes, but a common factor in each is the requirement of stimulation of the immune system in the absence of adults and thus the adults are the probable cause of the immunodepression (Behnke and Robinson, 1985). The observed neutralisation of established immunity by adult worms or a homogenate of them suggests that a product of the adult is responsible for affecting the induction and/or expression of a homologous immune response (Behnke and Parish, 1979; Hurley, Day and Mitchell, 1980; Pritchard and Behnke, 1983).

Price and Turner (1986a) provide evidence which would appear to rule out antigenic competition, modification of antigen distribution, proteolytic degradation of the antigen or the generation of T-suppressor cells as the causes of immunosuppression. It is, instead, postulated that the adult produces a substance which modulates the host's immune response, an immunomodulatory factor (IMF). If this factor is produced then this would explain the chronicity of primary infections and also the requirement of adults to prevent the development of potent larval immunity (Behnke *et al.*, 1983).

The transfer of lymphocytes and serum from immune mice to naive mice which can subsequently be infected suggests that the failure to affect adults *in vivo* is attributable to modulation of the efferent arms of the immune response by the

adults or their IMF (Behnke & Pritchard 1985). As noted before the larvae can survive for prolonged periods within intestinal cysts which may suggest an immunodepressive activity albeit by a different mechanism (Behnke and Parish, 1979).

Recent work by Monroy, Dobson and Adams (1989) has identified a low molecular weight component of adult *H. polygyrus* ES(MT) that suppresses spleen cell proliferation to mitogens. It may be that this is the IMF or at least a component of it. Crawford, Behnke and Pritchard (1989) have also found that the antigens of *H. polygyrus* can suppress the *in vitro* response of spleen cells from uninfected mice to heterologous antigens (SRBC) indicating that the IMF may be a component of the adult.

How the putative IMF exerts its effect is uncertain. It may be that, unlike *Fasciola hepatica* ES products, it is not directly toxic to lymphocytes. Rather, it may cause a defect in antigen presentation, the macrophages becoming less efficient in infected hosts, leading to the generation of suppressor cells (Pritchard & Behnke, 1985). However, the failure of 2 deoxyguanosine treatment to affect parasite survival during challenge with a heterologous antigen, that is as NSID is occurring, suggests that non-specific T suppressor cells are not the sole reason for parasite survival (Pritchard *et al., 1984).*

The parasite or its IMF also affects cell localisation and proliferation, possibly interfering with the sequence of events that lead to cell trapping in the lymph nodes. The parasite affects host cell localisation in the intestines of parasitised mice (Hagan & Wakelin, 1982; Behnke, 1987) and this may also be the reason why little inflammation is seen on *H. polygyrus* infection. This may be correlated with the peripheral leucocytosis suggesting a division of white blood cells (Jones and Rubin, 1974). The cell trapping is thought to involve complement activation with subsequent prostaglandin E_2 production (Zatz and Lance, 1971; Hopkins *et al.*, 1981). If complement production is affected by the parasite (it can stimulate

complement by the alternative pathway (Prowse *et al.*, 1979)) then cell trapping would be affected with its concomitant effects on immunity to the parasite.

If the IMF does exist it may be an anti-cholinesterase. Anti-cholinesterases have been implicated in the survival strategies of other helminths (Dobson *et al.*, 1985) but other work disputes their importance (Rothwell, 1989). Despite the identity of the IMF not being known, in order for the host to become immune then not only must it recognise the protective antigens but it must also overcome the effects of the IMF. This may be the reason why immunity develops in repeated infections.

Although the parasite may depress immunity to homologous and heterologous antigens, SID is different in mechanism and effect to NSID. For an IMF to be effective in increasing parasite survival it must affect specific immunity and the effect may or may not be local. The importance of NSID in parasite survival is not certain, especially as it can be shown in other host parasite systems in which it does not benefit parasite survival (Terry and Hudson, 1982). The effects of NSID are small even in heavily infected hosts, whereas SID is strong in its effects in lightly infected hosts (Behnke, 1987). It is suggested that NSID and maybe SID involve increased catabolism of immunoglobulins but work suggests that this is not the case (Behnke, 1987). The IMF may cause production of nonspecific suppressor cells but if they have any relevance it is likely to be in the later stages of an infection. In the earlier stages tissue damage and toxic parasite products may contribute to NSID and although it may not be of benefit to the parasite, it may be an inevitable consequence of infection or, more unlikely, a side effect of a mechanism which acts to suppress the expression of homologous immunity (Terry and Hudson, 1982).

The specific suppressor effect of *H. polygyrus* is not permanent and on abbreviation of a primary infection the suppression of immunity wanes, as judged by establishment of a challenge infection (Dobson *et al.*, 1985). Residual levels of

immunodepression were reported at up to 5 weeks post abbreviation of an infection and by extrapolation its effects are judged to last 7 weeks. The NSID reported in heavy infections is of much shorter duration lasting for 17 days (Behnke & Ali 1984). Crawford and co-workers (Crawford, Behnke and Pritchard, 1989), in agreement with Price and Turner (1986b), suggest that T-cells are unlikely to be mediators of NSID. Instead it is postulated that a non-specific, non-T suppressor cell is involved, the generation of which is antigen-specific.

1.5 Genetic variation in resistance

The genetic basis of susceptibility and resistance to parasite infection has been extensively reviewed (Wakelin, 1978, 1985), and a series of papers (Dobson, 1982) has described genetic variation in resistance to *H. polygyrus*. Consequently, only a brief overview of the host genetic control of immunity to helminth infections will be given here.

The variation in responsiveness to *H. polygyrus* reported by many authors is mainly due to genetically determined differences in the hosts to respond to parasite infection. Investigations into responsiveness have mainly been concerned with those differences apparent in acquired immunity (Behnke and Robinson, 1985).

The majority of parameters used to assess resistance have been shown to exhibit genetically determined host variation (Wakelin,1985). If it is accepted that this genetic determination of responsiveness is common to most host/parasite systems, and that it plays a part in determining the distribution of a parasite in a host population, then studies in the laboratory using inbred stains of mice are of relevance to the question of the dynamics of parasites in the wild. Furthermore, the use of inbred strains, which differ in their response phenotype, may allow the identification of responses that are relevant to immunity or at least correlate with greater resistance.

Work on *H. polygyrus* and inbred strains of mice has shown that the response phenotype is indeed influenced by MHC genes and also by background genes, although their relative importance is not yet certain (Behnke and Robinson, 1985). The initial recognition of the antigen, which causes the release of lymphokines that may have an effect on cells that need not have specificity for the antigen, is under the control of the MHC. The translation of this lymphocyte mediated component to the myeloid cell mediated component is primarily linked to non-MHC genes (Wakelin *et al.*, 1986). It has been suggested that one way in which these background genes influence the response is by determining the susceptibility of bone marrow and stem cells to factors of lymphocyte origin (Wakelin and Donachie, 1983).

It is thought that the level of susceptibility, as determined by the MHC of the host, is related to the Ir gene product that the parasite antigen is presented with (Degwert *et al.*, 1987 ; Wassom et al., 1984).

Enriquez *et al.*, (1988) present evidence that two H-2 genes affect the level of resistance shown. They suggest that these genes act in much the same way as the Ts-1 and Ts-2 genes that are involved in the response to *T. spiralis*. The strains used in this study are CBA/Ca, a low responder to *H. polygyrus* with the haplotype $H2^k$, and NIH which is a high responder with the haplotype $H2^q$. These haplotypes have been correlated with resistance to other parasites (Behnke and Robinson, 1985; Enriquez *et al.*, 1988) although it should be stressed that high resistance to one parasite is not necessarily correlated with a high level of resistance to another.

1.6 The role of T-cells in anti-helminth responses

Wakelin (1985) states that the phenomena mediating resistance are, without exception, all T-cell mediated (the subset involved is generally CD4+). Thus, genetically determined variation in T-cell function could underlie the variation in response. This is therefore part of the rationale for this study. The products of the

major histocompatibility genes are known to regulate T-cell function (Behnke and Robinson, 1985). However, definite involvement of the MHC in parasite infections has been shown in very few cases (Wakelin, 1985).

Infection by most infectious agents, including gastro-intestinal nematodes, is known to be strongly controlled by T-cell dependent mechanisms of immunity (Miller, 1984). Other studies have also stressed the importance of the T-cell in immunity to gastro-intestinal helminths (Ruitenberg and Elgarsma, 1976; Catty and Ross, 1976; Dineen and Adams, 1971; Ferguson and Jarret, 1975; Gore, Burger and Sadun, 1970; Colley, 1981; Dehlawi and Wakelin, 1988; Vos *et al.*, 1983; Wakelin and Selby, 1974; Grencis and Wakelin, 1982; Wakelin, Grencis and Donachie, 1982).

The role that T-cells play in resistance and immune responses has been investigated using nude and thymectomised mice (For *T. spiralis*: Vos *et al*, 1983; Gore, Burger and Sadun, 1970. For *Schistosoma mansoni*: Colley, 1981. For *Trichuris muris*: Wakelin and Selby, 1974; Lee, Wakelin and Grencis, 1983; Parmentier *et al.*, 1987. For *H. polygyrus*: Prowse, Mitchell, Ey and Jenkin, 1978) or by depleting T-cells using complement and anti-lymphocyte serum (For *T. spiralis*: Ruitenberg and Elgersma, 1976; Gore, Burger and Sadun, 1970). Other studies have shown T-cell involvement in immunity by the adoptive transfer of cells from infected host to a naive host the level of resistance conferred being assessed by challenge of the recipient (For *Trichostrongylus colubriformis*: Dineen and Wagland, 1966; For *T. spiralis*: Gore, Burger and Sadun, 1970; Larsh, Race, Coulson and Weatherby, 1966; Grencis and Wakelin, 1982; Alizadeh and Wakelin, 1982. For *T. muris*: Wakelin and Selby, 1974. For *H. polygyrus*: Cypess, 1970).

All these studies indicate the importance of T-cells in the initiation and maintenance of the host's responses to the parasite. However, it should be noted that despite the observation of T-cell dependency, the T-cells or their products need not act directly on the parasites. Rather, it is likely that immunity is achieved by

the co-operation of T-cells, antibodies and, if relevant, inflammatory cells and their mediators. For example, Parmemtier *et al.* (1987) suggest that T-cells mediate expulsion of *T. spiralis* by releasing lymphokines that play a part in the generation of the acute inflammatory response.

The immune response to the parasite *H. polygyrus* has been intensively studied (see Behnke, 1987). It has been established that the parasite can be the target of an efficient host immune response (see section 1.3) despite its capability to immunodepress its host (see section 1.4). The mechanisms of this immunity are thought to involve macrophages and neutrophils. However, the major effector mechanism is thought to be IgG1 dependent eosinophilic killing (Pritchard and Behnke, 1983). IgG1 production is known to be T-cell dependent (Rawalho-Pinto and Smithers, 1981) and the activation of cells in the monocyte-macrophage series involves T lymphocytes (Butterworth, 1984)

More direct evidence of the T-cell dependence of immunity to *H. polygyrus* has been shown in thymus depletion experiments (Prowse *et al.*, 1978). In mice which have had their T-cells depleted in this way there is no inflammatory response and the worms show no delay in maturation even in repeated infections. However, homozygous nude mice (which lack thymuses and whose cells show no response to T-cell mitogens (Vos *et al.*, 1983)) do show some immunity and thus there must be a T-cell independent mechanism.

Despite the evidence for the involvement of T-cells in immunity to *H. polygyrus* and other helminths (Wakelin, 1986), as well as the applicability of the mouse/*H. polygyrus* system as a model for human infections, only a few studies have investigated the role of T-cells (Prowse *et al.*,1978; Bartlett and Ball 1974; Enriquez *et al.*, 1986; Price and Turner, 1986).

1.7 Assays for the activity of lymphocytes

For the study of the role of T-cells a suitable assay must be chosen and its protocol defined. However, it must be noted that the suitability of *in vitro* assays to

assess the importance of T-cell dependent mechanisms in resistance to parasites has been questioned. Phillips *et al* (1977) argue that as immunity is the result of a complex series of interactions of both cellular and humoral mechanisms, no single *in vitro* or *in vivo* determination can (or has been shown) to accurately reflect the *in vivo* situation. This argument is countered, somewhat, by Colley (1979) who suggests that even lacking a correlation between immune responses active against antigens and protective capabilities, it is perhaps important to remember that this does not rule out the possible participation of such responses in resistance. It may only mean they are not, of themselves, sufficient for its expression. Valid points concerning the use of *in vitro* assays have also been made by Butterworth (1984). Firstly they allow analysis of the mode of action and the nature of *in vivo* cellular mechanisms. Secondly, apart from anecdotal studies on individuals with innate or acquired immune defects, they provide the only approach to the identification of the mechanisms of (human) immunity. However, it should again be stressed that the *in vitro* mechanism may not accurately reflect or be relevant to the *in vivo* situation.

Despite the doubts about the use of these assays, an *in vitro* assay was used to study the role of T-cells in immunity to *H. polygyrus*. Lymphocyte function can be assessed by evaluating *in vitro* lymphocyte blast transformation in response to a stimulus. This trigger can be a non-specific one or, more usefully, a specific one which in this case would be antigens of *H. polygyrus*. This *in vitro* measure of antigen specific proliferation primarily detects T-cell responses (Mishell and Shiigi, 1980). More specifically the response to the antigens of *H. polygyrus* has been shown by antibody and complement depletion of Thy-1 cells (cells carrying the T-cell surface antigen) to primarily detect T-cell division (Price and Turner, 1986b).

The mechanisms of stimulation of cells in response to a specific signal (e.g. antigen) or to a non-specific one (e.g. mitogen) are thought to be the same, the differences in the response being quantitative not qualitative (Waithe and

Hirschhorn, 1978). Thus work on mitogen-stimulated proliferation is relevant to antigen-specific proliferation.

Proliferation in response to a mitogenic lectin (polyclonal activation) has been the method of choice to study the activation, growth and maturation of lymphocytes (Lane *et al.*, 1986)).This polyclonal (and, therefore, specific) activation normally induces both proliferation and maturation to effector function (judged by interleukin-2 production) and the *in vitro* situation does mimic the *in vivo* situation (unlike *in vitro* B-cell proliferation stimulated by anti-Ig immunoglobulin during which there is DNA but not immunoglobulin synthesis). However, Severinson and Larson (1986) suggest that activation, although it gives an indication of the ability of the cells to respond, gives "little insight" into the ability of the cells to differentiate into effector cells. If this limitation of the assay is accepted it is still a useful indicator of the ability to respond (that is, a measure of recognition).

It was decided to investigate the role of T-cells in immunity to *H. polygyrus* by using the *in vitro* lympho-proliferative assay. In this assay dividing cells incorporate radioactive isotope into their DNA. The amount of isotope taken up can be measured and is proportional, at least in the early stages of the assay, to the number of dividing cells (Olivotto *et al.*, 1982).

This *in vitro* measure of *in vivo* immune functioning may allow changes in the hosts response to the parasite to be correlated with concurrent changes in the worm burden during repeated infection. However, Wakelin (1985) notes that often it is difficult to correlate the observed response with *in vivo* resistance.

The level of proliferation observed during a lymphocyte proliferation assay can be affected by variables such as cell density, culture vessel geometry and media composition as well as by length of culture and procedural differences (Waithe and Hirschhorn, 1978). This necessitates a rigid, repeatable protocol and the use of controls. Controls are especially important as it is virtually impossible to establish

normal ranges for proliferation values as they will fluctuate from laboratory to laboratory and even within a given laboratory from time to time (Lane *et al.*, 1986).

The results obtained from the assay are in the form of counts per minute. The experimental results are performed in triplicate and should vary by less than 20% from the mean. A greater variation than this is indicative of technical problems such as bacterial contamination (Oppenheim and Schecter, 1980). The data is often expressed as raw counts per minute or as a stimulation index (counts per minute in the presence of antigen/counts per minute in the absence of antigen) and for this reason in this study both forms of data representation are used. However, as Oppenheim and Schecter (1980) report, although the use of SIs normalises the data to some extent, it is based on the assumption that the degree of proliferation in the control and experimental cultures will be affected by variables in the same way and remain proportional. Unfortunately this is not always the case and this can result in artificially lowered ratios. Consequently, it is recommended that controls as well as experimental data should be shown.

The variability seen when proliferation is considered is in part due to the process of cell growth. The cell population increases in size exponentially (Olivotto *et al.*, 1982). As a consequence there are large changes in the magnitude of isotope incorporation with increasing duration of incubation (Oppenheim and Shecter, 1980). Furthermore, the size of the population at the time of sampling is dependent on the number of cells at the beginning of the population growth and the growth rate of these cells. Due to the exponential growth rate, these small differences in growth rate and population size will become magnified with time resulting in a large variance and a non-normal distribution of the data. For proper statistical assessment the data must first be log transformed (Oppenheim and Schechter, 1980). The effect of starting conditions on the size of the final population and the resulting variance (if the proliferation values of a number of mice are combined) can be shown mathematically:-

(a) Size of population: If N_0=population at time $t=0$; N_t=population at time t; a=birth rate; u=death rate; $r=a-u$, then the number of cells at a given time,t, is given by: $Nt=e^{rt}$.

(b) Variance of population: The variance is also dependent on the size of the population at the start and also increases with time, the greater the size of the response the larger the variance is likely to be. (variance at time $t=[N_0(a+u)/r]exp^{rt}[e^{rt}-1]$) Anderson and Gordon, 1982).

It can be seen that the system is sensitive to the starting conditions, i.e. the value of N_0. Furthermore, as time progresses much variability is to be expected in the growth of cell populations in different replicates due to the instance of heterogeneity in experimental conditions. It should be stressed that the large variances are an unavoidable facet of the assay. The variances should be considered during any analysis of the results as the expansion of variance with time and size of response makes statistical analysis less sensitive.

The number of cells can be affected by the accuracy of pipetting (Mishell and Shiigi, 1980; Lane *et al.*, 1976) and the proportion of responding cells in the population (about 20% of cells respond to Con A (Olivotto *et al.*, 1982)) and of cells in the body about 0.01% are antigen sensitive (Zatz and Lance, 1971)) may also alter.

A further caveat is that activated lymphocytes release a number of factors which can modify the response. As the measured event is both temporally and physically far removed from the initial activation of the responding cells then this may obscure the quantitative nature of the original response (Severinson and Larson, 1986).

It is the aim of this study to investigate aspects of the cellular and humoral basis of immunity and how they change with parasite burden and time since initial infection in different strains of mice.

Chapter 2: Method and materials

2.1 Mouse maintenance

All mice strains (CBA/ca, NIH/ola, MF1) were purchased from Harlan Olac (Bicester, UK) or obtained from small breeding stocks maintained at the Department of Pure and Applied Biology, Imperial College. The mice were housed under standard animal house conditions in cages lined with wood chippings. This bedding was changed at weekly intervals to avoid infection with *Aspiculuris tetraptera* and *Syphacia obvelata*. The mice were fed on pelleted animal diet (Oxoid 4B) and watered *ad libitum*.

2.2 Parasite maintenance

The parasite *Heligmosomoides polygyrus* was obtained from Dr John Lewis (Royal Holloway and Bedford New College, London) by Dr Malefane Maema. It has been passaged in MF1 or CBA/ca mice at Imperial College for the past seven years. Previously it was passaged in ASH, CB1 or MF1 mice at the Wellcome laboratories, Kent.

2.3 Parasite culture

Infected mice in groups of up to 10 were housed overnight without food in wire bottomed faecal collection cages. Their faeces, containing the parasite eggs, were collected on a tray lined with moist tissue paper to prevent desiccation.

After the period of collection, all the faecal pellets were placed in a clean universal container (Sterilin, UK) and the worm eggs cultured according to the following modification of the MAFF method (MAFF, 1977) as suggested by Maema (1987). The faeces were crushed into a smooth paste using a pestle and mortar, adding distilled water as required. The paste was then washed through a fine muslin or wire mesh (mesh size 250 um) with distilled water into a large specimen jar. The contents of the flask were then allowed to settle for at least 2 hrs, the

debris (largely undigested vegetable matter) that remained on the mesh was discarded.

After settling the supernatant was discarded and the sediment poured into 15 ml polypropylene, round bottomed centrifuge tubes (Sterilin UK) and spun at 800g for 5 minutes. The supernatant was discarded and the resulting pellet spread onto the filter paper of a culture dish prepared as described below.

Three strips of Whatman 54 filter paper 3-5 cm wide were placed over a small inverted petri dish (diameter 4.5 cm) and their ends tucked under the dish. The filter paper and dish were then placed in a larger petri dish (diameter 14.0 cm) and sufficient distilled water added to ensure that the filter paper was moist and a shallow layer remained in the dish when it was incubated.

Once the faeces had been smeared onto the filter paper, the petri dish's lid was replaced and the dish and contents incubated for 7 days at 25°C.

After this period any hatched larvae either migrated from the faecal sediment into the water or remained attached to the edge of the filter paper. The free larvae were pipetted off into a 30 ml centrifuge tube (Sterilin UK). Those that remained attached to the filter paper were detached from it by gentle rinsing with distilled water and then pipetted into the centrifuge tube. The larvae were then allowed to settle under gravity and the supernatant pipetted off. The larvae were then washed in distilled water by removing the supernatant and resuspending them in distilled water. The washing was repeated two or three times and had the effect of removing most of the faecal debris and bacteria which adversely affect larval survival on storage (Maema, 1987). The larvae were then stored at 4°C in a small volume of distilled water.

2.4 Host infection

Mice were infected by gavage (stomach intubation). A known number of larvae in 100 ul of distilled water was given to the mouse via a syringe and a 35 mm 19

gauge needle (B&D UK) modified to take a blunt tip and plastic "blob" (the "blob" reduces the risk of injury to the mouse). The mouse was held by the scruff of the neck using the thumb and forefinger to prevent head movement. The tail was restrained gently by the little finger. This method of handling allowed the tube to be introduced into the mouse's mouth, and subsequently its oesophagus, whilst reducing the risk of introducing the larval suspension into the trachea.

2.5 Mouse chemotherapy

The drug of choice for clearing both the larval and adult stages of *H. polygyrus* from infected mice was Ivermectin (Merck and Sharpe Ltd., UK). This drug has an efficacy of greater than 95% against adults and 100% efficacy against larvae on the sixth day of infection (Sayles and Jacobson 1983). The drug was administered by gavage at a dose of 10 mg/kg body weight (diluted from the original stock solution in propan-1-2-diol) in a volume as close to 100 µl as possible.

2.6 Faecal egg collection and counting

Faecal collection cages, as described for the culture of the parasite, were used to collect faeces for egg counting. The mice were housed individually with food and water. The faeces were collected in a tray lined with moist tissue paper for 16 hrs. The length of time over which faecal collection was performed was 16 hrs as Dr Maema reported that the amount of eggs produced in this period was directly proportional to the amount produced in 24 hrs (pers. comm.). The shorter period of collection had the advantage of reducing the stress to the mice; especially important when faeces were collected from heavily infected mice which ate and drank little during their time in the cages.

The faecal pellets were collected in a pre-weighed conical bottomed test tube (Sterilin UK) and their weight determined. A small amount of saturated magnesium sulphate (specific gravity 1.25) was added to the pellets which were

then ground into a thick paste in the container in which they were collected (so as to reduce any possible loss). The suspension was then washed through a muslin or wire mesh (mesh size 250 um) into a large centrifuge tube using up to 30 ml of saturated magnesium sulphate per gram of faeces, a note being made of the volume used.

The tube containing the salt solution, eggs and faecal debris was shaken to ensure an uniform distribution of eggs. A small volume of the suspension was then pipetted into a MacMaster slide and the slide allowed to stand for two minutes before counting. Those eggs which had floated up to underneath the slide's grid were counted using the 40 times objective of a compound stereo microscope. The number of eggs per female or per 16 hrs was calculated assuming that the volume under the grid was 0.15 ml and knowing the volume of salt suspension from which the sample in the counting chamber was taken.

2.7 Organ removal

2.7.1 Blood collection

All mice were killed by cervical dislocation. If blood was to be collected, it was first withdrawn from the neck cavity using a 19 gauge needle attached to a 1 ml syringe, introduced via a small incision in the skin of the neck. When sufficient blood, along with any large clots, had been collected from the neck, blood was extracted form the thoracic cavity. This was done by piercing the chest wall and puncturing the heart with the needle, any blood being drawn up into the syringe.

The blood was put into a 1.5 ml reaction vial and allowed to clot at room temperature for 1 hour and then left overnight at 4°C. It was then centrifuged at 2000 rpm for 5 min at 4°C. The serum was pipetted off, placed in a labelled 1.5 ml reaction vial and stored at -20°C until required

2.7.2 Spleen and mesenteric lymph node removal.

The use of the spleen and mesenteric lymph nodes (MLN) in proliferation assays, described below, required that their removal was as sterile as possible. Thus, after killing by cervical dislocation the mouse was dipped in 70% ethanol. This sterilized the surface of the mouse, to some extent, but also reduced the possibility of fluff and dander becoming airborne. The mouse was then placed in a sterile laminar flow hood and its skin flayed using a pair of scissors which had previously been dipped in 70% ethanol. Using another pair of scissors and forceps, which had also been dipped in alcohol and then allowed to dry in a flow of sterile air, the peritoneal wall was cut and flayed.

The MLN was exposed by reflexing the gut. This lymph node is visible as firm translucent bumps in contrast to the soft fat in which it is embedded and is located within the mesentery of the ascending colon (Mishell and Shigii 1980). Using a pair of scissors, which had previously been dipped in 70% alcohol and allowed to air dry, the mesenteric lymph node was removed by cutting it away from the surrounding tissue. Care was taken not to cut the intestine as this would have probably have given rise to contamination on culturing its cells. On its removal the MLN was placed in warm culture medium (described in appendix 4) and used in the lymphocyte proliferation assay as soon as was possible.

The spleen is located in the upper left region of the abdominal cavity and was exposed by folding back the peritoneum and then displacing the intestines. Using forceps and scissors dipped in alcohol and allowed to air dry, the spleen was removed by cutting the anchoring connective tissue and surrounding vessels whilst taking care not to rupture its capsule. It was then placed in warm sterile culture medium and used as soon as possible in the lymphocyte proliferation assay.

2.7.3 Intestine removal

After the spleen and MLN had been removed, the intestine was removed by cutting if below the pyloric sphincter and above the ileo-caecal junction. Its

subsequent treatment depended on whether the worms contained within were to be counted or used as a source of antigen.

2.8 Counting of parasites

If the worm burden was to be determined then the intestine was placed in a 7 ml bijou flask (Sterilin UK) containing 5 ml of 10% formol saline and stored at room temperature until the number of worms could be determined. To count the worms the intestine was bathed in physiological saline to reduce the preservative fumes and then cut longitudinally. The intestine was examined under a stereo dissecting microscope and the worms removed using a pair of fine forceps (Inox. No. 5 forceps, Inox UK Ltd.). The worms were scored as male or female or as a pair *in copulo*. The sexes were identified by their distinctive posterior ends. This feature facilitated the identification of fragmented worms.

2.9 Recovery of parasites for use in antigen preparation

If the intestines were used as a source of parasite material then on their removal from infected mice they were placed in petri dishes containing physiological saline. If adults were to be extracted the intestine was cut along its length. If, however, larval stages (from the sixth day of infection) were to be removed then the intestine was ligatured at both ends with fine cotton thread to prevent spillage of its contents (Ey, Prowse and Jenkin, 1980)).

After the appropriate treatment the guts were incubated for 1-2 hrs at 37°C. Following this incubation the petri dish and contents were examined under a dissecting microscope and any worms which had migrated away from the intestine were removed from the saline using a wide bored pasteur pipette (reducing the risk of damage to the worms) and placed in a 1.5 ml reaction vial. The parasites which remained attached to the wall of the intestine were removed using fine forceps.The worms were then washed in RPMI 1640 containing 10 times (10 ug/ml)

gentamycin and 10000 ui/ml penicillin/streptomycin. After the final wash with non-sterile medium the worms were washed in sterile antibiotic supplemented medium and then snap frozen and stored at -70°C until required.

2.10 Antigen preparation from life cycle stages of *H.polygyrus*

2.10.1 Third stage larvae antigen preparation

The larvae were collected from faecal culture as previously described. They were then washed in distilled water until as much as possible of the contaminating debris had been removed. For the final wash the larvae were resuspended in RPMI 1640 supplemented with 10 times gentamycin and 10000 µi/ml of penicillin/streptomycin (i.e. antibiotic supplemented medium).

The larvae were then homogenised on ice using either a Down's Tissue Homogeniser or a sonicator (LKB Ltd., UK) The worms were sonicated at a wavelength of 8 um in short bursts (15 seconds) with intervals of at least a minute to prevent heating of the suspension and possible micelle formation, both of which would have lead to protein denaturation. Homogenization was judged to have been completed when the majority of the larvae were no longer intact.

The homogenate was then centrifuged at 14000 rpm in a bench top centrifuge at 4°C for 30 minutes. The supernatant was then removed and the pellet discarded. The supernatant was sterilized by filtration through a 0.22 µm membrane filter (Millivex USA) into a sterile 1.5 ml reaction vial (Scotlab Ltd., UK). The protein concentration was determined by a Bradford protein assay (Biorad UK). The L3 homogenate was then stored at -70°C until required.

2.10.2 Adult and D6L4 homogenate antigen preparation

The parasites in antibiotic supplemented medium were homogenised as described above. The resultant preparation was then decanted into sterile 1.5 ml reaction vials and centrifuged at 14000 rpm at a temperature of 4°C for 30 minutes. The supernatant was pipetted off, under sterile conditions, into fresh 1.5

ml reaction vials. The protein concentration of the homogenate was determined by a Bradford protein assay and it was stored at -70°C until required.

2.10.3 L3, adult and D6L4 ES(MT) collection and preparation

After washing in antibiotic supplemented medium, freshly collected live worms were placed into sterile 1.5 ml reaction vials containing antibiotic supplemented medium (RPMI 1640 with 10 µg/ml gentamycin and 10000 µi/ml penicillin/streptomycin) to which had been added glucose (at 1% w/v). In each reaction vial about 20-40 worms were placed so that for each worm there was 40-100 µl of medium. The 1.5 ml reaction vial and contents were then incubated at 37°C in a CO_2 incubator at 100% relative humidity and 5% CO_2. The medium was collected every 12-14 hrs and replaced with fresh medium, the collected medium being stored at -20°C until required. On changing the medium any vials which had a majority of dead worms (as judged by a lack of motility) were discarded. Collection of the medium was stopped after 3 days of incubation and all the worms discarded.

The worm products in the supernatants were concentrated by ultrafiltration. The solution was filtered through a fine membrane (PM10, Amicon USA) under 3.7 atmospheres of nitrogen. The membrane retains any molecule whose size is greater than 10 kilodaltons, thus allowing the concentration of the ES(MT) in which the molecules of interest are probably greater than 10 kd (Pritchard *et al.*, 1984; Pritchard, 1986). The solution underwent ultrafiltration until it had been concentrated at least twenty fold. The resultant concentrates were then sterilized by passing them through a 0.22 µm membrane filter and stored at -70°C until required. The protein concentration of the preparations was determined by a Bradford protein assay.

2.11 *In vitro* lymphocyte proliferation assay

The technique used was an adaptation of an in vitro assay, developed by Corradin, Etlinger and Chiller (1977) with modifications suggested by Mishell and

Shigii (1980), to measure antigen induced proliferation of murine cells. Organs removed aseptically from mice killed by cervical dislocation were placed in a bijou containing 5 ml of culture medium. (For details of the medium see appendix 2). Under sterile conditions single cell suspensions were prepared from the organ by crushing it through a sterile stainless steel gauze (mesh size 32 by 32, Locker Wire Weavers, UK, Ltd.) using the plunger of a sterile 5 ml syringe (BDH UK) as a pestle. The cells were pushed through the mesh into a petri dish containing approximately 5 ml of medium and the mesh then rinsed with a small amount of medium to wash off any remaining cells. The petri dish's contents were left to allow any large cell clumps and debris to settle. After a few minutes the supernatant, containing mainly single cells, was aspirated into a 13.5 ml conical bottomed test tube (Sterilin UK) and then spun at 200 g for 5 minutes at room temperature.

If a single cell suspension was to be prepared from the mesenteric lymph node the supernatant was discarded and the (pellet of) cells resuspended in warm medium. If, however, a single cell suspension was to be prepared from the spleen then after discarding the supernatant the cells were briefly resuspended in 2 ml of hypotonic lysis solution (details in appendix 4). Suspension of the preparation of spleen cells in the lysis solution had the effect of lysing any red blood cells which may interfere with the counting of the white cell details. Ten ml of medium was then added and the cells were then washed twice in warm medium. After the second wash the concentration and viability of the cells was determined by Trypan Blue Dye exclusion (Mishell and Shigii, 1980).

The concentration of the cells was then adjusted to that required by the experimental design and the cells dispensed in 100 ul aliquots into the wells of a microtitre plate (Nunc 96F, Gibco Ltd., UK). To each of the wells was added 100 ul of antigen or mitogen at the required concentration or medium alone. Each

treatment, whether medium alone or the appropriate concentration of antigen or mitogen, was carried out in triplicate.

The covered plates and contents were then incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C for the length of time determined by the protocol. After this period (referred to as the pre-pulse period) 50 ul of medium containing 0.1 µCi of the isotope 5-Iodo-2-deoxyuridine ^{125}I (Amersham Ltd., UK) was added to each well. The plates were then incubated for a further 18 or 24 hours (the pulse period) before the cells they contained were harvested. The cells were harvested onto glass fibre filter paper (934AH, Whatman Ltd., UK) using an automated cell harvester (Multimash 2000, Dynatech Ltd., UK) by sequential washing with 0.85% NaCl, 5% trichloroacetic acid and absolute methanol. The cells were then air dried. Discs corresponding to individual wells were placed in LP3 tubes (Luckham UK) and the amount of radioactive iododeoxyuridine incorporated was assessed by counting the radioactive disintegrations in a gamma counter (1275 Minigamma LKB Ltd., UK). The results were expressed as either counts per minute (cpm) or as a stimulation index (SI, calculated as the ratio of cpm cells cultured with antigen or mitogen to cpm of cells cultured with medium only).

2.12.1 Serological analysis by ELISA.

Serum samples from mice were analysed for antibodies against *H.polygyrus* using the Enzyme Linked Immunosorbent Assay (ELISA). The assay was used to test for the levels of total immunoglobulin and the immunoglobulin subclass IgG1 using horse radish peroxidase conjugated rabbit immunoglobulins with specificity to mouse immunoglobulin (Dakopatt, UK Ltd.) and to the light chain of IgG1 (Nordic UK Ltd.) respectively. The protocol used was an adaptation of that given by Lillywhite, Burgess and Stewart (LSHTM int. pub.).

The parasite antigen (D6L4 or adult homogenate) was diluted to the appropriate concentration in carbonate coating buffer (see appendix 2). 100 ul of this solution was placed into all the wells, except those in the first column, of a 96 well microtitre plate (Linbro, Flow Ltd., UK). In the first column coating buffer only was added and this served as a control to assess non-specific binding. The plate and contents were then incubated overnight at 4C.

After the overnight incubation the unattached antigen was then removed by washing three times with PBS-Tween (appendix 4). 100 ul of 2% Bovine Serum Albumin was added to each well to block non-specific binding and the plate then incubated for 2 hours at room temperature before washing three times with PBS-Tween to remove any unattached BSA. 100 ul of serum diluted in incubation buffer (appendix 2) were then added to each well and the plate incubated for 2 hrs at room temperature. The unattached serum was then removed by washing.

A volume of 100 µl of the appropriate horse radish peroxidase conjugated immunoglobulin (anti-IgG1 conjugate from Nordic Immunological Ltd., UK; anti-total immunoglobulin from Miles Ltd., UK) diluted in incubation buffer was added to each well. The plate was then incubated for 3 hours at room temperature. Unattached conjugate was then washed off and 100 ul of substrate buffer added to each well. The plate was covered with foil to prevent photo-decomposition of the orthophenyldiamine in the substrate and incubated at room temperature for 30 minutes. The reaction was then stopped by the addition of 50 µl of 2 molar H_2SO_4 to each well. The plate was read spectrophotometrically at 490 nm by an automated ELISA reader (Flow Ltd., UK), the machine being blanked on the first column.

2.12.2 Determination of the antigen and antibody dilutions used to assay immunoglobulin levels in serum samples from repeatedly infected mice.

To determine the dilutions of antigen and antibody required a checkerboard titration was performed. The antigen was double diluted down the rows of a

microtitre plate from an initial concentration of 10 ug/ml through to 0.078 ug/ml and the plate then incubated. After washing and then blocking with 2% bovine serum albumin, positive sera (pooled from 3 NIH mice which had received 50 L3/week for 15 weeks) was doubly diluted across half the plate from an initial dilution of 1/100 through to 1/1600. Negative serum (pooled from 5 naive NIH mice) was similarly diluted across the other half. The protocol outlined above was then followed.

From the results obtained the concentration at which the antigen was no longer in excess was 2.5 µg/ml and the serum dilution at which the positive and negative sera could be distinguished was 1/300. Thus these dilutions were used in all assays to determine antibody levels in serum from infected mice.

2.12.3 Serological analysis of serum from mice given repeated low-level infections of *H.polygyrus*.

A 1/10 dilution of the sera was made in 50% glycerol in PBS and stored at -20°C until required. The 50% glycerol was used as a diluent as it does not freeze at the storage temperatures used and thus reduces damage caused by the repeated freeze/thawing that the repeated assaying of the samples would otherwise require (Kemeny and Chantler, 1988). The glycerol diluent has been found to have little effect on the reactivity of human sera in the ELISA system even after prolonged storage (M.J. Cox pers. comm.). A working dilution of 1/300 was obtained by adding 10 µl of the 1/10 dilution to 90 µl of incubation buffer in the appropriate well of the microtitre plate. The antigen dilution was prepared from a stock solution as required.

CHAPTER 3: Cellular responses in naive and infected mice.

3.1 Introduction

The mouse/*H.polygyrus* system is often used as a crude model of human hookworm infection (Bartlett and Ball, 1974). It has been used to investigate many aspects of infection although it is the immunological one which has received the most attention.

It is widely held that resistance to helminths has a large T-cell component (Miller, 1984; Wakelin, 1986), as discussed in the first chapter. If this is the case then lymphocyte responsiveness can be studied to investigate the role of genetically determined variation in T-cell function that underlies resistance. To this end, conditions must be established to measure *in vitro* lympho-proliferative responses to preparations of *H. polygyrus* proteins.

Once the assay has been established it can be used to determine whether or not the ability of different strains to develop resistance can be correlated with their responses to the parasite's antigens. The statement, by Price and Turner (1986b), that this approach is especially valid in view of the fact that the likelihood of the development of host-protective vaccines may depend on the characterisation of specific cellular responses to the parasite's antigens, is relevant to this study.

The assay was used to measure lymphocyte blast transformation in response to specific (e.g. *H. polygyrus* antigen) and non-specific stimuli (e.g. Con A). The *in vitro* measure of *in vivo* immune functioning was correlated with changes in parasite burden during single, repeated and anthelmintic abbreviated infections in two strains of mice.

The aim of this chapter is to describe the experiments performed to establish the conditions required to assay the host's cellular response to various life cycle stages of *H.polygyrus*. Thus, the variability of the response to the mitogen Con A is

considered (section 3.2). Once the appropriate ranges for the proliferative response have been established, the ability of antigens from the parasite to effect proliferation are investigated: the non-specific proliferative properties of the preparations are considered (section 3.3); the response of cells from mice which have experienced a primary infection (section 3.4), an immunising regime and the effect of varying the length of incubation on the proliferative response of cells from immunised mice are determined (section 3.5). The responses of cells from both the mesenteric lymph node and the spleen to antigens of *H. polygyrus* are investigated (section 3.6).

3.2 Mitogen induced proliferation

3.2.1 Introduction and aims

Lymphocyte proliferation assays are useful *in vitro* measures of *in vivo* immune functioning. However, as well as being affected by the degree of *in vivo* activation, the amount of proliferation observed may vary due to culture conditions and procedural differences (Lane *et al.*, 1986). The use of a rigid repeatable protocol reduces the effect of the latter sources, the only other source of non-specific variability being that due to sampling from an exponentially growing population (Anderson and Gordon, 1984).

To allow delineation of what was normal immune functioning, given that absolute ranges are impossible to determine, the range of proliferation values in response to a non-specific stimulus was established by repeating a rigid protocol. Mitogen induced proliferation was used to establish the conditions as it is widely used as a model of immunocyte response to antigen stimulation (Gibbs, Potts, Brown, Robertson and Swanson-Beck, 1982).

3.2.2 Method

A range of dilutions of Con A was prepared by double diluting the mitogen in medium from an initial concentration of 100 ug/ml through to 0.5 ug/ml. 100 ul of male CBA mesenteric lymph node (MLN) cells at a concentration of $5x10^6$ cells/ml

was then added to 100 ul of the appropriate concentration of the mitogen Concavalin A (Con A). Each dilution was made in triplicate in the wells of a microtitre tissue culture plate. Cells were added in a volume of 100 ul to 100 ul of the appropriate dilution of Con A giving a final concentration of the mitogen half that indicated in the figures. The cells were then cultured in a CO_2 incubator for 48 hours before the addition of the isotope and harvested at 72 hours (i.e. 48 hours pre-pulse, 24 hours post-pulse). The level of proliferation was judged by measuring the amount of isotope which had been incorporated by the cells (assayed by counting the gamma emissions from individual discs corresponding to individual wells). This assay was repeated a number of times to allow a range of proliferation values to be obtained.

3.2.3 Results

It was found that in the majority of cases, the maximum proliferative response occurred at a dilution of Con A corresponding to 12.5 ug/ml (actual concentration

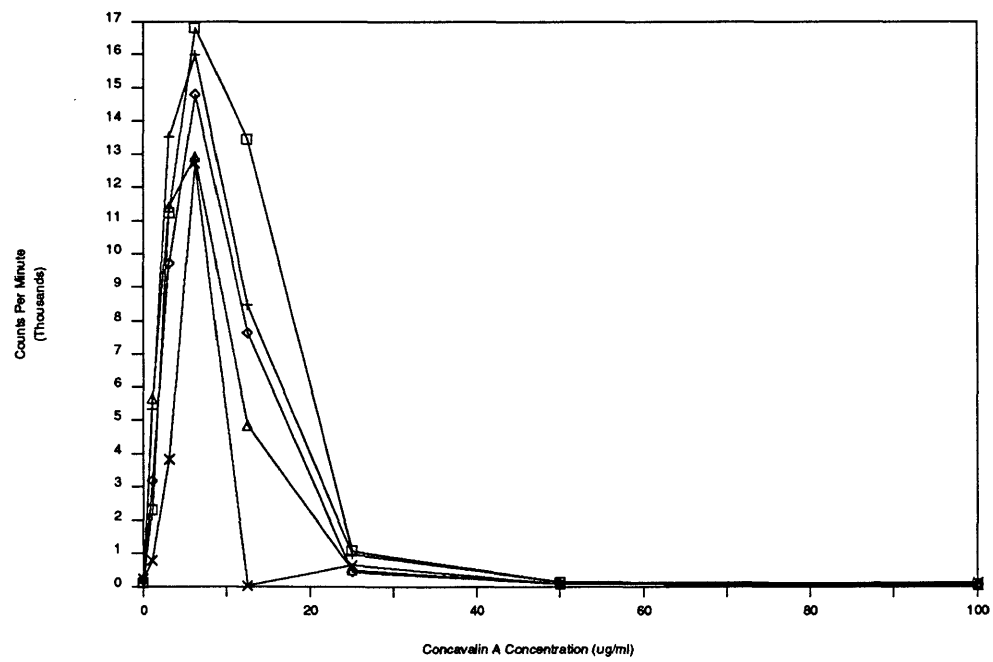


Figure 3.1 *The* in vitro *response of mesenteric lymph node cells of 5 CBA female mice to the mitogen Concavalin A.* Each line represents the response of an individual mouse.

in well being 6.25 ug/ml). In the few other cases the peak was at 25 µg/ml or 6.25 µg/ml or very rarely at 3.125 µg/ml of Con A (e.g. Figure 3.1).

The amount of proliferation, as judged by the stimulation index, was observed to vary between 40 and 450 and the actual counts obtained ranged between 3000 and 45000. Analysis showed that in the majority of cases there was relatively little variation between the responses of cells from the MLN of individual mice within one experimental period. For example, for the results illustrated in figure 3.1 there was only one mouse whose cells' response to Con A was different from that of all the others (statistical analysis is given in appendix 1.1). However, there was often, but not invariably, a significant difference in the response between experimental periods (see appendix 1.1)

3.3 Non-specific proliferative properties of *H. polygyrus* antigens.

3.3.1 Introduction and aims

"Antigens" prepared from the various life cycle stages of *H.polygyrus* may stimulate non-specific proliferation. This ability to cause proliferation in cells from naive mice is also shown by the antigens of other parasites such as *P. falciparum* (Golenser *et al.*, 1985) and *N. brasiliensis* (Price and Turner, 1986). If the extract has strong non-specific proliferative properties then the response it stimulates may obscure any changes that correspond to an *in vivo* alteration in responsiveness (Corradin *et al.*, 1977).

3.3.2 Non-specific proliferative properties of adult *H. polygyrus* antigen.

3.3.2a Method

To determine whether the antigens of *H.polygyrus* had non-specific proliferative effects MLN cells from five 6-8 week old CBA female mice, bred under standard laboratory conditions, were added at different cell densities ($3,4,5,8,10,20 \times 10^6$ cells/ml) to a range of concentrations of adult homogenate (10

ug/ml double diluted through to 0.08 ug/ml) and to medium alone (a control for spontaneous proliferation). The cells were incubated for 72 hours before addition of the isotope and then harvested 18 hours later.

3.3.2b Results

The results of the experiment are shown in figure 3.2 and the statistical analysis given in appendix 1.2 It was observed that, compared to the proliferation of cells from naive mice in the absence of antigen, cells from the same mice when cultured with adult homogenate did not show a significant increase in cell division at any antigen concentration. There was a significant increase in background or spontaneous proliferation with increasing cell density .

3.3.3 Non-specific proliferative properties of adult and D6L4 homogenates.

3.3.3a Method

The concentration of adult homogenate used may have been insufficient for any non-specific effect to have occurred. Thus, cells from naive CBA mice were exposed to a smaller range of concentrations of adult homogenate. The mitogenicity of D6L4 homogenate was also investigated by adding the cells to a range of dilutions of the preparation. Both the antigens were double diluted from 200 ug/ml through to 0.16 ug/ml. Due to the difficulty in obtaining sufficient parasite material at the time of the assay, a cell density of only $5x10^6$ cells/ml was used. Incubation pre-pulse was for 72 hours and for 18 hours post-pulse.

Figure 3.2 In vitro *response of mesenteric lymph node cells of naive CBA mice at different densities to adult* H. polygyrus *homogenate. The lines represent the isotope incorporation of the cells at the following densities (concentration expressed as cells/ml): $2x10^6$ (square); $4x10^6$ (+); $5x10^6$ (lozenge); $8x10^6$ (triangle); $10x10^6$ (x); $20x10^6$ (inverted triangle).*

Figure 3.3 *The proliferation of mesenteric lymph node cells of uninfected CBA mice to adult homogenate (square) or D6L4 homogenate (+).*

Figure 3.2

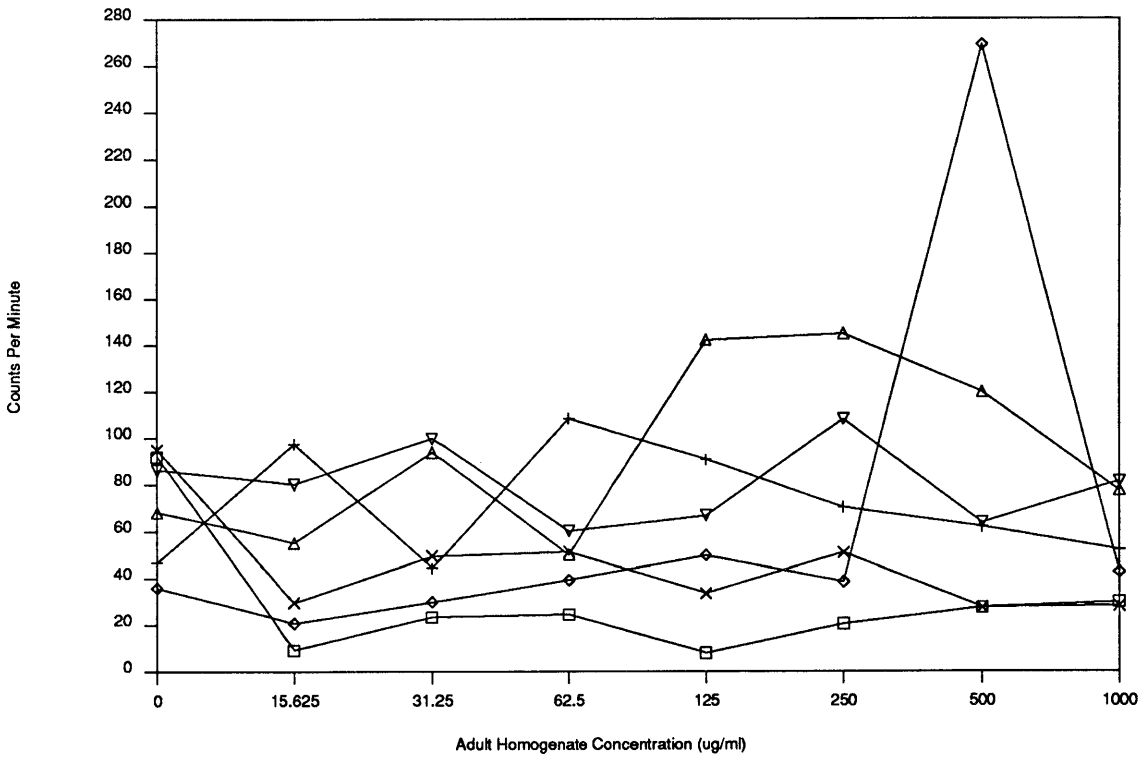
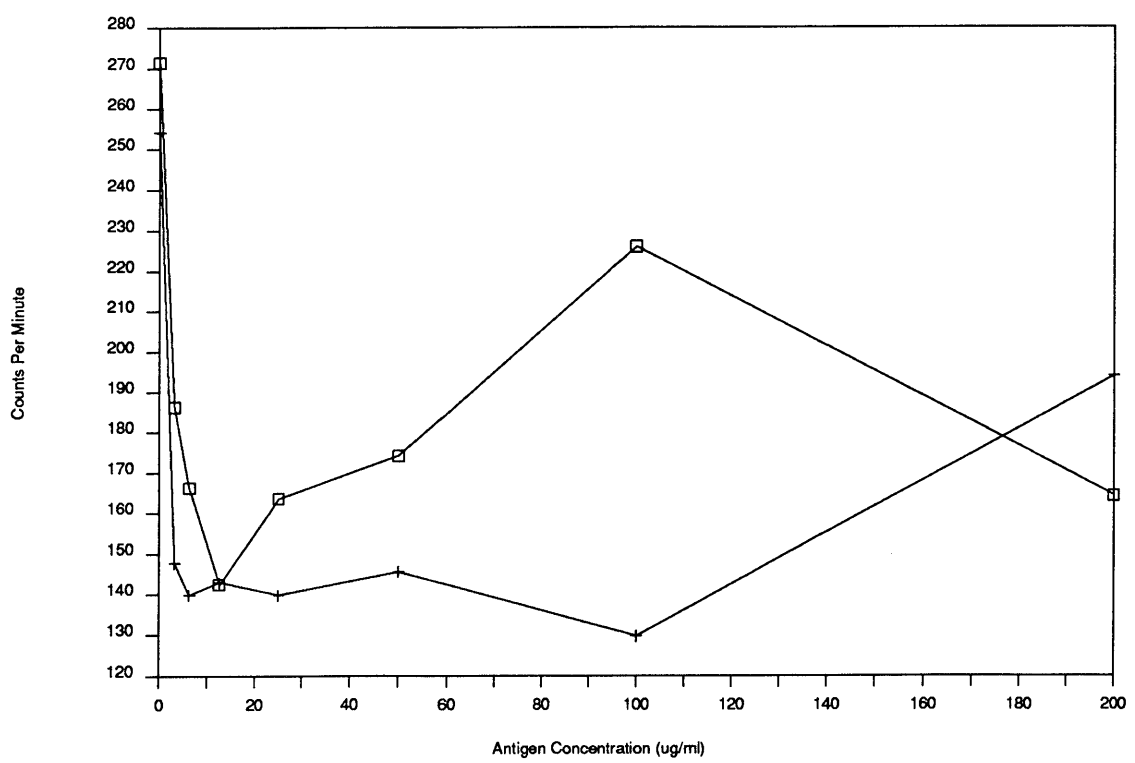


Figure 3.3



3.3.3b Results

The results are shown in figure 3.3 and the statistical analysis given in appendix 1.2. No non-specific proliferative effects were observed when higher concentrations of the adult homogenate were used, the response when cells were cultured with medium alone being greater than when they were cultured with antigen. The D6L4 homogenate also showed no significant proliferation when incubated with cells from naive mice.

3.4 Cellular responses in mice infected once

3.4.1 Introduction and aims

The preparations of parasite antigens had no non-specific proliferative effect and thus it thought likely that the response of cells from non-naive mice would not be obscured by non-specific reactivity. The system was therefore used to investigate the changes in lymphocyte activity that occurred on infection with the parasite.

3.4.2 Method

To determine the changes in lymphocyte proliferative ability that occur as a result of a single infection with *H.polygyrus*, MLN cells from mice which had only been infected once with the parasite were incubated with D6L4 homogenate and adult homogenate. Single cell suspensions were prepared from the MLN of 3 CBA female mice which had been infected with 150 larval *H.polygyrus* three months earlier. The cells were pooled and incubated at a concentration of $5x10^6$/ml with a range of dilutions of either adult or D6L4 homogenate, both antigens being double diluted in medium from an initial concentration of 200 ug/ml through to a final concentration of 3.13 ug/ml. The procedure was repeated using cells from naive mice rather than infected mice. Cells from both naive and infected mice were also incubated with a range of dilutions of Con A (50 ug/ml double diluted through to 6.25 ug/ml) to act as positive controls, the cells from naive mice acting as

inter-experimental controls. The cells were incubated for 72 hours pre-pulse and 18 hours post-pulse before harvesting.

3.4.3 Results

The results obtained are shown in figure 3.4 and the statistical analysis given in appendix 1.3. The cells from infected mice incubated with adult homogenate did not proliferate any more than did cells from naive mice incubated with the same homogenate. Cells from infected mice when incubated with D6L4 homogenate also proliferated significantly more than did the cells from naive mice. The proliferation of the cells from naive mice to the D6L4 homogenate was greater than it was to the adult homogenate.

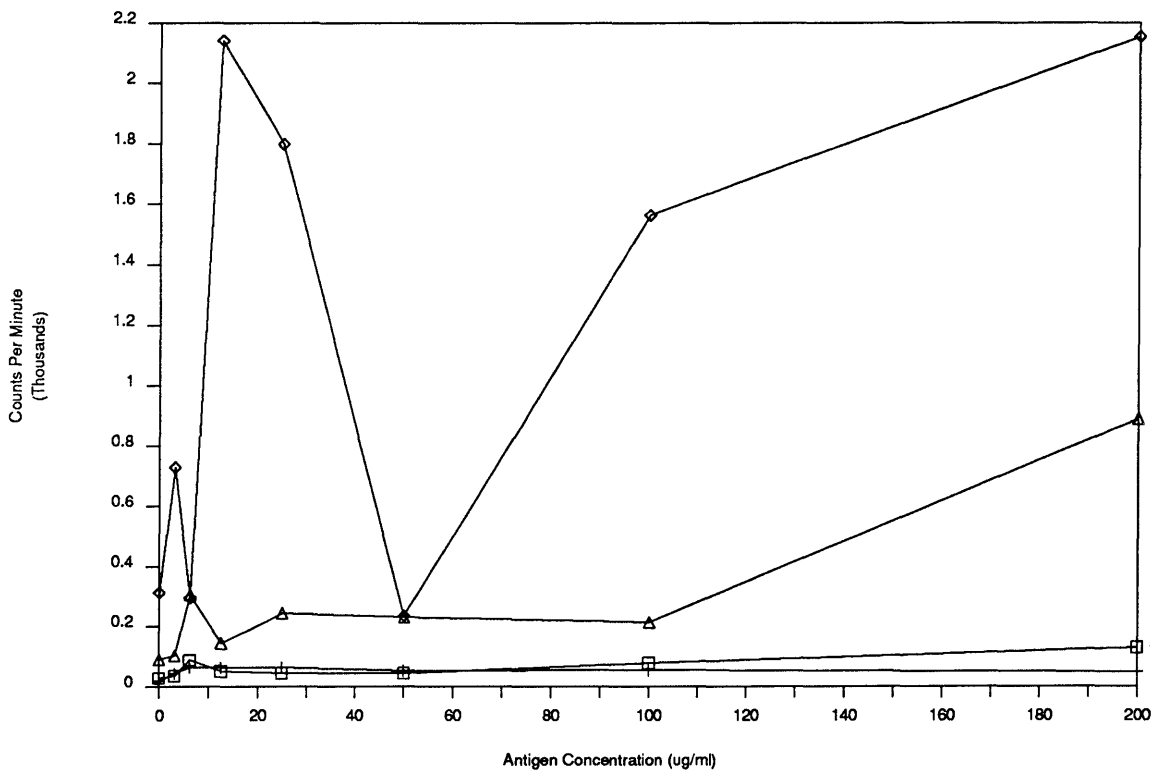


Figure 3.4 *Dose-response profile of mesenteric lymph node cells of uninfected female CBA mice to adult homogenate (Control) and D6L4 homogenate (triangle) or cells of mice given 150 L3* H.polygyrus *to adult homogenate (square) or D6L4 homogenate (Lozenge).*

3.5 Cellular responses in immunised mice

3.5.1 Introduction and aims

The proliferative response of cells from non-naive mice may vary according to the mouse's experience of infection, especially as the adults are immuno-suppressive and the larvae immunogenic (Pritchard *et al.*, 1984). Thus, to determine whether differences due to different infection patterns (i.e. "immunising regimes") could be detected mice were "immunised" by various protocols.

It should be noted that the above experiments, for reasons of convenience, were performed using CBA/ca mice. The CBA strain is a low responder to *H.polygyrus* and for this reason the generation of protective immunity is laborious (Behnke and Robinson, 1985). Consequently, a high responder mouse strain, NIH, was used in all the protocols.

3.5.2 Method

Age-matched (6-8 week old) NIH mice were used as controls to assess non-specific proliferative effects and 6-8 week old CBA mice were used as inter-experimental controls. The CBA MLN cells were incubated with Con A diluted from 50 ug/ml to 3.125 ug/ml for 72 hrs pre-pulse and 18 hrs post-pulse.

For each protocol the cells were incubated with *H. polygyrus* antigen or Con A at the concentrations given in table 3.1

Table 3.1 *Concentration of antigens and mitogens incubated with cells from immunised and naive mice*

Antigen	Dilution	
adult homogenate:	250 ug/ml double diluted to	16.25 ug/ml
adult ES(MT)	50 ug/ml	3.13 ug/ml
D6L4 homogenate	250 ug/ml	16.25 ug/ml
D6L4 ES(MT)	10 ug/ml	0.63 ug/ml
L3 homogenate	10 ug/ml	0.63 ug/ml
Con A	50 ug/ml	3.13 ug/ml

as well as medium alone.

The protocols for each infection regime and their controls are shown in table 3.2. The mice were given 200 L3 in 100 ul unless otherwise stated and dosed with ivermectin at a dose of 10 mg/kg body weight. The larvae (donated by Dr. M. Maema) used in the "irrad" protocol were irradiated with 25 Krad according to the method of Hagan, Behnke and Parish (1981).

The cells from all mice, except those given the irradiated larvae were incubated 72 hours pre-pulse and 24 hours post-pulse. The cells from mice given the irradiated larvae were cultured for different lengths of time before addition of the isotope and subsequent harvesting.

The S&J1 protocol was used to investigate the effect of one anthelmintic abbreviated regime on proliferative ability of the cells. This S&J1 protocol is one

Table 3.2 *Protocols used to raise resistant ("immune") mice*

	TIME (Day)							
TREATMENT								
	-9	0	6	9	12	19	26	39
S&J1	L3	I.		LPA				
S&J3		L3	I.	L3	I.	L3		LPA
BEHNKE 9 DAY		$L3^2$	I.		LPA			
IRRAD		$L3^3$		I.		LPA^T		
RESID. CONTROL		I.		LPA				
NAIVE CONTROL				LPA	LPA	LPA		LPA

NOTES: L3= 200 L3 in 0.1 ml *per os*.

$L3^2$ = 250 L3 given in 0.1ml *per os*.

$L3^3$ = 300 irradiated L3 given in 0.1 ml *per os*.

I. = Dosing with ivermectin (10 mg/kg body weight).

LPA = Lymphocyte proliferation assay, cells harvested after 96 hours incubation.

LPA^T= Lymphocyte proliferation assay, cells harvested after the incubation periods: 24, 28, 72, 96, 120, 144 hours.

half of the Sayles and Jacobson (1983) regime used to obtain resistant mice. The S&J3 protocol in which mice immunised by the Sayles and Jacobson regime were given another dose of larvae to assess their resistance and the effect of a further parasite challenge on the proliferative ability of the cells. The "resid" protocol was used to determine whether any effect of ivermectin on cellular proliferation could be observed. The other protocol used was as given by Behnke and Robinson (1985).

The full Sayles and Jacobson protocol was performed as was one in which mice were given larvae at times corresponding to the times they were given during the S&J3 protocol but were not treated with anthelmintic. However, due to equipment failure it was not possible to assess the cellular proliferation of cells from mice given these treatments and the protocols could not be repeated due to a number of constraints.

3.5.3 Results

The results are shown in figures 3.5 to 3.9 inclusive and the statistical analysis of these results given in appendix 1.4. Briefly, apart from the regime used to test for the residual effect of ivermectin all the regimes gave rise to MLN cells which proliferated to both the D6L4 preparations (figures 3.5c to 3.9c for the D6L4 homogenate and figures 3.5d to 3.9c for the D6L4 ES(MT)).

None of the treatments gave rise to cells that proliferated significantly to the adult homogenate. There was also no significant proliferation to the L3 homogenate (figures 3.5e to 3.9e).

Figure 3.5 *Lympho-proliferative response to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of mesenteric lymph node cells of naive NIH mice (square) or NIH mice given ivermectin at 10 mg/kg (+).*

Figure 3.6 *Kinetics of the in vitro cellular response to adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of mesenteric lymph node cells of NIH mice given larvae according to the "irrad" protocol. The lines represent the response after different lengths of culturing: 24 hr (square); 48 hr (+); 72 hr (lozenge); 96 hr (triangle); 120 hr (x); 144 hr (inverted triangle).*

Figure3.5 a

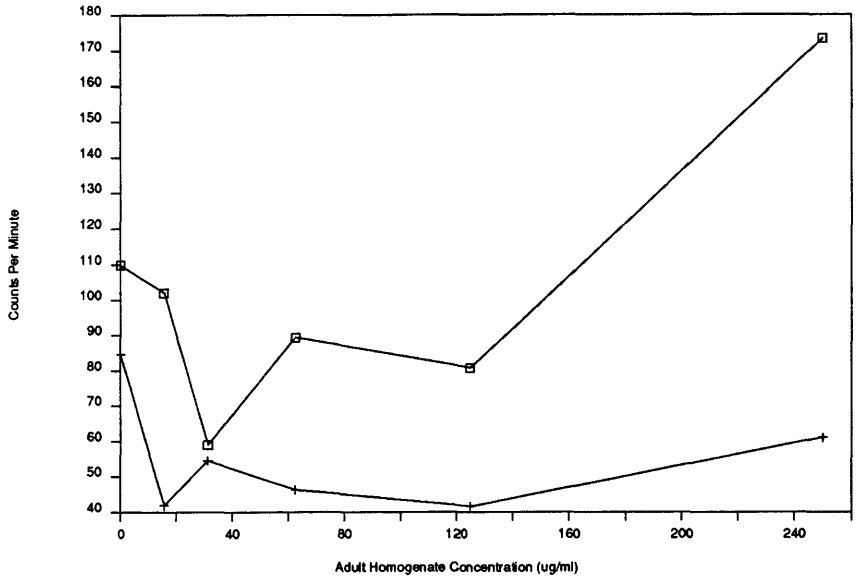


Figure 3.5 b

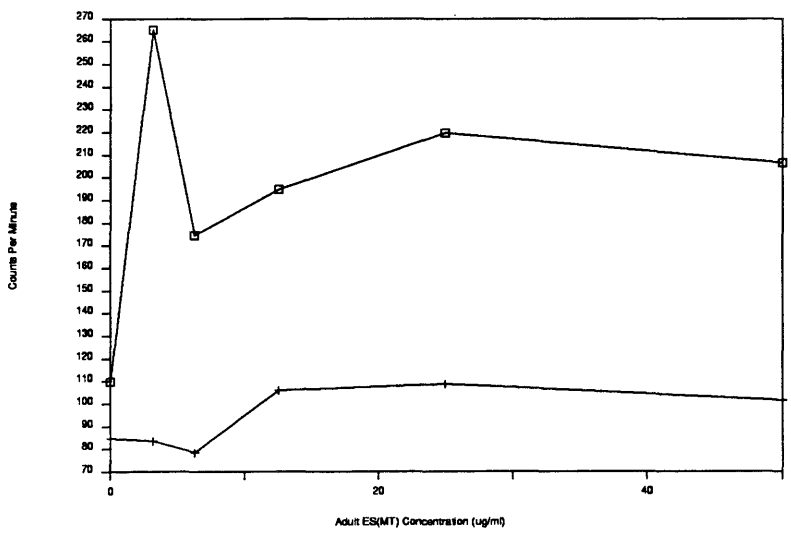


Figure 3.5 c

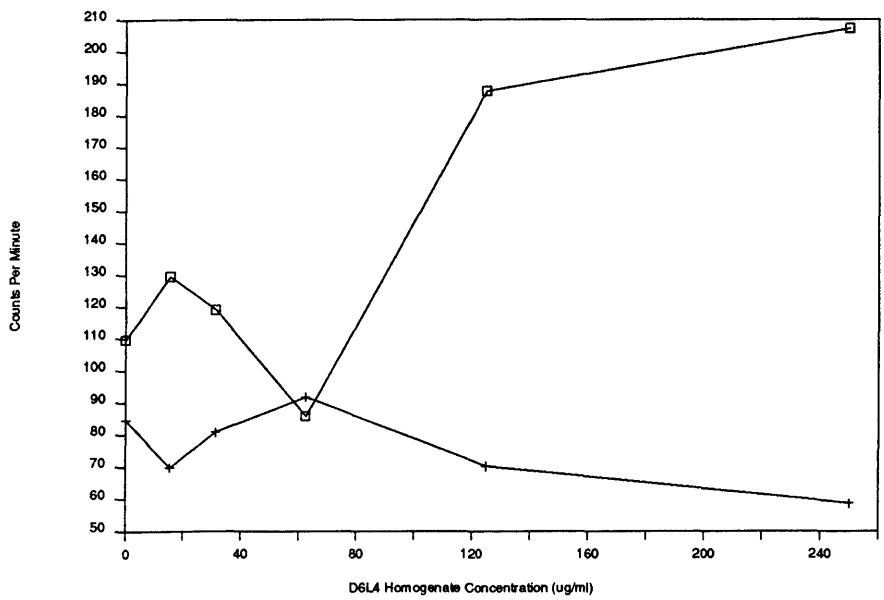


Figure 3.5d

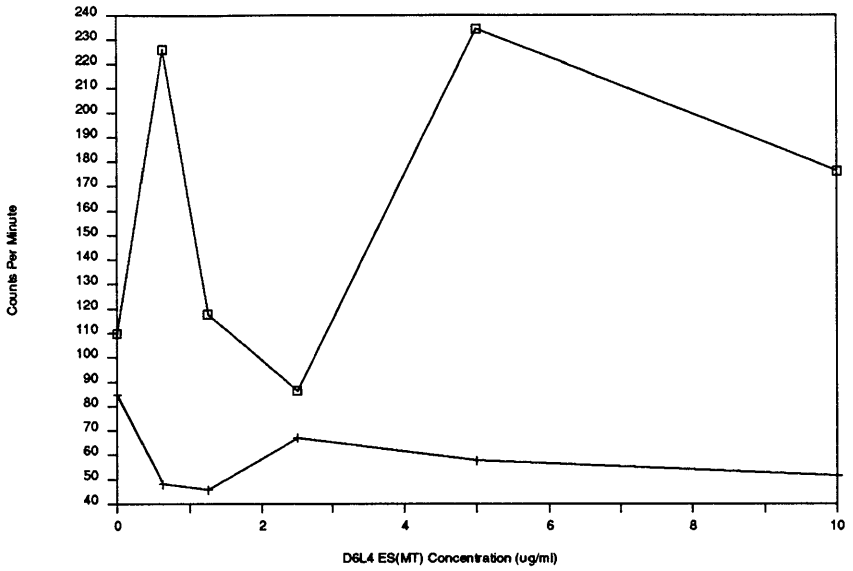


Figure 3.5 e

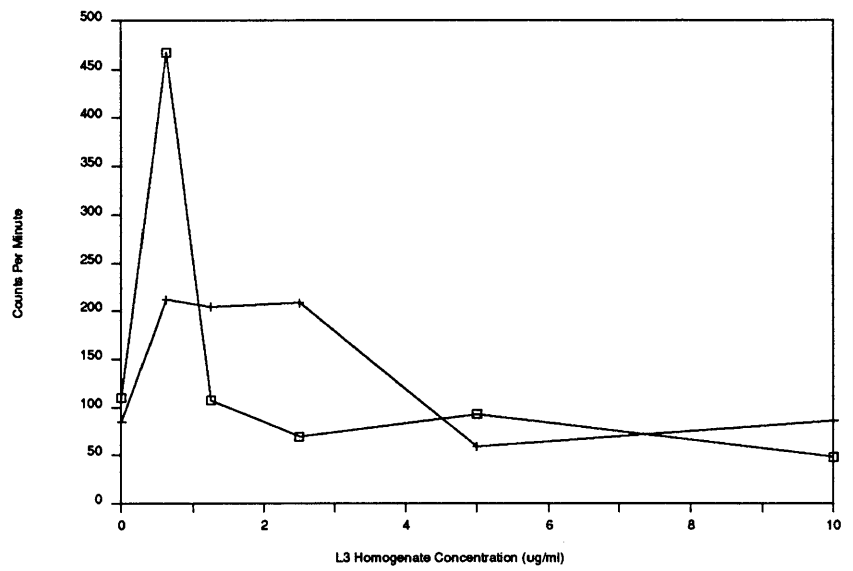


Figure 3.6 a

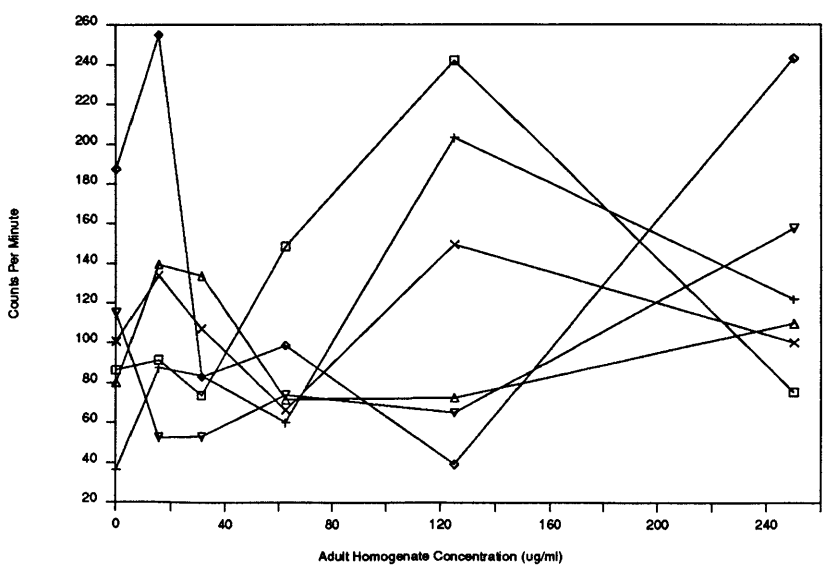


Figure 3.6 b

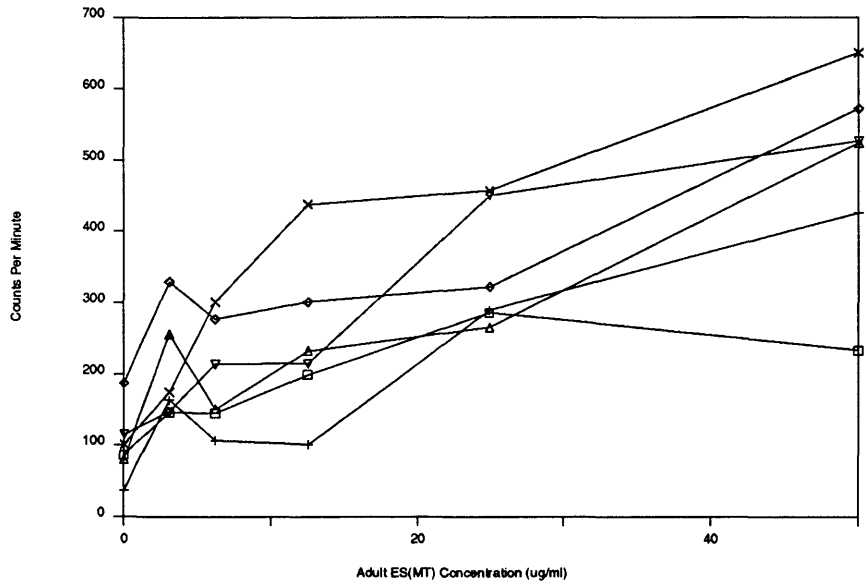


Figure 3.6 c

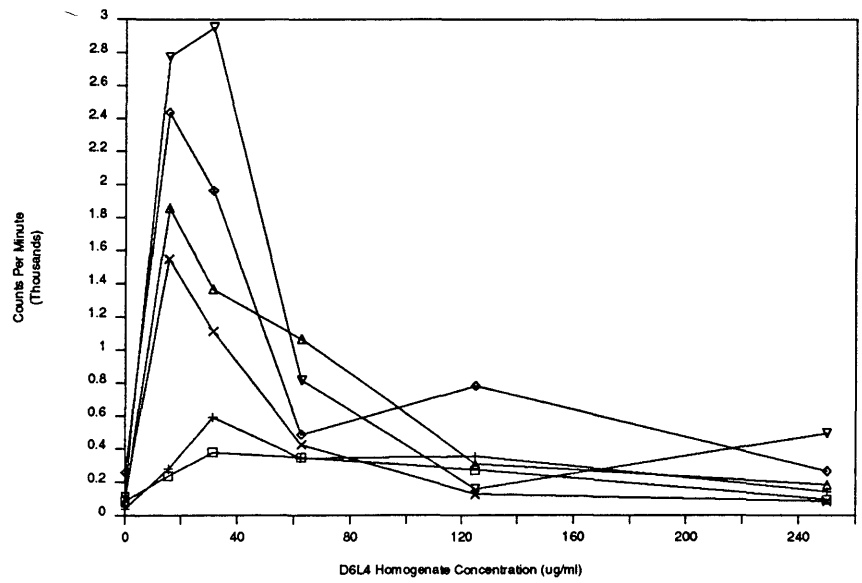


Figure 3.6 d

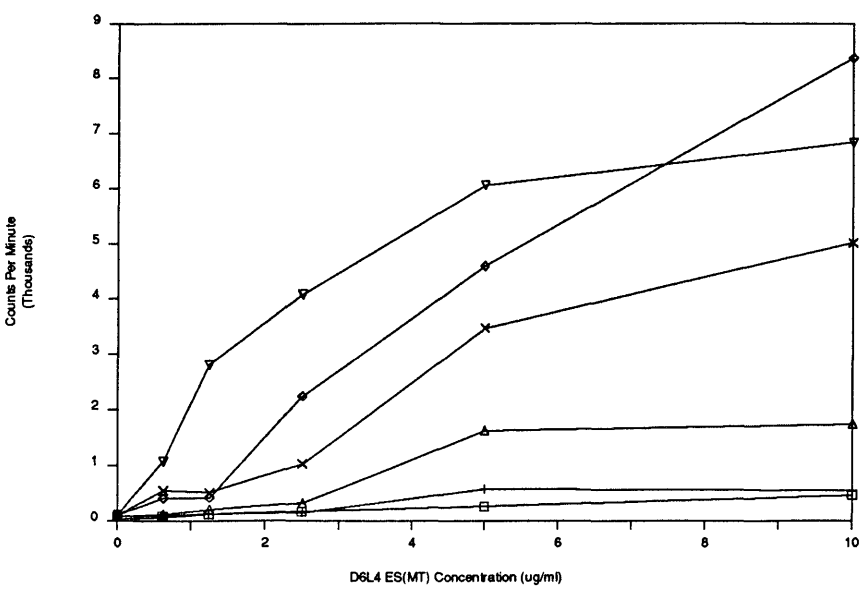
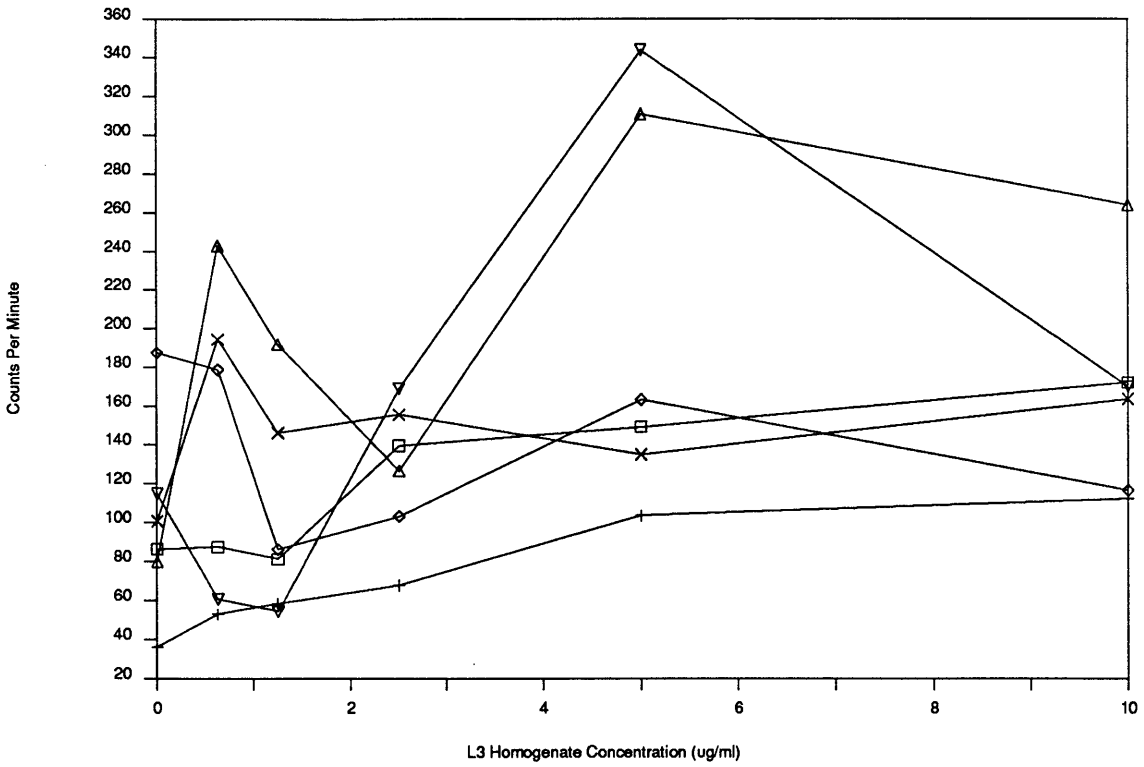


Figure 3.6 e



The response of the cells of "irrad" mice to the D6L4 homogenate was similar to that seen for mitogens in that a single peak of proliferation was apparent.There was no significant peak in the proliferation of these cells to the D6L4 ES(MT), the peak of response occurring over a wide range of antigen concentrations. For both D6L4 preparations the shape of the response was maintained when the length of pre-pulse incubation was varied, the magnitude of the response increasing with time.

The S&J3 protocol and Behnke protocol both gave rise to cells which proliferated to the adult ES(MT) (figures 3.7b and 3.9b respectively). As in the other regimes, on examination of the intestines of the mice, no larvae or adults were recovered.

Figure 3.7 *Antigen-specific proliferation of mesenteric lymph node cells of NIH mice immunised according to the abbreviated infection regime of Behnke and Robinson (1985) (i.e. the Behnke 9 day protocol) to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT).*

Figure 3.7 a

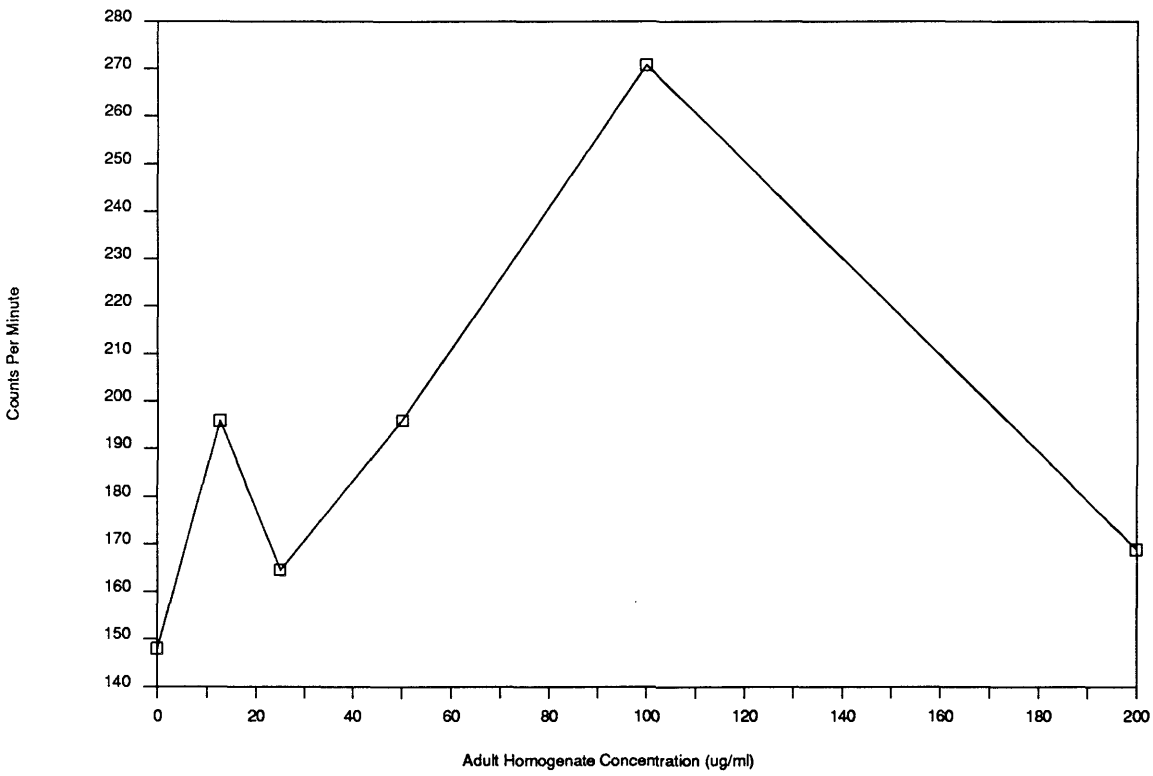


Figure 3.7 b

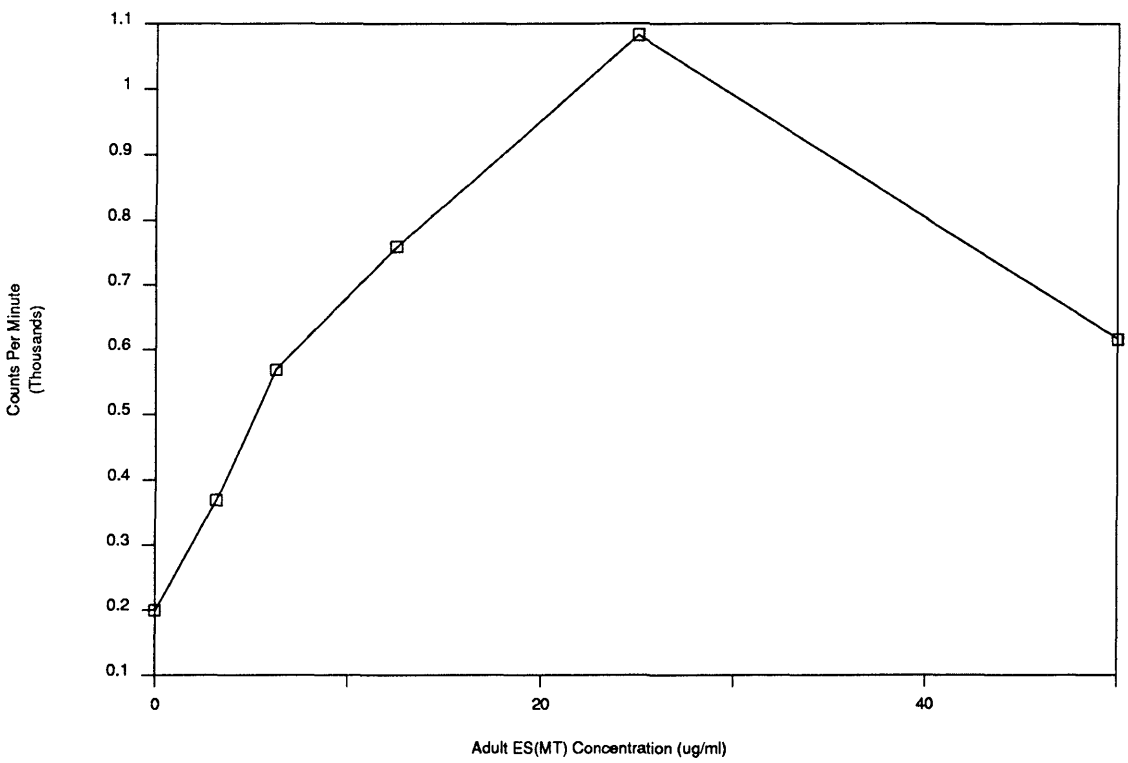


Figure 3.7 c

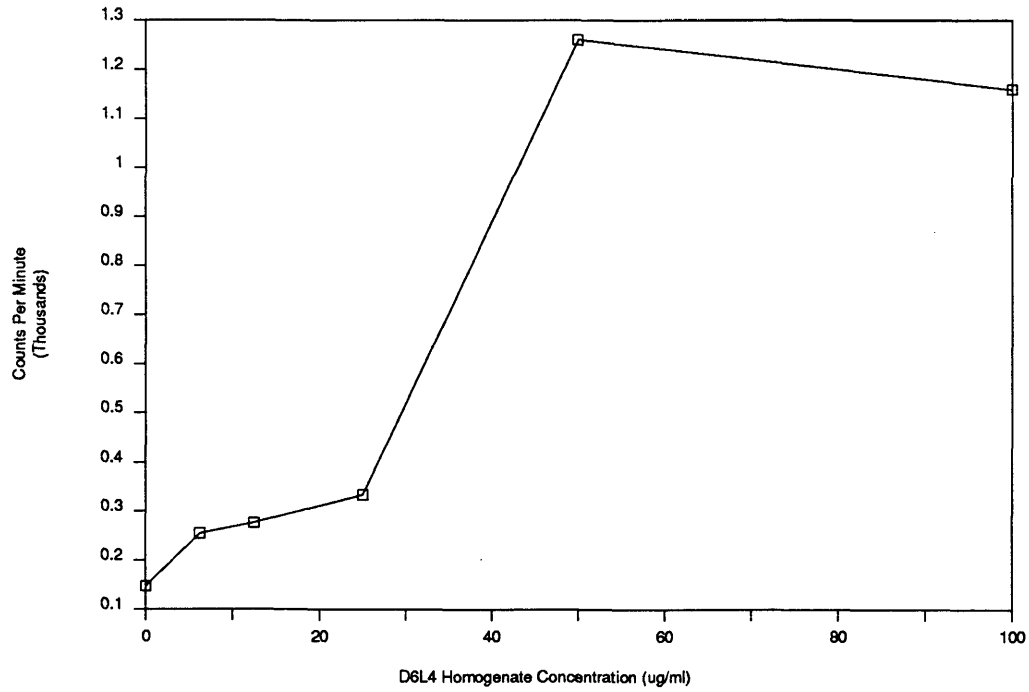


Figure 3.7 d

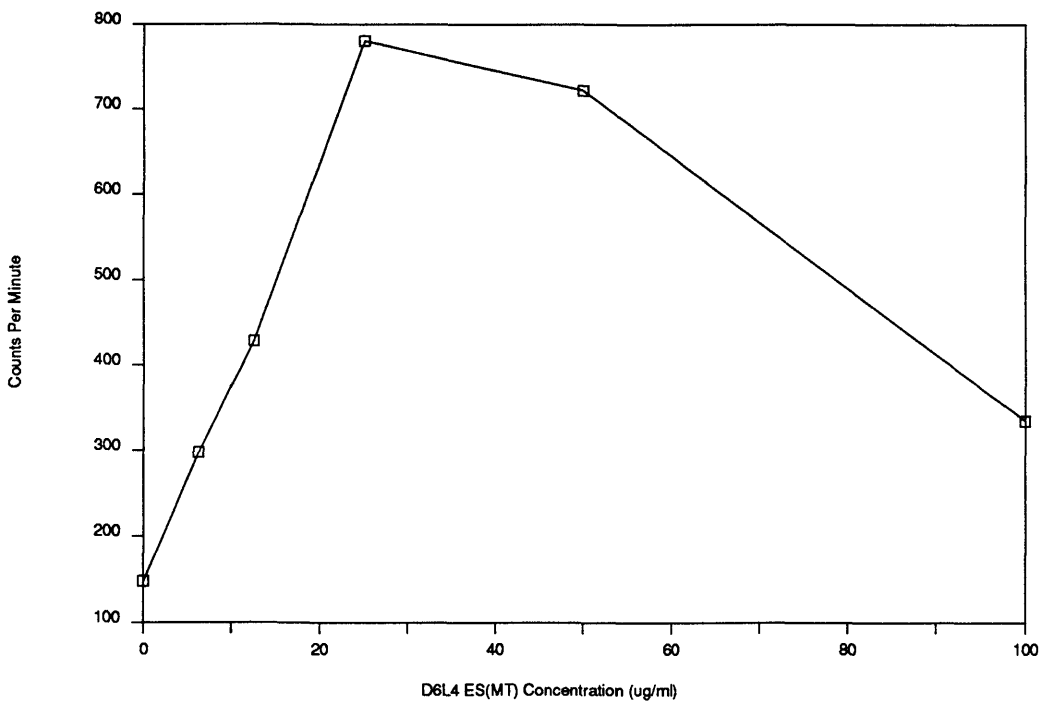
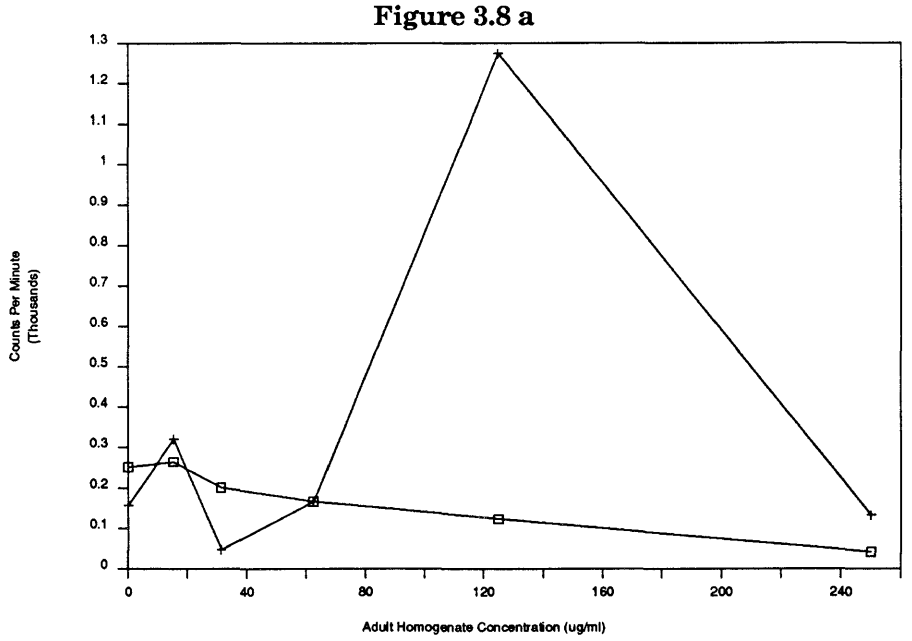


Figure 3.8 In vitro *cellular response of mesenteric lymph node cells of NIH mice given 200L3 followed after 6 days by ivermectin (square) and MLN cells of naive mice (+) to (a)adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate.*

Figure 3.8 a



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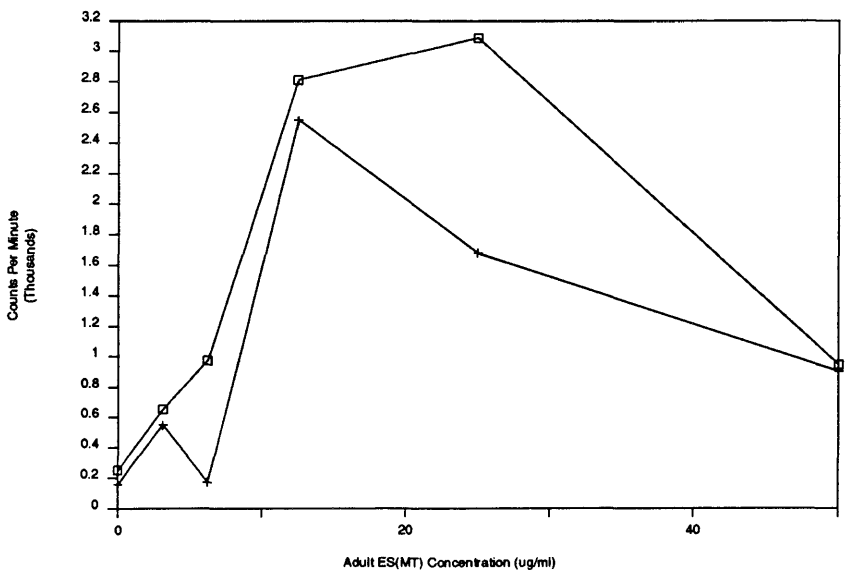


Figure 3.8 c

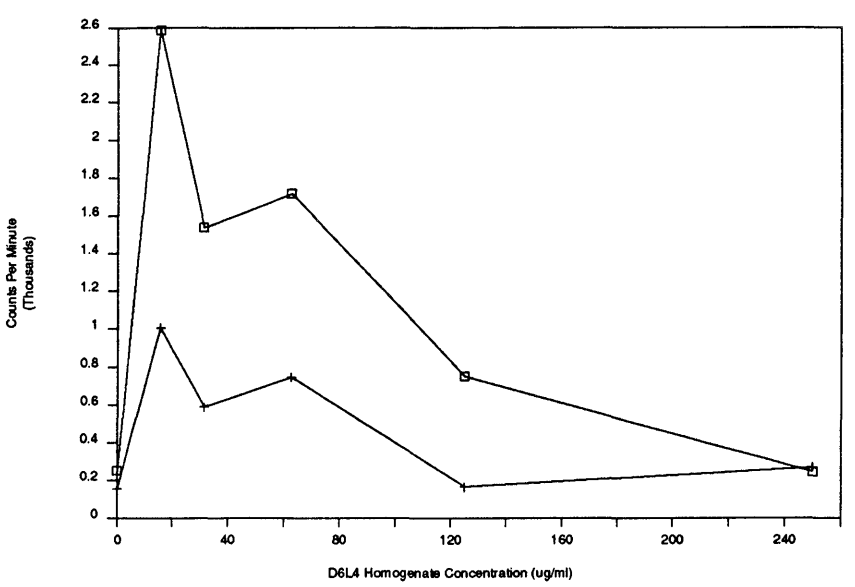


Figure 3.8 d

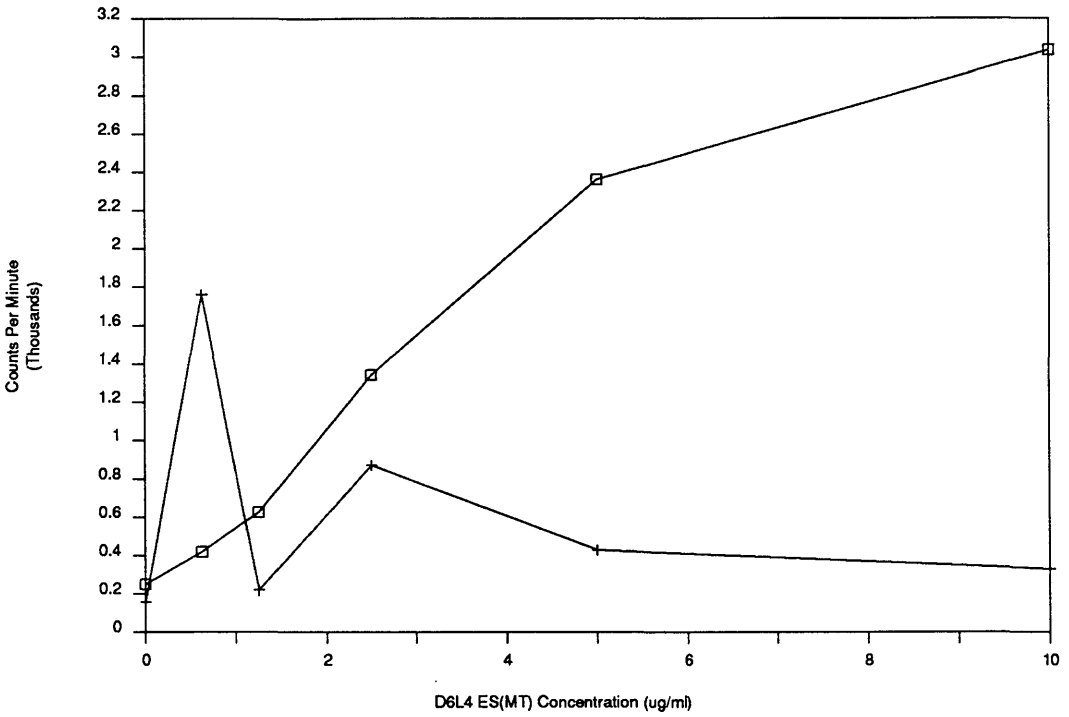


Figure 3.8 e

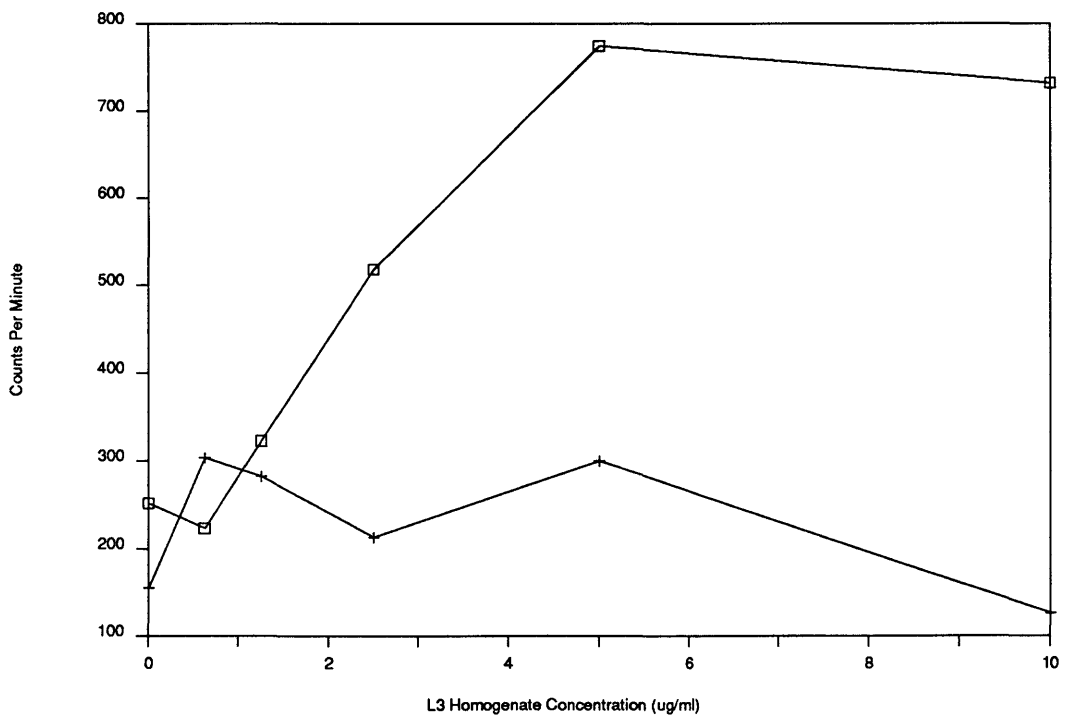


Figure 3.9 *Proliferation to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of mesenteric lymph node cells of mice immunised according to the S&J3 protocol.*

Figure 3.9 a

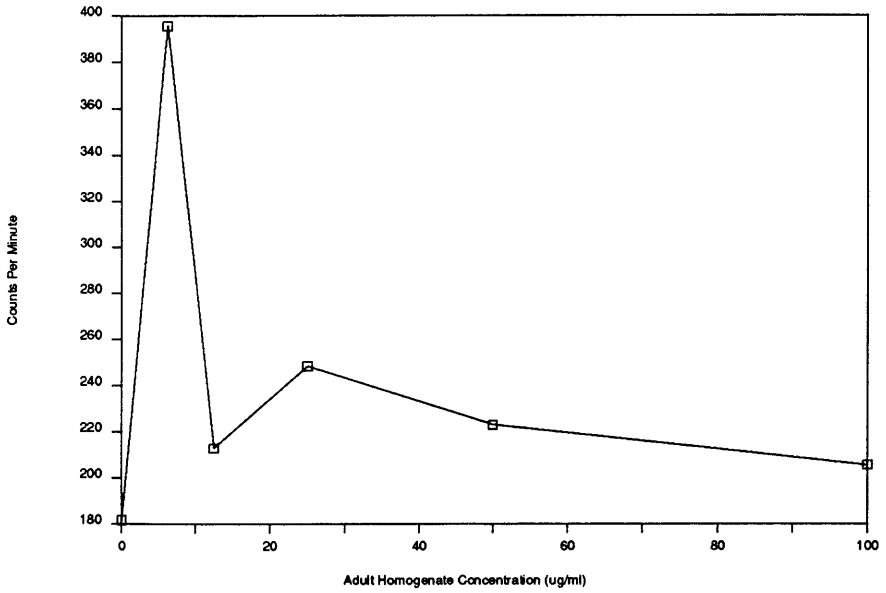


Figure 3.9 b

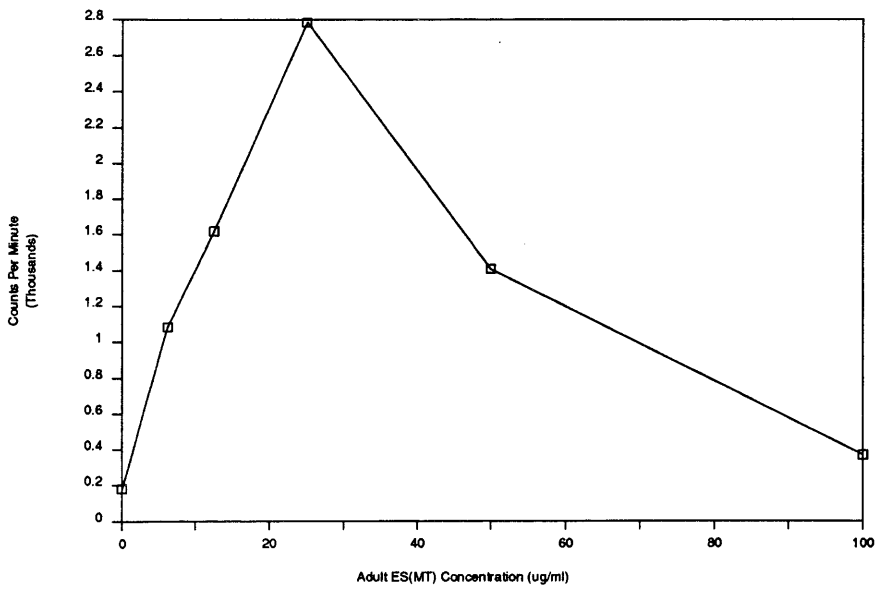


Figure 3.9 c

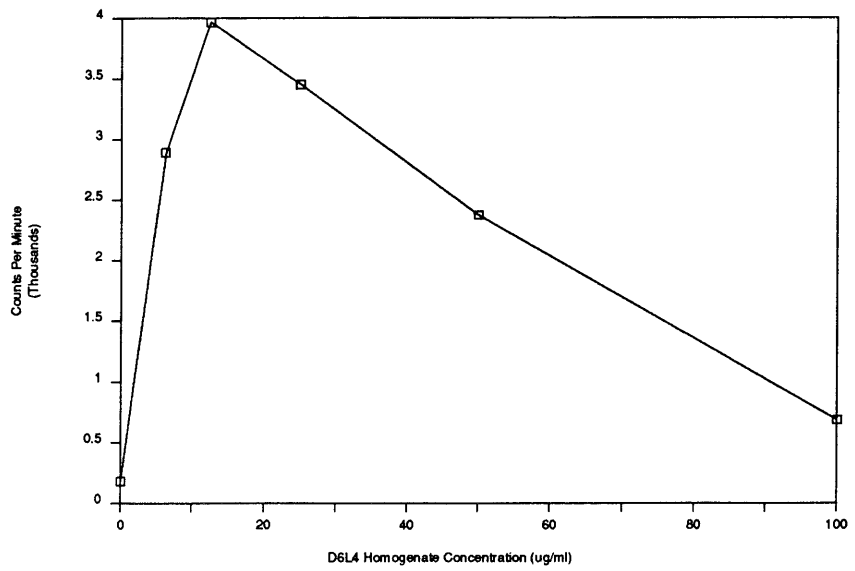


Figure 3.9 d

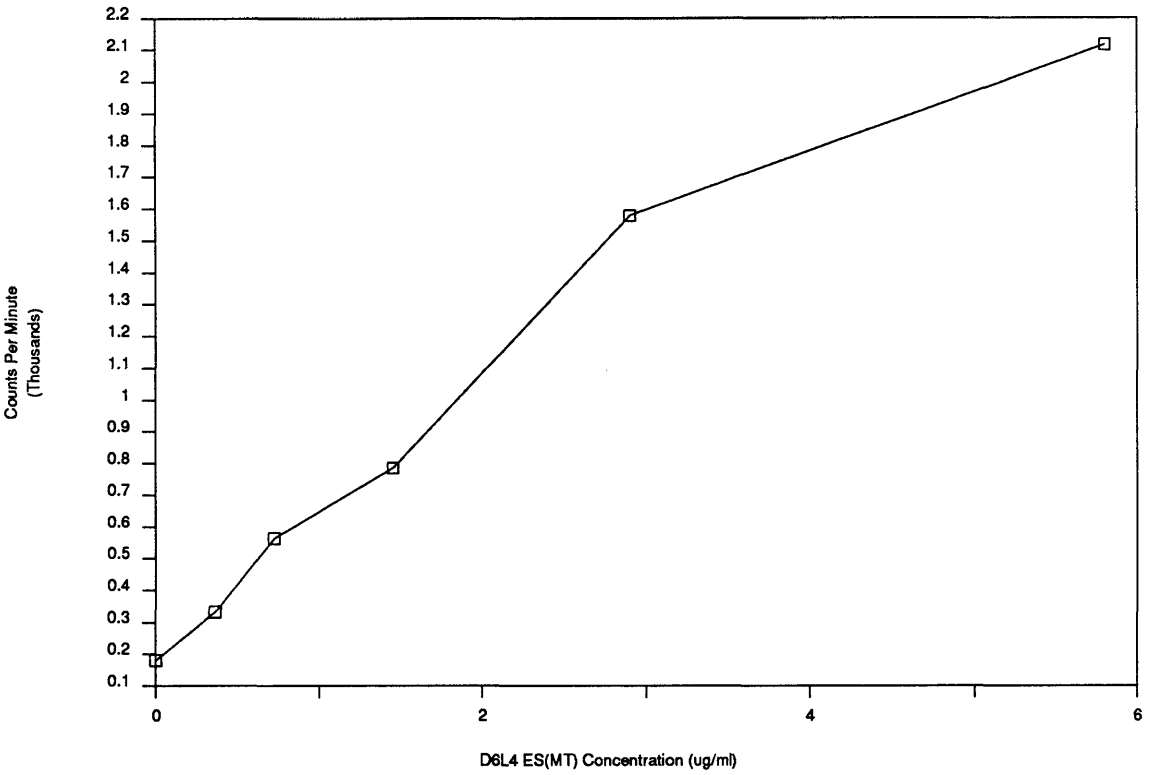
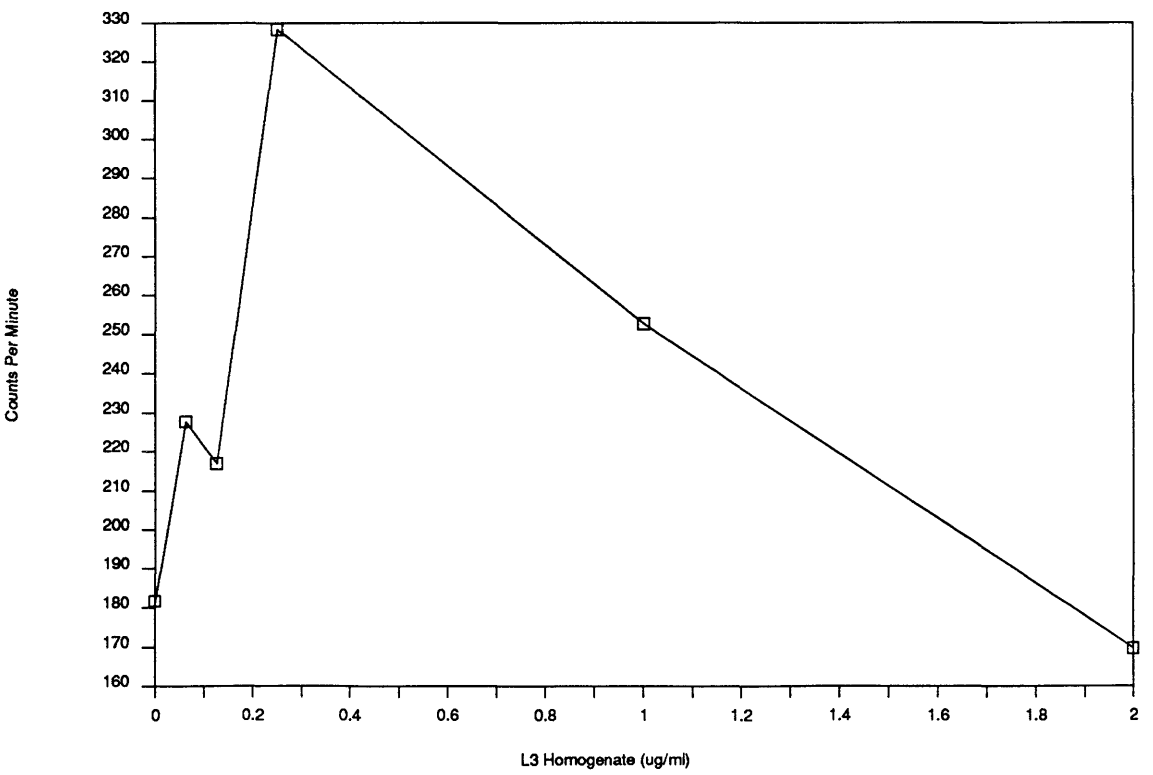


Figure 3.9 e



For each regime the spontaneous proliferation of the cells was greater than that of their age matched controls. The amount of spontaneous proliferation also increased with increasing length of incubation.

3.6 Response of spleen and mesenteric lymph node cells from immunised mice

3.6.1 Introduction and aims

The MLN node is the major drainage organ for the small intestine, the spleen having a secondary role in dealing with the products of an *H. polygyrus* infection (Ali and Behnke, 1981). The response of cells of both organs to *H. polygyrus* antigens was investigated to determine whether the relative importance of the organs is reflected in their cells' ability to proliferate.

3.6.2 Method

Six to eight week old NIH females were immunised according to the Behnke 9 day protocol (Behnke and Robinson, 1985). The mice were killed 12 days after anthelmintic abbreviation of the infection and the cells from the spleen and MLN used in a lymphocyte proliferation assay. The cells were incubated for 72 hrs pre-pulse and 24 hrs post-pulse with the following antigens used at the concentrations given in table 3.3

Table 3.3 *Concentrations of the antigens incubated with spleen and mesenteric lymph node cells of NIH mice*

Antigen	Dilution	
adult homogenate:	200 ug/ml double diluted to	12.5 ug/ml
D6L4 homogenate	150 ug/ml	8.25 ug/ml
D6L4 ES(MT)	100 ug/ml	6.25 ug/ml
L3 homogenate	100 ug/ml	6.25 ug/ml
Con A	50 ug/ml	3.13 ug/ml

as well as medium alone.

3.6.3 Results

The results are shown in figure 3.10 and the results of the statistical analysis given in appendix 1.5. It was observed that the MLN cells showed proliferation to the D6L4 ES(MT) and to a lesser extent the D6L4 homogenate and L3 homogenate. The spleen cells showed almost the opposite pattern of reactivity. The spleen cells did not proliferate to the D6L4 ES(MT), proliferated to the D6L4 homogenate and showed a response to the L3 homogenate. The cells from the spleen proliferated on incubation with the adult homogenate whereas those from the MLN did not.

It was noted that the level of spontaneous proliferation was greater for the spleen cells than it was for the MLN cells. The level of proliferation shown by cells from the two sources to Con A was not markedly different.

On repeating the experiment a similar set of results was obtained.

Figure 3.10 In vitro *lymphoproliferative response to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of cells of NIH mice immunised according to the protocol of Behnke and Robinson (1985). The lines represent the response of: spleen cells*

Figure 3.10 a

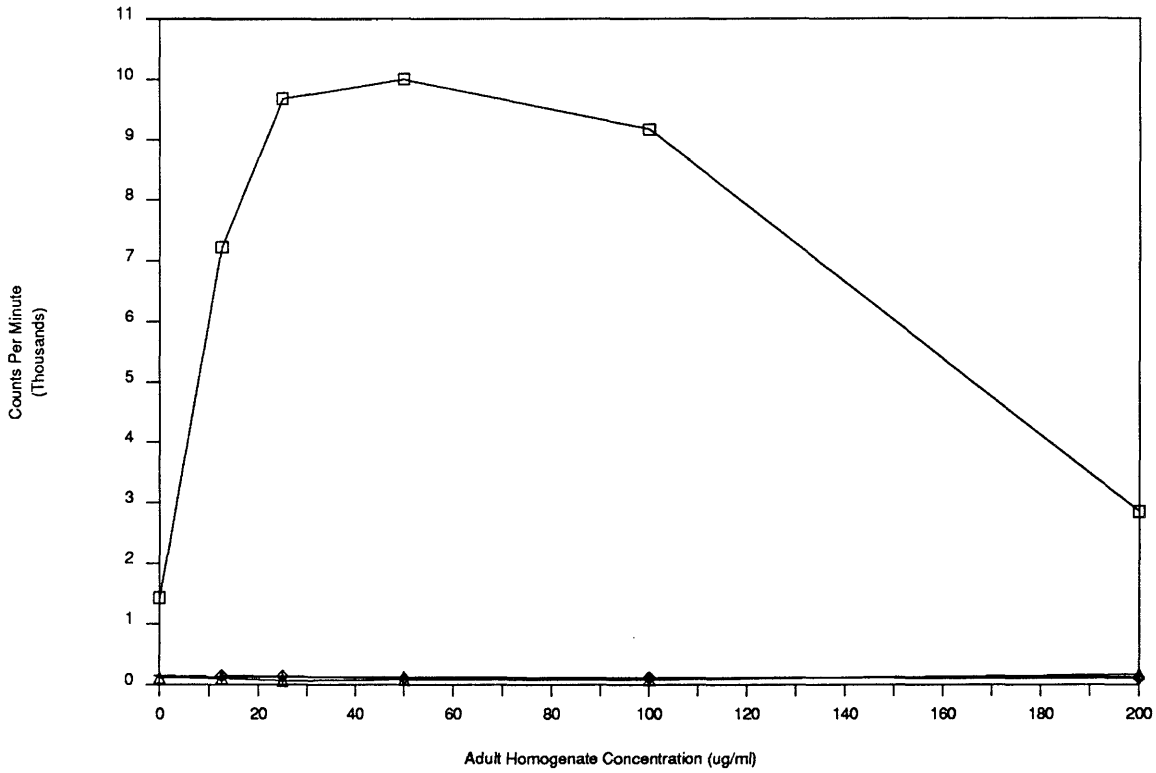


Figure 3.10 b

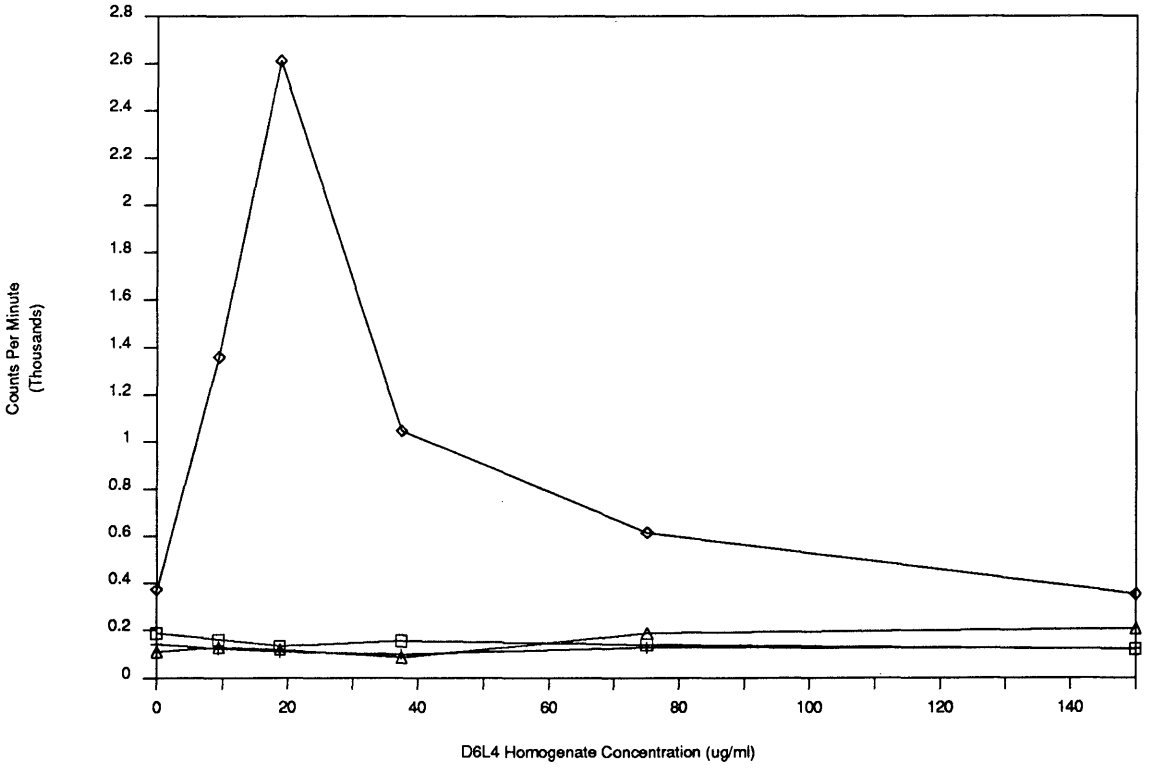


Figure 3.10 c

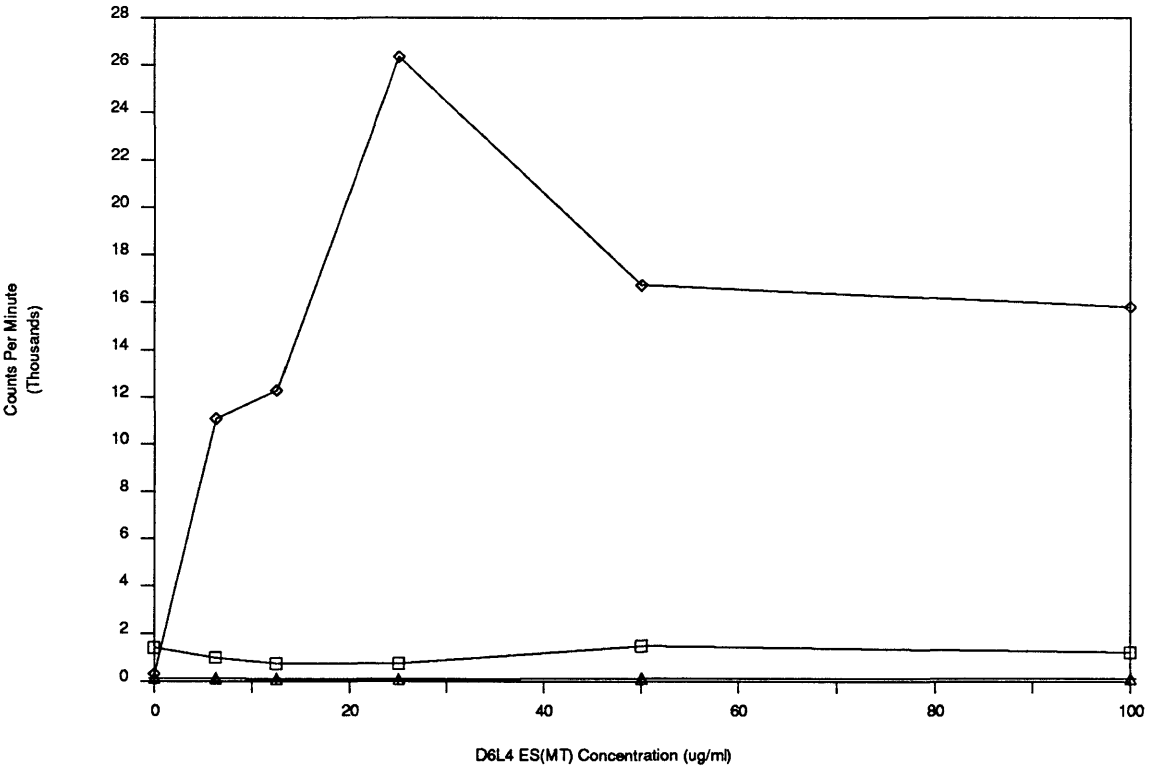
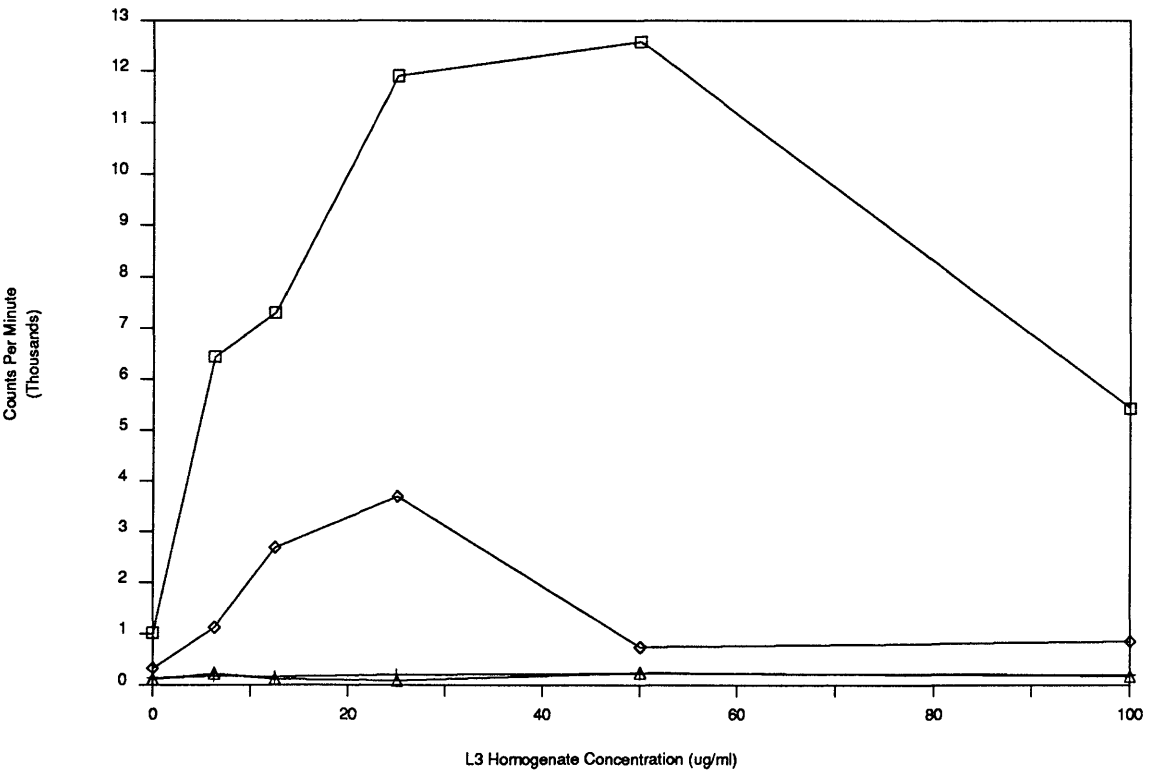


Figure 3.10 d



3.7 Discussion

The results reported here are those obtained on developing the *in vitro* assay of Corradin *et al.* (1977) to measure the proliferation.of lymphocytes cultured with antigens of *H. polygyrus.*

3.7.1 Mitogen induced proliferation

The repeated culturing of cells from naive mice with a range of dilutions of Con A (section 3.2) allowed the reproducibility of the technique to be ascertained. The results indicate that response was variable. This often leads to different responses at different times of assaying, even though within one time period there was little difference in the response of different mice. Similar patterns of variability have been seen for antigen specific proliferation in this study and by other authors (Colley *et al.*, 1977; Maddison *et al.*, 1979; Gibbs *et al.*, 1982; Castes, Moros, Martinez, Trujillo, Castellanos, Rondon and Convit, 1989).

The difference between the periods may have been due to the use of different batches of foetal calf serum (FCS) (Corradin *et al.*, 1977). This component of the cell culture medium, which is necessary for cell growth, is an undefined fluid which exhibits lot-to-lot variability in its biochemical composition and culture performance. The serum components can both stimulate and inhibit the desired cell response (Jayme, Epstein and Conrad, 1988). The proliferation of cells may be affected as the foetal calf serum may contain xeno-antigens. When taken up by antigen presenting cells and presented to the lymphocytes they may have a stimulatory effect (Knight, 1987). This proliferation, which has the same kinetics as antigen specific proliferation, may be sufficient to mask the *in vitro* response to the antigen (Corradin *et al.*, 1977). This source of variability was reduced by selecting a batch of foetal calf serum that could support cell growth yet had low non-specific properties and using this in the assays.

The process of cell growth is also a source of variability. As discussed in the introduction, the cell population increases in size exponentially and thus the size of the final population is sensitive to the starting conditions (Mishell and Shiigi, 1980) such as the number of cells, the proportion of responding cells and the viability of the cells (Olivotto *et al.*, 1982). This large amount of inter-experimental variability emphasises the need for controls to assess proliferation at different times. In this case mitogen induced proliferation is used as a control for antigen specific proliferation as it is likely that the difference between the two responses is quantitative not qualitative (Waithe and Hirschhorn, 1976). A more suitable control for the comparison of the response at different times would have been to use PPD as this causes less proliferation than Con A. The peak at the later time would mean that the cells would be healthier, the variance lower and the comparisons more accurate.

The differences shown emphasise the need to consider this source of variability in the design of any experiment comparing the proliferative response at different times. However, it should be noted that these controls do not take account of circadian rhythms of DNA synthesis. Thus it may be that the practice of comparing this multiple time point data obtained from treated animals to a single control time point is misleading. (Burns, 1981). It would be more accurate to carry out a control each time an experimental procedure was performed but this would be too impractical.

The technique was also shown to be sensitive enough to detect changes in the size of the proliferative response and the concentration of mitogen at which it occurred for different cell densities and lengths of culturing, although the results are not given here.

3.7.2 Non-specific proliferative properties of *H. polygyrus* antigens

Gastro-intestinal nematodes release an undefined array of molecules into their environment. These antigens are generally classified as somatic or

excretory/secretory depending on their source (Pritchard, 1986). Of these antigens some may be highly immunogenic whereas others may be irrelevant in the initiation of protective responses (Pritchard *et al.*, 1985) whilst others could be immunosuppressive (Crawford *et al.*, 1989; Monroy *et al.*, 1989). Some antigens by virtue of their molecular complexity are likely to be highly mitogenic (Chapman *et al.*, 1979). This mitogenicity may benefit parasite survival as this polyclonal activation may result in maturation until a state of exhaustion, and hence functional depletion, is reached. On a more practical note, the mitogenicity of the parasite's antigens may obscure any *in vitro* change that corresponds to an *in vivo* alteration in immune responsiveness.

It was found that on culturing the MLN cells of naive CBA mice with D6L4 and adult homogenate no non-specific proliferation occurred (see section 3.3). In other experiments when similar cells were cultured with the other preparations no proliferation was observed (see section 3.5). On incubation with spleen cells of CBA mice given repeated low level infections all but the adult antigen caused non-specific proliferation (see section 4.11). In most, but not all assays, when the same antigens were cultured with spleen cells from NIH mice all, with the exception of the adult homogenate, had a significant non-specific proliferative effect (see section 4.6).

The immunogenicity of the parasite preparations may affect their mitogenicity. For example, it is difficult to detect antibody on L3 and more adult ES(MT) proteins are precipitated by immune sera than are adult homogenate proteins (Pritchard, Behnke and Williams, 1984). However, Day *et al* (1979) and Hurley, Day and Mitchell (1980) have demonstrated that adult ES(MT) is an inefficient immunogen.

The difference between the organs, strains and the different time periods may be due to the inherent variability of the technique, the ability of the medium to support proliferation or because of differences in the preparation of the different

batches of antigen. However, it is likely that the difference reflects the activity of the cells in each organ. The spleen cells exhibited higher levels of spontaneous activity than cells from the MLN. This may be due to localisation of (non-specific) suppressor cells within the MLN (Male, Champion and Cooke, 1987).

Preparations of *H. polygyrus* antigens have been reported to have non-specific activity, the mitogenic effect of the adult ES(MT) being greater than that of the adult homogenate Price and Turner, 1986). Other studies investigating lymphocyte responses during *H. polygyrus* infection have reported that the antigens have no non-specific effect (adult ES(MT), Day *et al.*, 1979 and Monroy *et al.*, 1989; adult homogenate, Enriquez *et al.*, 1986). Day *et al.* (1979) suggested that the observed mitogenicity of parasite preparations is due to contamination with bacteria. This supposition is at odds with the results from this study. It was seen that antigens prepared from adults retrieved from the bacteria rich gut were less mitogenic than the products from the D6L4 from the muscularis mucosa.

Other studies have reported mitogenic effects of other parasite preparations. Wakelin *et al.* (1986) reported that antigens of *T. spiralis* were mitogenic and that the effect was strain dependent (it was less in the higher responder NIH than in C57bl/10). It is also, interesting to note that different levels were seen at different experimental periods.

Mitogenicity has been reported for soluble preparations derived from malaria parasites (Freeman and Parish, 1978; Golenser *et al.*, 1985) and for particulate sub-cellular preparations from *Trypanasoma brucei* and *Trypanasoma congolense* (Clayton *et al.*, 1979). The work by Golenser *et al.* (1985) raises two points. The first is that the parasite extracts are only mitogenic in the permissive host, which suggests a possible mechanism for differentiating between immunogenic and mitogenic responses. The second point is that different preparations have different antigen dose-response curves, suggesting that the preparations have different factors, of which more will be said later.

3.7.3 Cellular responses of mice infected once

The proliferation to the D6L4 homogenate of MLN cells from mice infected once with *H. polygyrus* (section 3.3) suggests that the chronicity of the infection is not due to a failure to recognise the parasite (a hypothesis proposed by Pritchard and Behnke, 1985). The low level of proliferation against the adult homogenate is in agreement with the lack of response observed by Enriquez *et al.* (1986) who measured the response in high and low responders.

The time at which the cells were sampled may have been important as to whether or not a response was observed. The response was assayed three months after the initial infection in this study and six days after infection by Enriquez and co-workers (Enriquez *et al.*, 1986). It has previously been shown that cells secreting IL2 to parasite products are found in the MLN five days after infection (Prowse, 1982). This, along with the fact that the population that responds to *T. spiralis* remains in the MLN for only a very short period of time, (Wakelin *et al.*, 1982) may mean that the adult reactive cell population was not sampled at the time it was sequestered. However, cells which were reactive to the larval homogenate were detected and thus, unless the cells that respond to different antigens have different dynamics, the explanation of time dependent sequestering is unlikely to be sufficient.

Price and Turner (1986b) noted a response in the MLN of C57Bl/6J mice given 300 L3 *H. polygyrus*. The response may have occurred as a larger dose was given by Price and Turner (1986b) than in this study and there is likely to be a dose dependence in the proliferative response. Alternative explanations are that the response to the adult homogenate is suppressed or that cells capable of responding to it are localized elsewhere. Experiments performed discussed later (section 3.7.5) suggest that this is why no response is seen.

3.7.4 Cellular responses in immunised mice

The immunising regimes employed in this chapter to generate resistant mice all gave rise to mice whose cells reacted, *in vivo*, to the antigens of *H. polygyrus* (section 3.5). A common feature of each method was the stimulation of the immune system in the absence of adults. This is probably related to the fact that the adults are immunosuppressive (Behnke, Hannah and Pritchard, 1983) and as a consequence interfere with the immunity that is stimulated and acts against the larval stages in the intestinal mucosa (Pritchard *et al.*, 1983) and anthelmintic treatment removes the source of immunosuppression. The balance between the immunosuppressive and stimulatory effects of the parasite may explain why some procedures in which adults develop may take over a month to generate immune mice (e.g. the regimes of Leuker and Hepler, 1975; Behnke and Wakelin, 1977).

The amount of proliferation that the various regimes induce is not the same, the cells of the "irrad" protocol and the S&J3 protocol was greater than that of cells from the Behnke 9 day protocol. This may be because the first two regimes kill larvae in the gut wall. There is, therefore, a depot effect which allows prolonged access of the immune system to the relevant antigens, an effect not seen in the other regimes.

The depot effect may also be the reason why Behnke and Robinson (1985) find that their protocol is not as effective at inducing immunity as infection with irradiated larvae (the latter gives rise to immunity in high and low responder strains whereas the former does not).

The S&J3 protocol was the only infection regime in which the mice were given a dose of larvae which was not then followed with ivermectin and it is possible that, as a result, adult worms developed. However, on examination of the intestines of the mice no adults were detected. This is probably due to the high level of resistance that this regime confers on NIH mice, it being unlikely that

the worms were lost before examination of the intestines (Sayles and Jacobson, 1983).

This observation that both the S&J3 and the Behnke 9 day protocols both give rise to cells which proliferate against the adult ES(MT) suggests that even if adults do mature their presence is not essential for the cells to react against their metabolic products.

The increased spontaneous activation of cells from infected mice in the absence of any exogenous stimuli has been observed in other infections (e.g. for *T. spiralis*: Ljungstrom and Sundqvist, 1979; for *Leishmania major*: Solbach *et al.*, 1987). It has been attributed to a macrophage dependent activation of T-cells by Ljungstrom and Sundqvist (1979). The phenomenon of increased activation is probably due to antigen carry over into the organs (P. Kaye pers. comm.). It is likely to occur in *H. polygyrus* infections as antigens have been detected in the spleen and other organs (Behnke, 1987). It has been suggested that studies of baseline DNA synthesis within organs reflects the initial stimulation of the immune system (Pelley, Ruffier and Warren, 1979).

3.7.5 Responses in the spleen and mesenteric lymph node of immunised mice.

The spleen and mesenteric lymph node of *H. polygyrus* infected mice do not show the same reactions to the parasite's antigen (section 3.6). For two of the antigens the response was complementary: the D6L4 ES(MT) caused proliferation of MLN cells but not spleen cells whereas the adult homogenate stimulated cells from the spleen to proliferate but had little effect on the MLN cells. The other antigens caused different amounts of proliferation with the cells from the different organs. A difference in the organ responses of C57bl/6J mice has been noted by Price and Turner (1986b) and in B10 mice by Enriquez et al. (1986): whilst the response in both organs of infected mice was augmented relative to the response in

naive mice, the spleen cells proliferated more to parasite preparations than the MLN cells.

The difference between the spleen and MLN may be due to the antigen and cell trapping hierarchy of the lymphoid system's organs. The lymphoid system is organised such that a lymph node drains a specific region and antigen from that region is processed in the mesenteric lymph node (Male, Champion and Cooke, 1987). Relating this to *in vitro* lymphocyte proliferation assay, de Batselier (1980) reports that antigen specific proliferation is restricted to the organ draining the site of stimulation.

The phenomenon of specific lymph node involvement has also been reported for parasite infections (Pelley *et al.*, 1976; Manson-Smith *et al.*, 1979; Lee, Wakelin and Grencis, 1983; Ali and Behnke, 1985). In *H. polygyrus* infections the MLN is thought to process a significant proportion of the parasite material. The spleen is only involved when the MLN is no longer capable of processing all the antigen (Ali and Behnke, 1985). The implantation of radio-labelled adults in the intestine of naive mice and subsequent discovery of the worm's antigens in the spleen (and liver) suggests that the MLN is not the only organ involved in antigen trapping (Behnke, 1987).

It is possible that the reactive lymphocytes are found in the spleen because they circulate throughout the host. This circulation of lymphocytes has been shown in parasitic infections by Love and Ogilvie (1977) and Manson-Smith *et al.* (1979). There appears to be homing to the site of stimulation (Duijvestjin and Hamman, 1989). Wakelin and Selby (1974) have shown that in a *T. muris* infection that cells injected intravenously became redistributed into patterns characteristic of their organs of stimulation. Also, Ottaway and Parrot (1980) report that in mice blast cells migrate from the MLN into intestinal grafts.

This recirculation is necessary for protection (Duijvestijn and Hamman, 1989). For example, if antigen trapping occurs in the MLN then for resistance to be

achieved, the cells must migrate to the lamina propria and muscularis mucosa. It is in the intestinal wall that T-cell dependent granuloma formation around the larvae (Prowse, 1982). However, although this explains why cells are found in both organs it does not explain why there is organ-specific antigen-specific proliferation.

This antigen-specific organ-specific proliferation in *H. polygyrus* has also been shown by Prowse (1982). Interleukin-2 secreting cells (activated T-cells) were found in both the spleen and mesenteric lymph node. It was suggested that as the superior MLN drains the duodenum, the antigens it responds to are those from the L3 exsheathment as well as parasite growth and development. He noted that the spleen was the major source of T-cells specific for parasite somatic antigen. This should be compared with the results of Chapman *et al.* (1977) who found more anti-adult antibody producing cells in the MLN than in the spleen. The difference between the two cell types may be that the B-cells remain in the site of stimulation whilst T-cells recirculate or alternatively because of the different migration patterns of the two cell types (Duijvestijn and Hamman, 1989).

Another reason as to why the organ-specific antigen-specific reactions take place may be the result of the route of antigen uptake. It is generally held that the recirculation of activated cells is restricted to the sites of antigenic insult (Duijvestijn and Hamann, 1989). The D6L4 antigens are released into the tissue whereas some of the antigens of the adults are released into the milieu of the intestine. The adult antigens in the intestine are taken up by the M cells and processed in the gut associated lymphoid tissue (GALT). The triggered cells differentiate and travel in the draining lymphatics to the regional mesenteric lymph node. Antigen presentation in GALT often leads to the prevention of antigen-specific responses (Male, Champion and Cooke, 1988).

If suppressor cells specific to the adult are generated and localise in the MLN then they will suppress the response in that organ (Male, Champion and Cooke, 1987) but proliferation will still occur in the spleen or other sites where the

suppressor cells have not localised. The difference in response may be because of (non-specific) suppression, in the same way that the response to heterologous antigens is suppressed (Crawford, Behnke and Pritchard, 1989). However, Enriquez *et al.* (1986) reported that they could not suppress *in vitro* proliferation of spleen cells to the adult homogenate by adding MLN cells. The difference may be because the trapping of antigen-specific cells occurs in one organ at the expense of another (Zatz and Lane, 1971). It is also possible that different T-cell populations in the two organs affect the response and possibly that these different lymphocyte subsets have their migratory pathways defined by different selective high-endothelial recognition (Duijevestijn and Hamann, 1989).

Delovitch, Semple and Phillips (1988) also proposed a model for organ-specific responsiveness. They noted that antigen uptake, processing and presentation to T-cells can be accomplished by independent cell populations. Thus, as not all antigen presenting cells may process antigens similarly and different organs may have different cell populations, this may explain the generation of organ-specific responsiveness. It may also be that T-cell mediated rejection mechanisms are limited *in vivo* by the availability of macrophages in each organ (Price and Turner, 1986).

The difference in the response of the two organs may be due to the molecular and antigenic properties of the antigens. Stage specific antigens have been reported in somatic (Pritchard *et al.*, 1984) and excretory (Pritchard, 1986; Adams, East, Monroy and Dobson, 1988)) preparations from the parasite. It is thought that the putative IMF factor is a constituent of the adult ES(MT) (Pritchard, 1986). A low molecular weight component (less than 26000 daltons) in the ES(MT) has been identified. This fraction of the ES(MT) can suppress the early stages of proliferation of B and T-cells to mitogen (Monroy, Dobson and Adams, 1989).

The differences in the antigens may affect how they are processed and with which component of the Ia gene products they are presented with (P. Kaye, pers.

comm.). That this may be the case is suggested by Renier *et al.* (1979) who report that the difference in the response of human splenic and blood lymphocytes is due to the localisation of antigen specific adherent helper cells in the spleen, and states that precedents exist for the interaction between regulatory cells in the spleen and antigen responding cells in the spleen. Furthermore, Degwert *et al.* (1987) showed that proliferation to BSA was dependent on antigen presentation in the context of Ia. Thus, the lack of an observable response to the antigens of *H. polygyrus* in a particular organ may be due to its context of presentation to the lymphocytes.

As well as showing organ-specific responses, the antigen-dose responses of the cell to the different antigens are not all the same. The single peak of proliferation seen with the D6L4 and adult homogenates suggests that only one factor is responsible for the response or that only one cell population responds (Golenser *et al.*, 1985). It could also be that there is more than one activating component but if so the responding cells must show the same kinetics. The proliferation to the L3 homogenate and D6L4 ES(MT) lacks a definite peak and this suggests that the antigens have more than one factor or that more than one population of cells responds. The small proportion of antigen-specific cells (0.01%, Zatz and Lance, 1971) implies that the existence of other responding populations is not unlikely.

An interesting point is that the mitogenic effect of the D6L4 homogenate was similar to the antigenic effect in terms of the shape of the dose response curve. This would tend to suggest that the difference between the two effects is quantitative not qualitative.

3.8 Summary

The exponential growth of cell populations in response to a stimulus means that the size of the population on sampling is affected by the starting conditions. The lymphocyte assay reflects the problem.

The antigens of *H. polygyrus* may possess mitogenic properties depending on the method of preparation, the source and condition of the cells and the time of assaying.

These antigens cause proliferation with the cells from infected mice. The level of proliferation is different for each antigen although they may show cross reactivity.

The proliferation to the antigens is also markedly organ specific. The reaction to the adult antigens is greater in the spleen than in the mesenteric lymph node whilst the reverse is true for the D6L4 products.

CHAPTER 4: Changes in the parasite population and the host's immune response during a chronic parasitic infection.

4.1 Introduction

Animals are exposed, in nature, to varying quantities of infective larvae (Behnke, 1987). Despite this, many studies of resistance to parasite infection are based on the results of a single infection with a subsequent challenge (e.g. Prowse, Ey and Jenkins, 1978; Butterworth, 1984) or the infection of the host with a few large doses of the parasite (Dobson, Brindley and Sitepu, 1982). In these studies resistance is recorded as the percentage reduction in challenged hosts as compared to the burden in previously uninfected animals (Crombie and Anderson, 1985).It is unlikely that these patterns of infection would occur in the "real" world (Keymer, 1986).

A few studies have attempted to mimic natural infections by giving the host repeated low level doses of the parasite's infective stage. Crombie and Anderson (1985) suggest that the trickle infection with frequent sampling of the host and parasite population has advantages over the conventional experimental design when attempting to interpret observed epidemiological patterns in the human community. The studies allow the effect of repeated exposure on the dynamics of the infection and the acquisition of immunity to be investigated.

A number of studies have investigated the dynamics of parasite populations in economically important species. The dynamics of *Trichostrongylus vitrinus* and *Trichostrongylus colubriformis* on repeated infection in sheep were investigated by Donald and Waller (1982). It was observed that the worms that matured persisted for long periods of time even in the face of resistance to larval infection. Other studies by Barger, Le Jambre, Georgi and Davies (1985) on trickle infections of *Haemonchus contortus*, a parasite of sheep, indicated that although parasite

survival was prolonged it was dose dependent, a fourfold increase in weekly dose resulted in the adults being expelled three weeks earlier.

In the laboratory a few studies have investigated parasite population dynamics during a trickle regime. Jenkins and Phillips (1971) gave repeated doses of *Nippostrongylus brasiliensis* to young rats and observed that the worms accumulated and survived for longer (12 weeks) than would otherwise be normal. Behnke and Wakelin (1973) reported that in laboratory bred house mice (*Mus musculus*) *T. muris* matured and persisted if its eggs were given in low level repeated doses (5 or 10 eggs at weekly intervals). However, Behnke *et al.*, (1984) notes that the establishment and survival of *T. muris* is dependent on the strain of the host. For example, strains such as CFLP or NIH do not have a stable parasite population on trickle dosing.

Using the non-gastrointestinal parasite *S. mansoni*, Crombie and Anderson (1985) reported that on trickling the pattern of parasite burden with time changed depending on the intensity of exposure. It was observed that on increasing the weekly dose the change in worm burden with time altered from a monotonic rise to a convex curve.

It has been suggested that more natural conditions (for infection) could be created if mice were kept in large communities and allowed to become infected by taking up the parasite's infective stage from the habitat (arena trials). However, this method of infection introduces a greater amount of variability than that seen on trickling with constant doses. Differences in worm acquisition are as likely to be due to the spatial distribution of the infective stages and differences in worm uptake due to host behaviour as they are to be the result of differences in effectiveness of the immune response. Thus when it is the change in the host's immune response which is of interest then it is most important to remove what may be confusing sources of variation.

The *H. polygyrus*/mouse system is a crude model of human hookworm infection patterns (Bartlett and Ball, 1974) and Miller (1979) suggests that hookworm infections are acquired by continual reinfection at low levels of exposure over long periods of time. Thus trickle infection of mice with *H. polygyrus* would appear to be a suitable model to mimic the natural conditions of human hookworm infection. Yet despite this, few studies have investigated the parasite's population dynamics or changes in the host's immune system on repeated infection with *H. polygyrus*.

In one of the few studies, Kerboeuf (1982) repeatedly infected male CD1 Swiss strain mice with 50 larvae but only sampled at one time and thus no information on the dynamics of the infection could be obtained. Keymer (1986), however, performed a trickle in which mice were given 50 +/-2 larvae every two weeks and the resultant worm burdens were determined at regular intervals. It was noted that the worm burden increased and then plateaued whilst at the same time the variance to mean ratio increased. The increase in the variance to mean ratio indicates a heterogeneity in response and this was attributed to genetic differences in immunological competence within the outbred (MF1) strain used.

Many studies have also reported genetically determined differences in responsiveness to *H. polygyrus* (e.g. Dobson, 1982). The genetic basis of immunity to helminths has been reviewed by Wakelin (1986). The review describes the genetic variation in immune responses and how these may be related to worm survival. It is suggested that although this research has been pursued at an academic level it does provide a conceptual framework for studies in man (although it must be noted that there is no proof of genetically determined resistance to intestinal helminths in man (Schad and Anderson, 1985)).

It was decided to investigate changes in *H. polygyrus* dynamics and host responses using two different strains of mice: NIH, a high responder to *H. polygyrus* and CBA/ca, a low responder to *H. polygyrus*. As suggested by James *et al.* (1988), the use of different responder strains is perhaps of most direct benefit

because it allows the requirement of the various immune responses in the development of resistance to be tested.

The chapter gives the results from experiments in which two strains of mice, one a high responder to *H. polygyrus* and the other a low responder, were given repeated low level doses of the parasite. This was done in an attempt to mimic the pattern of acquisition of the parasite that occurs in nature and allow an investigation of the murine immune response during this exposure. The use of the two responder strains may give information on the correlation of a response with effective resistance.

The chapter is organised into three main sections followed by a discussion. The first is a documentation of the results of preliminary experiments performed to allow the determination of the conditions required to assay the proliferative response during a trickle infection regime (section 4.2). The next two parts give the immunological and epidemiological results obtained on regular assaying of NIH mice (section 4.3) and CBA mice (section 4.4) respectively. The discussion that follows (section 4.5) relates these results to the biologies of the parasite and host.

4.2 Establishment of lymphocyte proliferation assay conditions.

4.2.1 Introduction and aim

The culture conditions required to assay changes in lympho-proliferative ability during repeated low level infections may differ from those needed to assay the changes in cells from mice "immunised" by anthelmintic regimes. To determine if this was the case and whether differences in cellular proliferation between mice trickled with different doses of *H. polygyrus* were detectable, NIH mice were infected at weekly intervals with different doses of the parasite. Cells from these mice were then tested for their ability to proliferate to preparations from different life cycle stages of the parasite.

Preliminary experiments indicated that the worm burden of NIH mice given 10, 25 or 50 L3/week peaked between the second and fourth week of infection and declined to zero by the tenth week, this observation being supported by the work of Maema (1987). Behnke and Robinson (1985) noted that NIH mice were most resistant to a challenge infection 12 days after the anthelmintic abbreviation of a 9 day infection. It was therefore decided to assay cellular proliferation 2 weeks after the peak in worm burden. However, it must be noted that the peak in worm burden may not be correlated with the onset of resistance and even if it is,lympho-proliferative ability may not correlate with resistance.

4.2.2 Method

To determine assay conditions and investigate differences in lympho-proliferative ability 15 six to eight week old female NIH mice were divided at random into 5 groups of 3. Each group was dosed at weekly intervals with one of the following doses of parasite larvae: 10, 25, 50, 200 or 0 l3. After 5 weeks of repeated infection the mice were killed, their intestines removed for worm burden determination and their mesenteric lymph nodes (MLN) extracted. The MLN cells of mice in each cohort were pooled and used in a lymphocyte proliferation assay performed under standard conditions. The concentrations of the antigens and Concavalin A are given in table 4.1.

Table 4.1 *The concentration of antigens used to determine conditions to assay the proliferative ability of cells of mice given low level repeated infection.*

Antigen	Dilution	
adult homogenate:	100 ug/ml double diluted to	6.25 ug/ml
Adult ES(MT)	50 ug/ml	3.13 ug/ml
D6L4 homogenate	100 ug/ml	6.25 ug/ml
D6L4 ES(MT)	50 ug/ml	3.13 ug/ml
L3 homogenate	10 ug/ml	0.62 ug/ml
Con A	50 ug/ml	3.13 ug/ml

as well as medium alone.

The experiment was then repeated.

4.2.3 Results

The results of the first preliminary experiment are shown in figure (4.1a-e) and statistical analysis is given in appendix 2.1.1.. The cells from mice given different weekly doses of the parasite could be distinguished by the amount of proliferation shown to the antigens of the parasite. However, the relationship between proliferation and *in vitro* antigen concentration was not a linear one, and neither was the relationship between dose of parasite and proliferative response. Furthermore, a response was not shown towards all the parasite preparations.

The antigens against which the MLN cells showed significant proliferation, as compared to the response in naive mice or to the response when the cells were cultured with medium alone, were the D6L4 homogenate and L3 homogenate. A significant response was also shown towards the adult ES(MT). No response was shown against the adult homogenate.

The cells which proliferated to the greatest extent against the D6L4 ES(MT) were from the mice given 50 L3/week followed by those from mice given 25 L3/week. The proliferation of cells from mice given 10 or 200 L3/week was not significantly different and these cells did not proliferate to the D6L4 ES(MT) any more than did the MLN cells from naive age-matched controls. The same pattern of response with dose was seen when the cells were cultured with the D6L4 homogenate. The response to the D6L4 homogenate was, however, less than the response to the D6L4 ES(MT).

Figure 4.1 *First experiment to investigate the lympho-proliferative response of mesenteric lymph node cells of NIH mice given infective L3 for 6 weeks to. (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate. The lines represent the responses of the cells of mice given the following weekly doses: 10 L3 (square); 25 L3 (+); 50 L3 (lozenge); 200 L3 (triangle).*

Figure 4.1 a

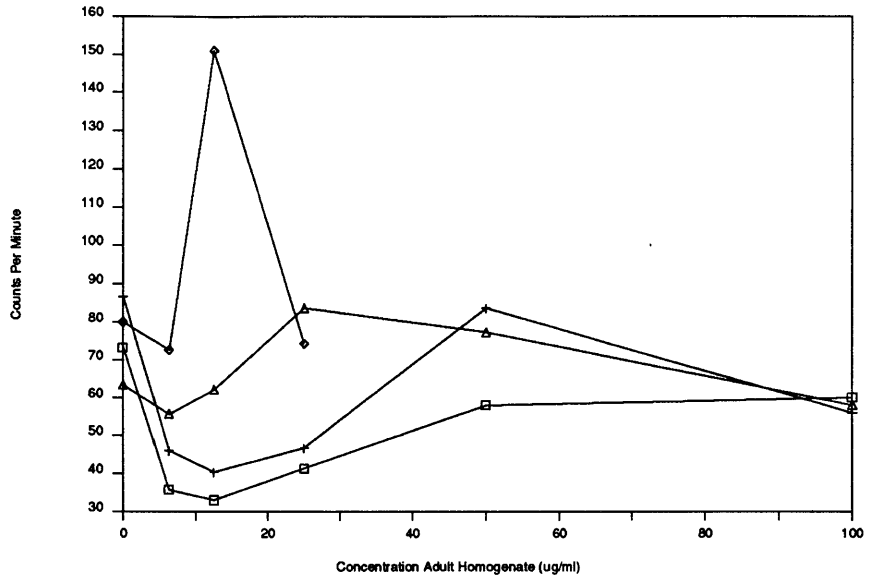


Figure 4.1 b

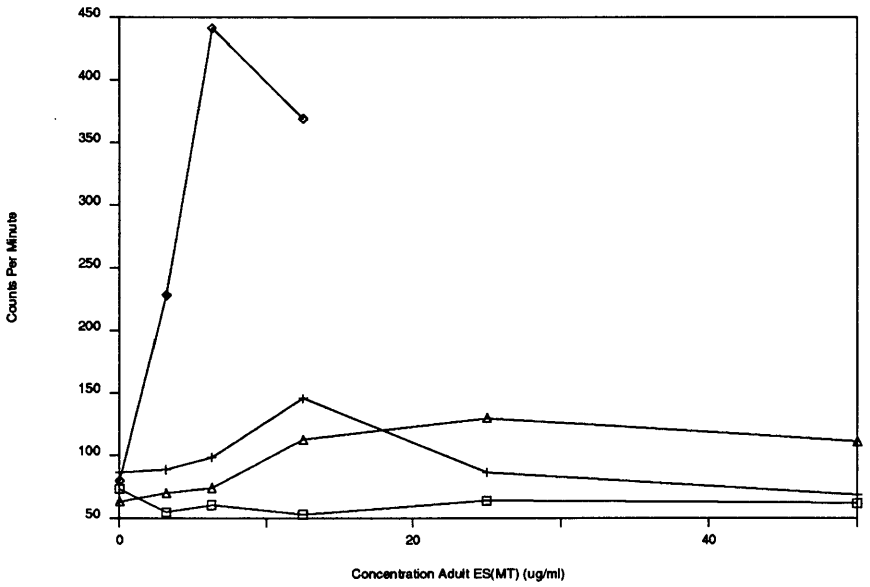


Figure 4.1 c

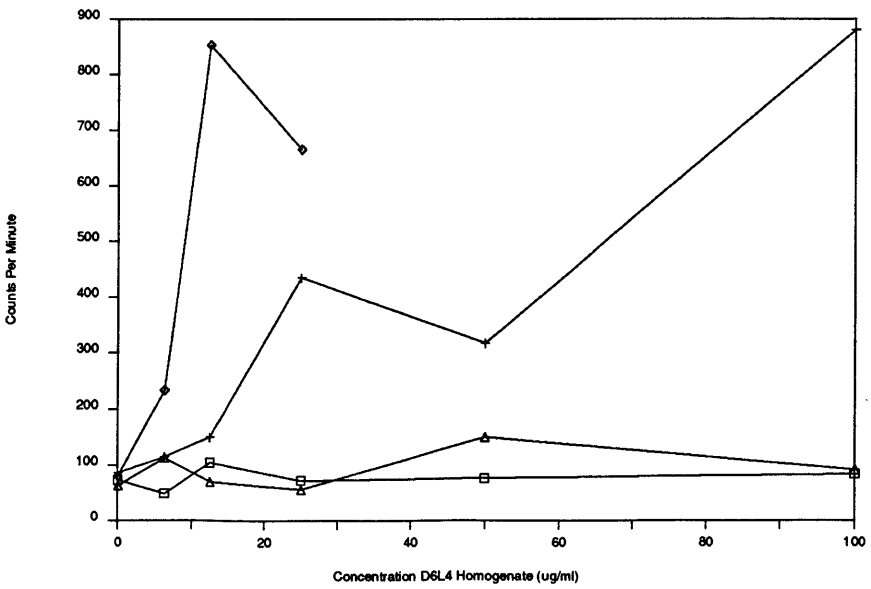


Table 4.1 d

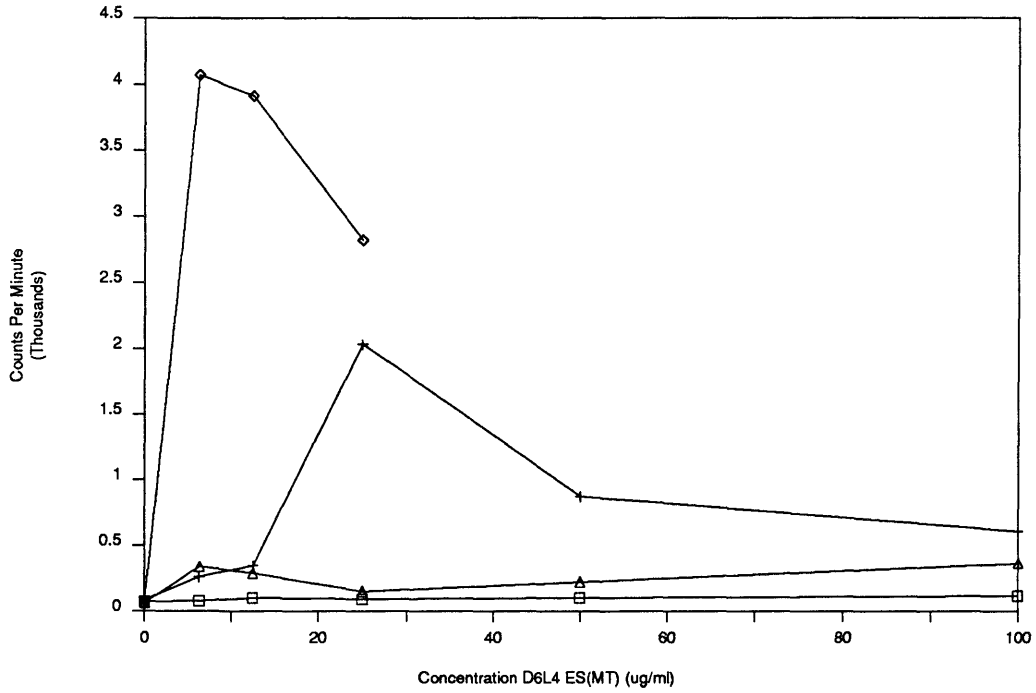


Table 4.1 e

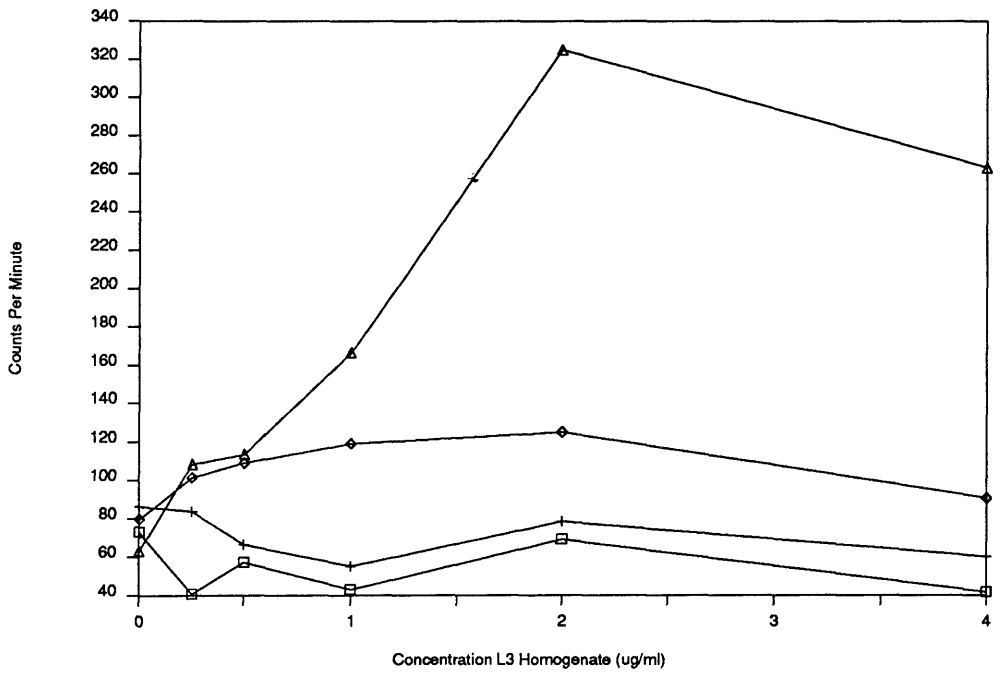


Figure 4.2 *Second experiment to investigate the lympho-proliferative response of spleen cells of NIH mice given infective L3 for 6 weeks to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate. The lines represent the responses of the cells of mice given the following weekly doses: 10 L3 (square); 25 L3 (+); 50 L3 (lozenge); 200 L3 (triangle).*

Figure 4.2 a

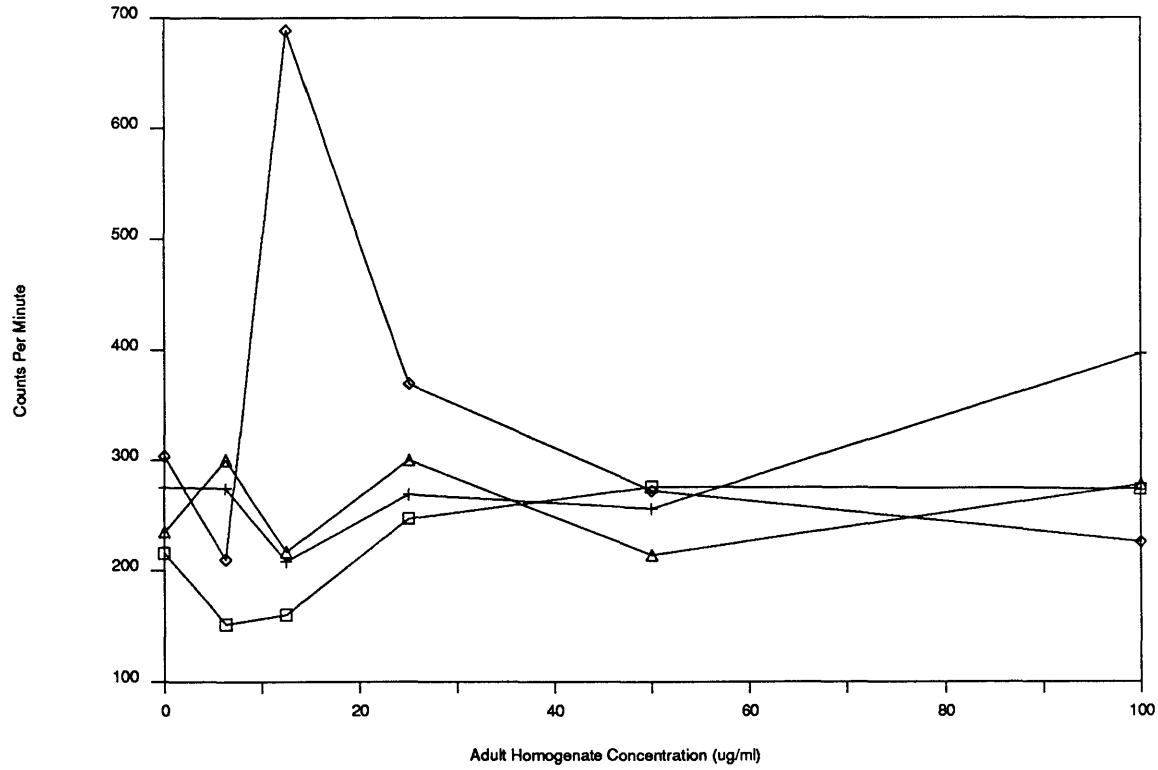


Figure 4.2 b

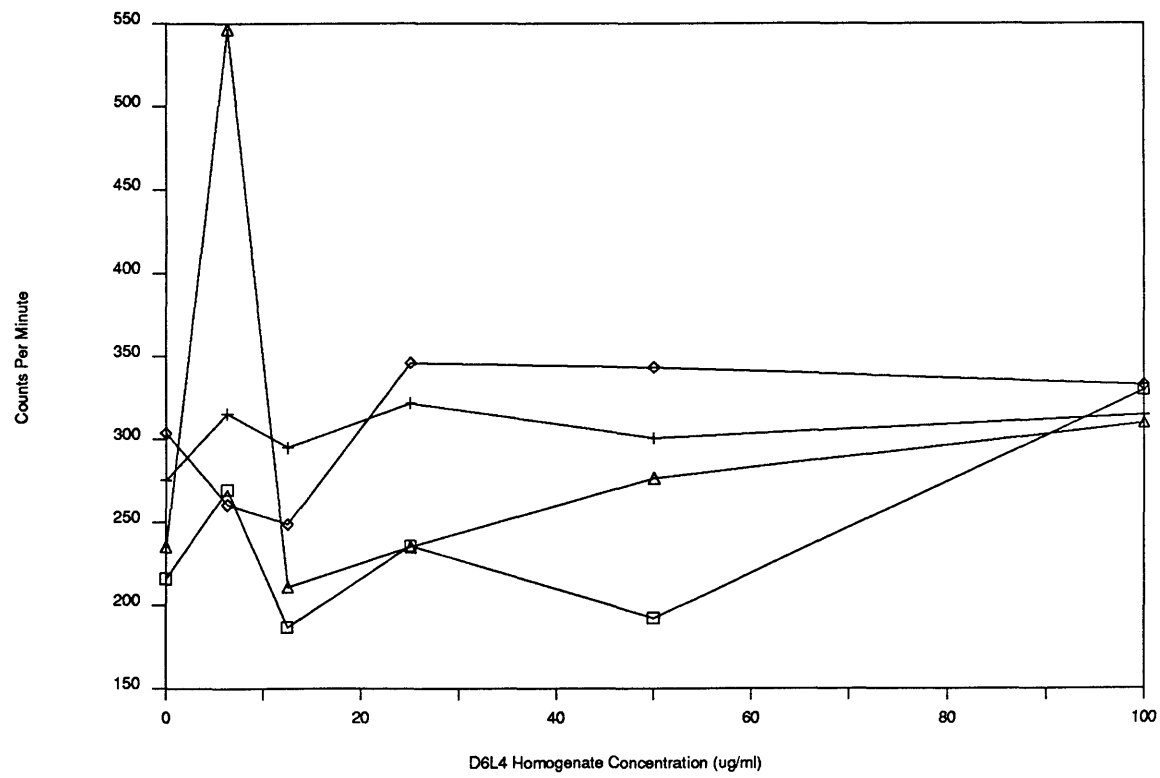


Figure 4.2 c

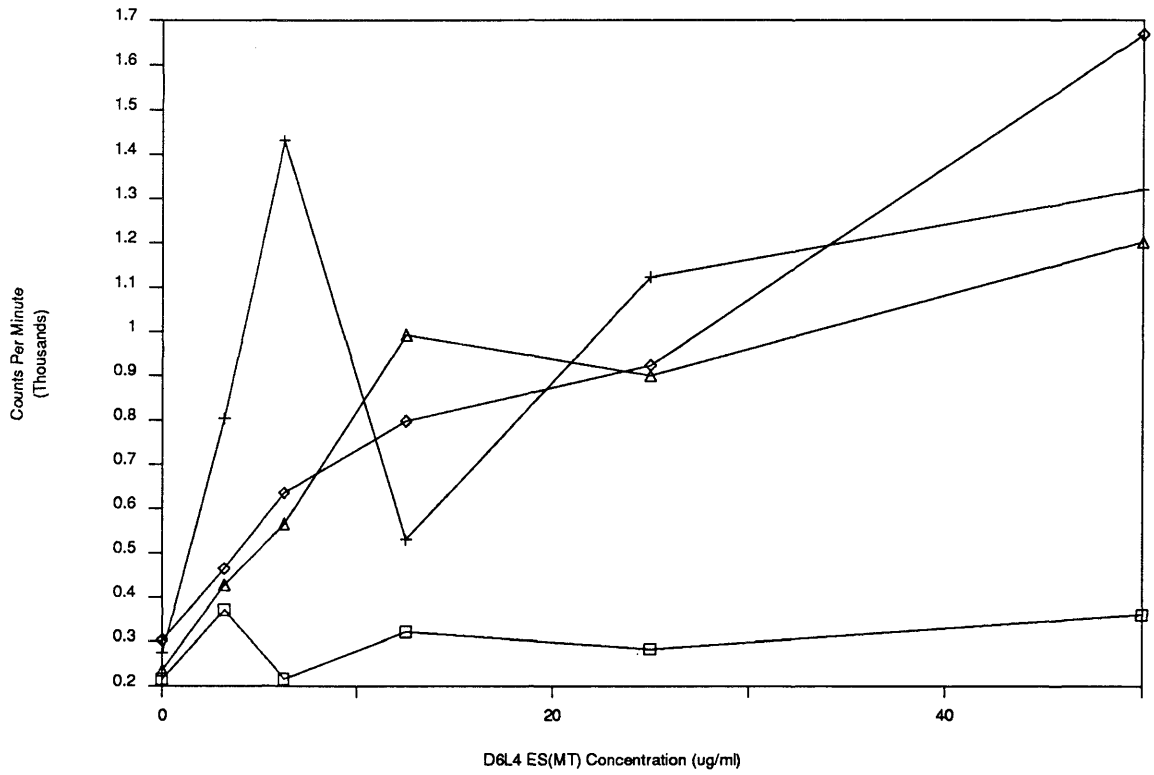
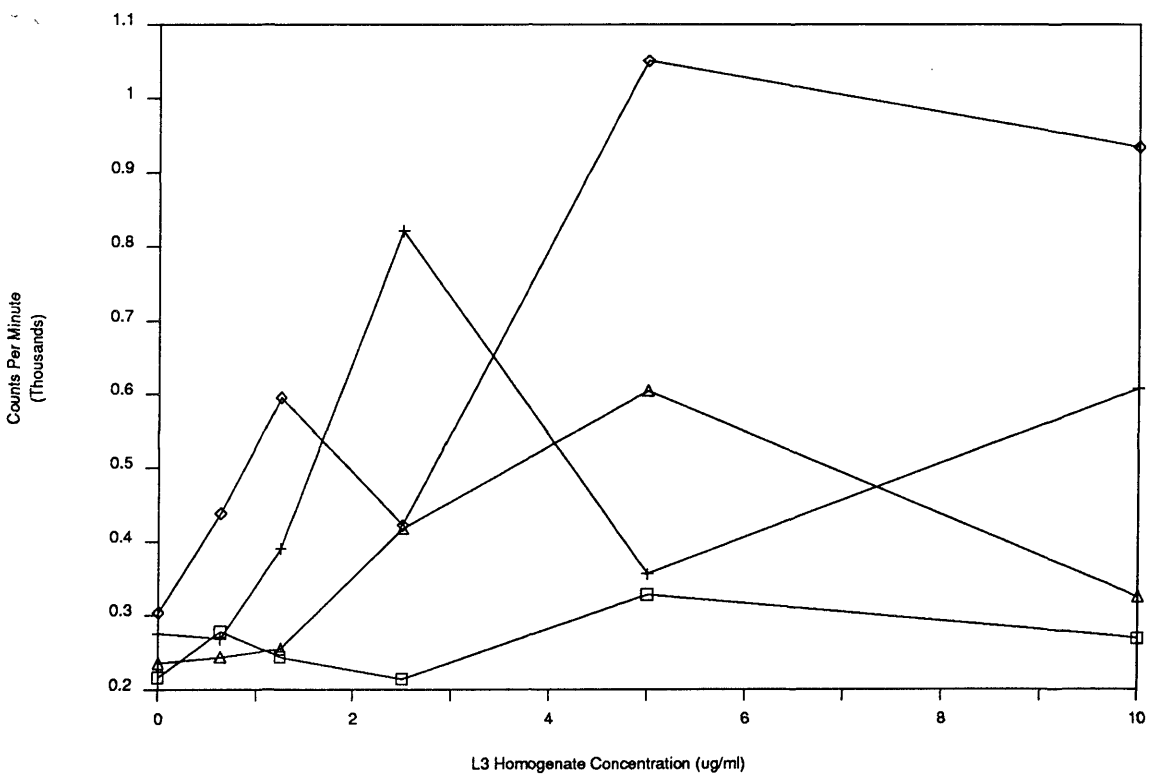


Figure 4.2 d



The trickle infection of mice with different weekly doses gave rise to MLN cells which showed significant proliferation to the L3 homogenate. However, the response to the L3 homogenate of cells from mice given different weekly doses was different to the response to the D6L4 preparations. When the cells were cultured with the L3 homogenate the cellular proliferation was greater the larger the weekly dose given whereas the order of the response to the D6L4 preparations did not follow the order of the weekly dose.

The response to the L3 homogenate was less than the response to the D6L4 ES(MT) but the same amount of proliferation was shown to this homogenate and the D6L4 homogenate. The amount of Con A induced lympho-blastogenesis was not significantly different between each of the doses.

The worm burdens of the mice were in broad agreement with those of Maema (1986).

On repeating the experiment a slight difference in the pattern of response was obtained (results shown in figure 4.2 (a-e) and statistical analysis given in appendix 2.1.2). Although proliferation was shown to the D6L4 ES(MT) and to the L3 homogenate and not to the adult homogenate, in this instance there was no response shown to the D6L4 homogenate. A further difference observed was in the reactivity of the cells from mice given different trickle doses. Whereas before the reactivity of the cells to the D6L4 ES(MT) from mice given 200 L3/week was less (but not significantly so) than the response for the cells from mice given 25 L3/week, in this case the reverse was true. As before, when the cells from mice given 10 L3/week were cultured with the D6L4 ES(MT) the proliferation observed was not significantly greater than that of the controls. A similar pattern of response was seen when the cells from mice given different weekly doses of larvae were cultured with the L3 homogenate. As before, the response of the cells from infected mice to the D6L4 ES(MT) was greater than to the L3 homogenate. It was

not possible to incubate the cells with the adult ES(MT) due to difficulties in preparing the antigen.

Despite these differences, the results from the two experiments do show that proliferation to the D6L4 ES(MT) is greater than to the L3 homogenate. Proliferation against the D6L4 homogenate was only shown in the first experiment. In neither experiment did the adult homogenate cause any significant proliferation.

The relationship between parasite dose and proliferative ability was similar in both experiments: the cells from mice which had received 10 L3/week proliferated least of all and those from mice given 50 L3/week proliferated to the greatest extent. Between these limits the cells from mice given 25 L3/week or 200 L3/week proliferated, the difference between the two was not significant.

4.3 Regular assaying during a trickle infection.

4.3.1 Introduction and aims

The results from the preliminary experiments indicated that it was possible to differentiate between the cellular responses of mice given different weekly doses of *H. polygyrus*. Thus, to investigate more fully the pattern of proliferation during repeated low level infection, high and low responder mice were given repeated low level doses of the parasite and the proliferative ability of their cells assessed at regular intervals.

4.3.2 Method

One hundred and twenty (120) six to eight week old female NIH mice were divided at random into four groups of thirty. One group of thirty (30) served as uninfected age matched controls for cells in the LPA and sera in the ELISA. Of the other three groups, one was dosed with 2 L3/week, another with 10 L3/week and

the remaining one with 50 L3/week (described as low, medium and high dose respectively). At three weekly intervals the infectivity of the parasite larvae was assessed by infecting 6-10 week old female NIH mice with 50 L3 and determining their worm burdens two weeks later.

The mice were infected for 13 weeks. At two weekly intervals five (5) mice were selected at random from each group and the number of eggs in their faeces determined. The mice were then killed, their intestines fixed in formalin for later worm burden determination, blood removed for antibody level determination and their spleens used in a LPA.

Cells from the spleen were used in an *in vitro* assay rather than cells from the MLN as they showed more consistent proliferation to a greater number of parasite antigen preparations. This greater range of responses may allow more information about immune changes to be obtained than if the responses in the MLN were investigated. Analysis of the changes in both the spleen and MLN would have yielded the most information about immune changes but for logistic reasons changes in only one of the organs could be studied. It should be noted that the spleen may be secondary in role to the MLN in dealing with the products of an intestinal parasite (Ali and Behnke, 1985). Thus any changes observed may not correlate with resistance, although it has been noted that cells from the spleen can transfer resistance adoptively (Cypess, 1970).

Table 4.2 *Concentration of antigens used in lymphocyte proliferation assays to assess change in proliferation during repeated low level infection*

Antigen	Concentration
adult homogenate:	50 ug/ml
Adult ES(MT)	20 ug/ml
D6L4 homogenate	25 ug/ml
D6L4 ES(MT)	20 ug/ml
L3 homogenate	10 ug/ml
Con A	50 ug/ml double diluted to 3.13 ug/ml
as well as medium alone.	

A further change, also made for logistic reasons, was that only one concentration of each antigen was used in the assay. The antigen concentration chosen was the one which gave the maximum response when cells from "immunised" mice were cultured with a range of dilutions of the antigens. This may have had the effect of losing information on the relationship of cellular proliferation at different *in vitro* antigen doses with time.

The concentrations of the antigens and mitogen used are given in table 4.1.

As positive (inter-experimental) controls, spleen cells from 6-10 week old female CBA mice were incubated with a range of concentrations of Con A (50 ug/ml double diluted through to 3.125 ug/ml).

The experiment was repeated using CBA mice instead of NIH mice.

4.3.3 Repeated low level infection in NIH mice: epidemiological and immunological data.

4.3.3.1 Worm burden

The average worm burden of mice receiving 50 L3/week reached a maximum of 19.4 adults per mouse after the second week post initial infection. (The results are shown in figure 4.3 and statistical analysis is given in appendix 2.2a). It remained at that level for a further two weeks (worm burden at week 4 was 19.2) before declining to zero by the sixth week. The worm burden of mice trickled with 10 L3/week showed a similar pattern, plateauing at an average of 5.2 adults per

Figure 4.3 *Worm burden of NIH mice given repeated low level doses of* H. polygyrus. *The lines represent the burdens of mice given: 2 L3 / week (square); 10 L3 / week (+); 50 L3 / week (lozenge).*

Figure 4.4 *The sex ratio (male / (male+female)) of* H. polygyrus *adults in NIH mice given repeated low level doses of the parasite. The lines represent the ratios of mice given the following weekly doses: 2 L3 (square); 10 L3 (+); 50 L3 (lozenge).*

Figure 4.3

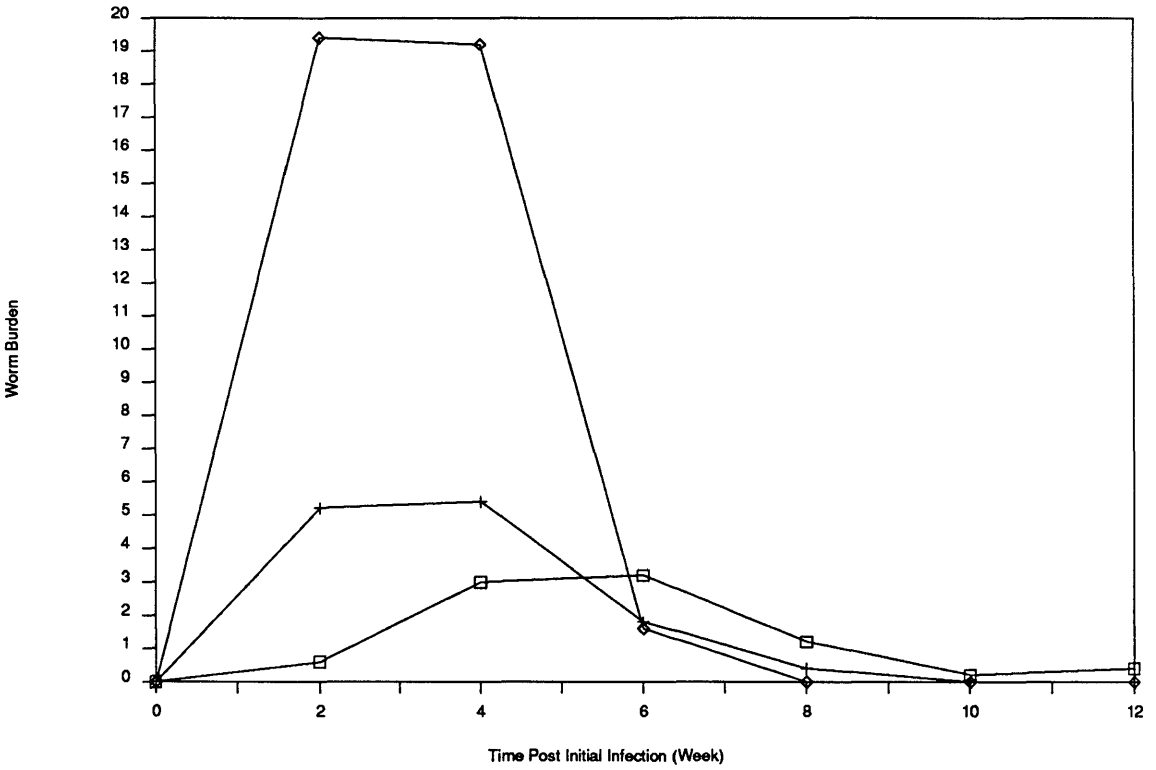
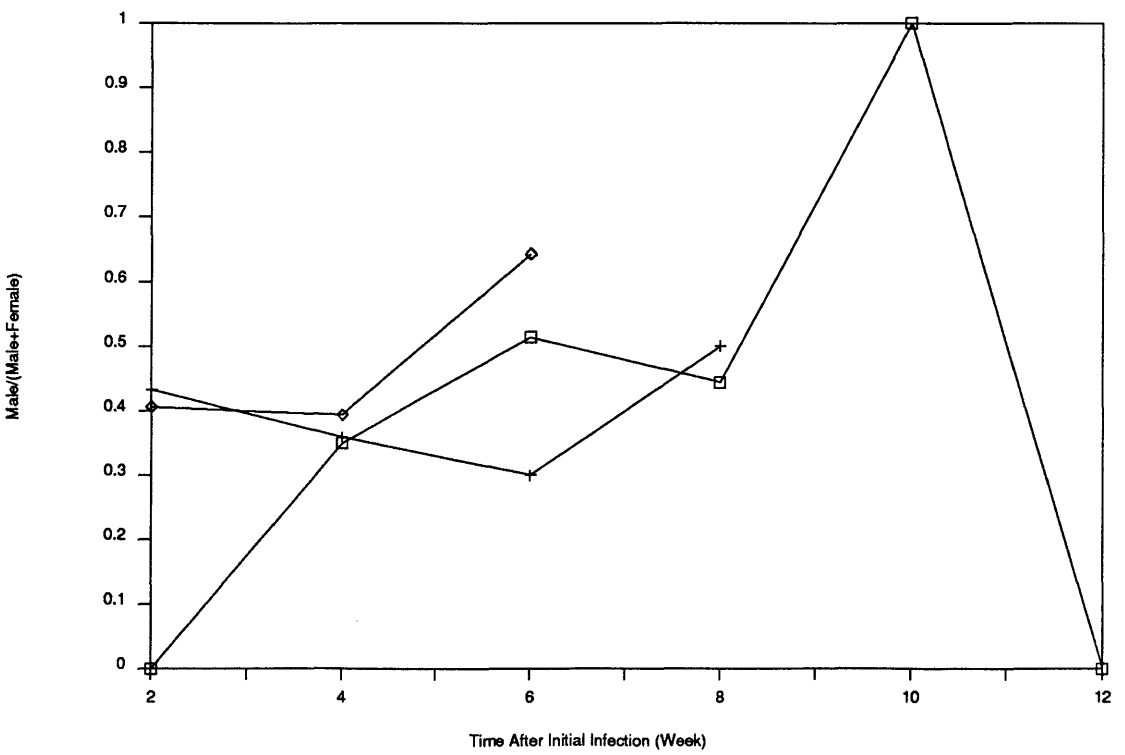


Figure 4.4



mouse between the second and fourth week of the trickle before declining to zero by the eighth week. The repeated infection of mice with 2 L3/week gave rise to a different pattern of worm burden with time. The worm burden plateaued at an average of 3.2 adults per mouse between the fourth and sixth week of the repeated infection before it declined to zero by the tenth week.

The change in worm burden with time was significant for each of the dose levels. The worm burden of the mice given 50 L3/week was significantly different from the worm burdens of mice given 2 L3/week and 10 L3/week. The worm burdens of mice given 2 or 10 L3/week were not significantly different. A difference in the pattern of worm expulsion between the three doses was noted: the greater the weekly dose of parasite given, the earlier all the worms were expelled.

The infectivity of the larvae did not alter significantly during the period of the experiment ($P<0.05$).

4.3.3.2 Sex ratio

The sex ratio of the parasites (number of males/total number of worms) was calculated for all the times at which worms were observed. The results of the analysis are shown in figure 4.4 and the statistics given in appendix 2.2b. However, statistical comparison of the ratio in mice receiving different weekly doses of the parasite could only be made when worms were recovered from all infection levels, that is for weeks 2 to 6.

The male to female ratios for mice receiving 2, 10 or 50 L3/week were not significantly different for the first 6 weeks of infection and there was no significant change in the ratio with time. The parasite populations did show a bias towards females.

After six weeks the mice given 50 L3/week no longer harboured any worms. Although it was not possible to analyse the change in the ratio after this time the ratio increased, the males constituting a greater proportion of the worm burden.

4.3.3.3 Faecal egg counts

The results of the investigation into the fecundity of female parasites are shown in figure 4.5 and the statistical analysis given in appendix 2.2c. Female worm fecundity did not remain constant throughout the course of the trickle. The egg production per female per 16 hours peaked at the fourth week of the trickle for the worms in the mice receiving 10 or 50 L3 /week. The fecundity was slightly, but not significantly, greater for the females in the 10 L3/week mice. A different pattern of egg production was seen for the female worms in the 2 L3/week mice, the peak of production was at week 8 not week 4 as for the other doses. There was also a difference in the time of cessation of egg production with dose, the time at which no eggs were detected in the faeces was earlier the larger the weekly dose given. Although the change in fecundity with time was significant for each dose, the number of eggs per female was not significantly different.

Figure 4.5 *The fecundity of* H. polygyrus *female adults (eggs per female per 16 hr) in NIH mice given repeated low level doses of the parasite. The lines represent the ratios of mice given the following weekly doses: 2 L3 (square); 10 L3 (+); 50 L3 (lozenge).*

Figure 4.6 *The number of cells from the spleens of NIH mice given repeated low level doses of* H. polygyrus. *The lines represent the ratios of mice given the following weekly doses: 2 L3 (square); 10 L3 (+); 50 L3 (lozenge);* 50 L3 (triangle).

Figure 4.5

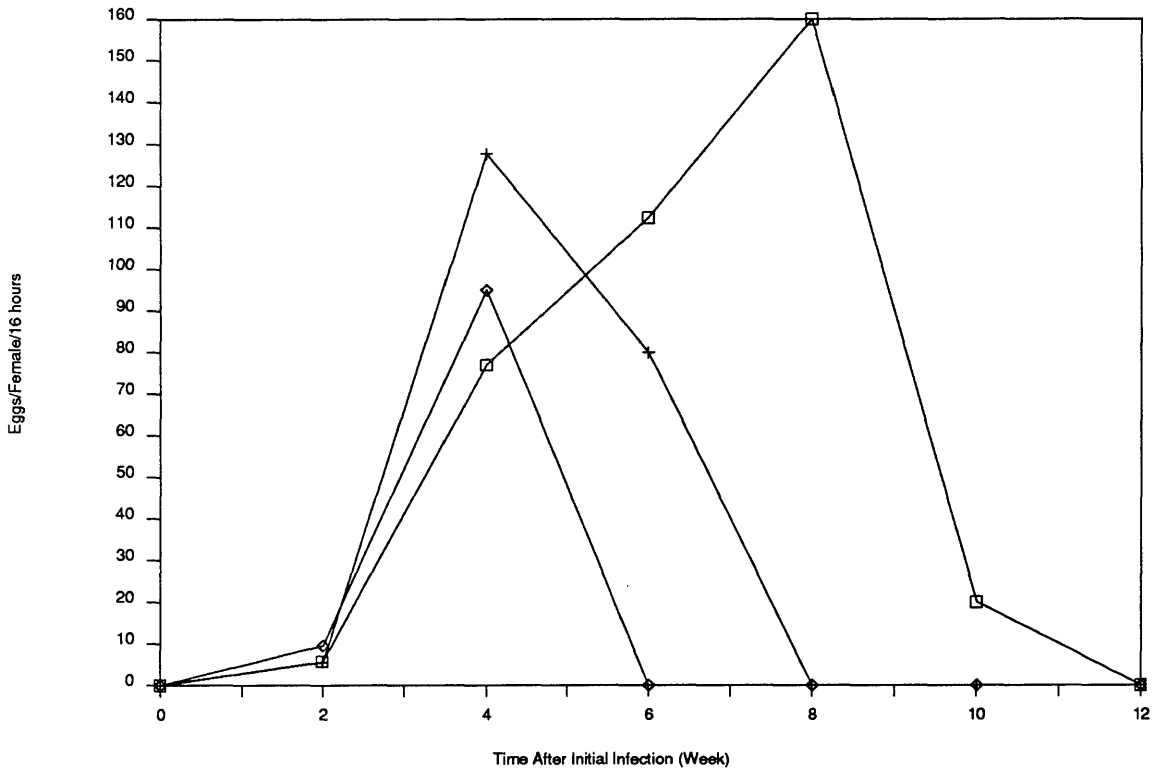
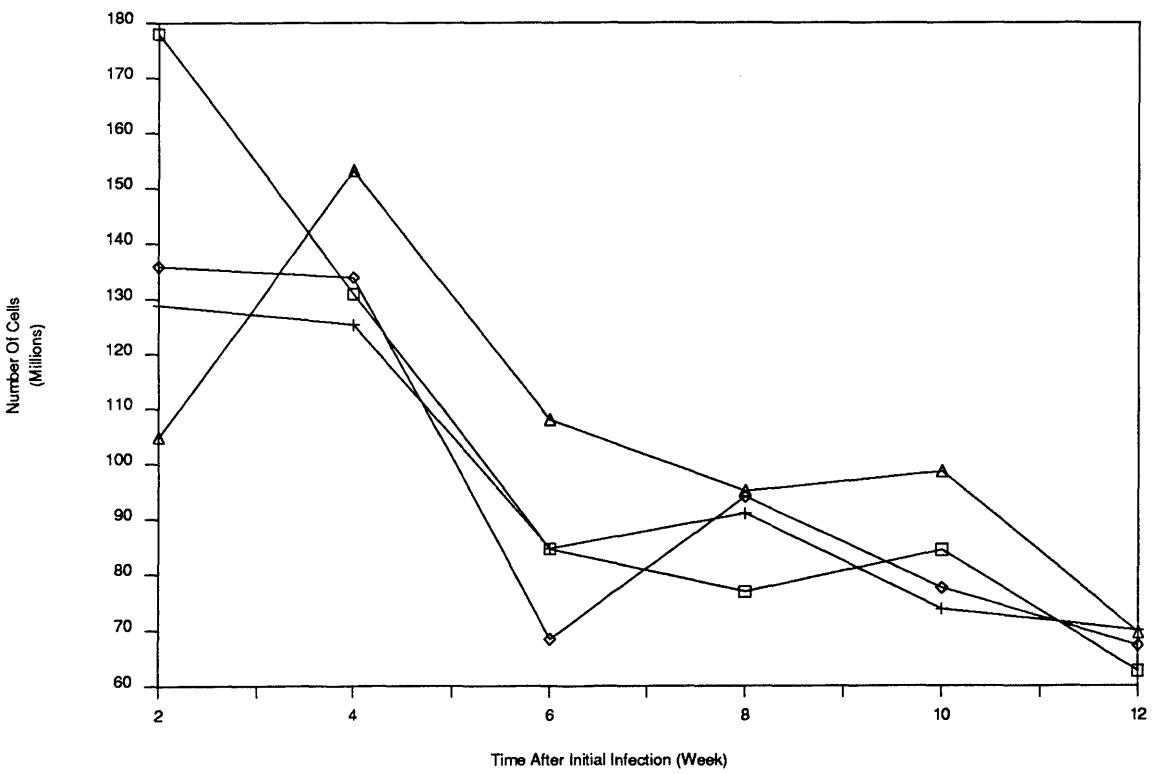


Figure 4.6



4.3.3.4 Spleen cell counts

It was observed that none of the infected mice's cell counts were greater than those of the controls (see figure 4.6 for results and appendix 2.2d for statistical analysis). This means that the observed decrease in spleen cell counts is unlikely to be as a result of the parasite infection.

4.3.3.5 Immunoglobulin levels

The levels of total immunoglobulin and IgG1 antibody specific to the D6L4 or adult homogenate in sera from infected mice were determined by ELISA. The statistical analysis of the results is given in appendix 2.3a and 2.3b for the total immunoglobulin and IgG1 levels respectively.

4.3.3.5a Total antibody levels

There was no significant difference in the amount of antibody of all classes to the two homogenates (see figure 4.7a & 4.7b for responses to adult and D6L4 homogenate respectively). There was a significant difference in the amount of antibody in the mice which had received different weekly doses of the parasite: the larger the weekly dose, the greater the amount of antibody. The increase in antibody concentration with increasing number of infections (increasing length of time) was significant although the level plateaued between weeks 10 and 12.

Figure 4.7 *The humoral response of NIH mice. The serum samples from NIH mice were assayed for their total immunoglobulin to (a) adult homogenate and (b) D6L4 homogenate. The lines represent optical densities for the sera of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.8 *The humoral response of NIH mice. The serum samples from NIH mice were assayed for their IgG1 to (a) adult homogenate and (b) D6L4 homogenate. The lines represent optical densities for the mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.7 a

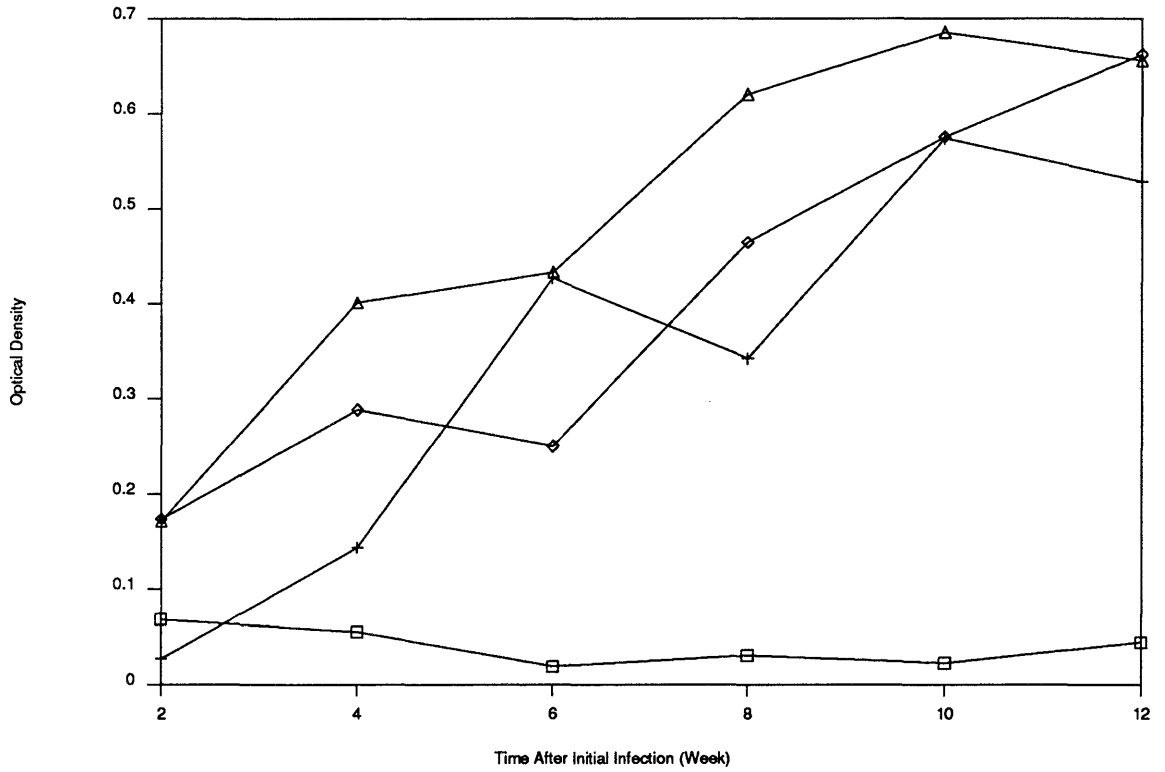


Figure 4.7 b

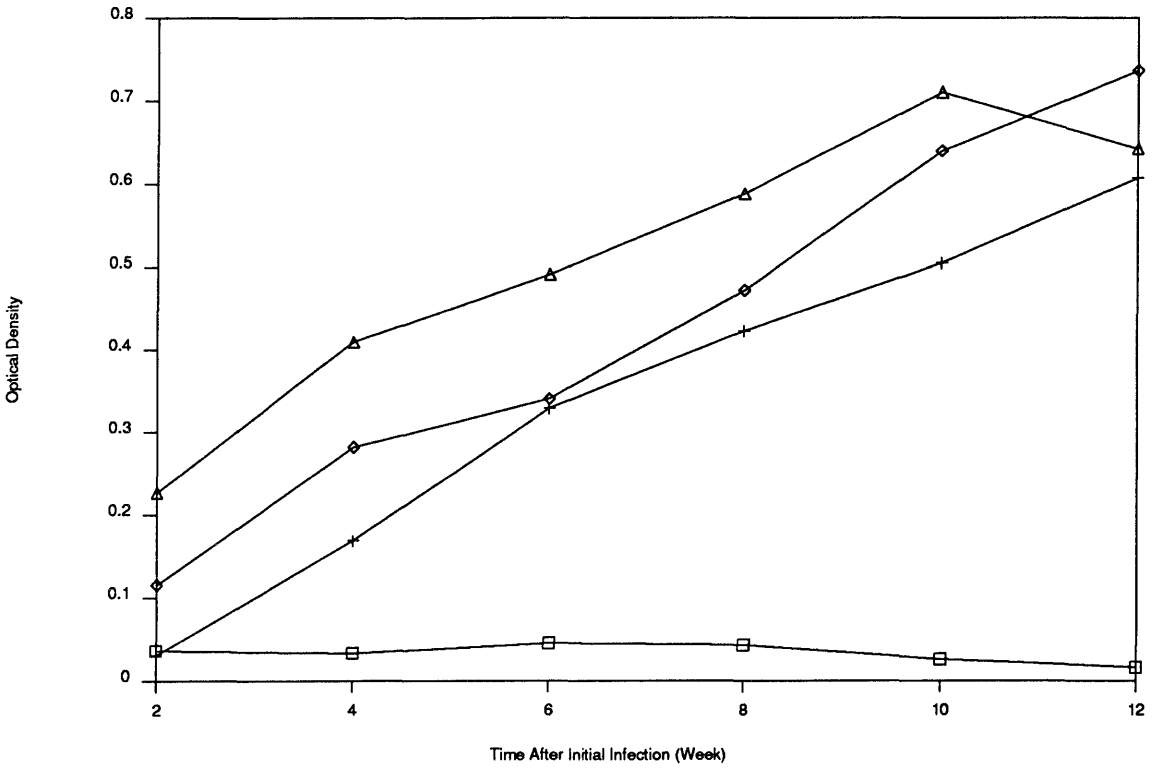


Figure 4.8 a

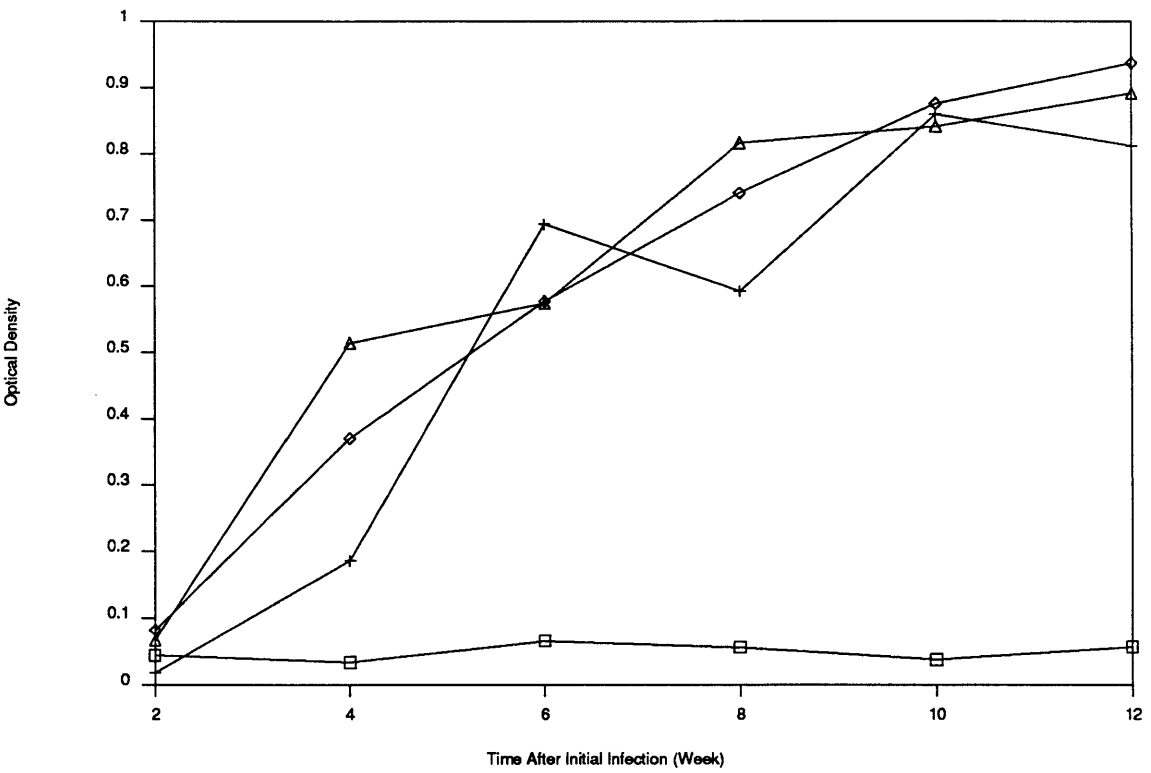
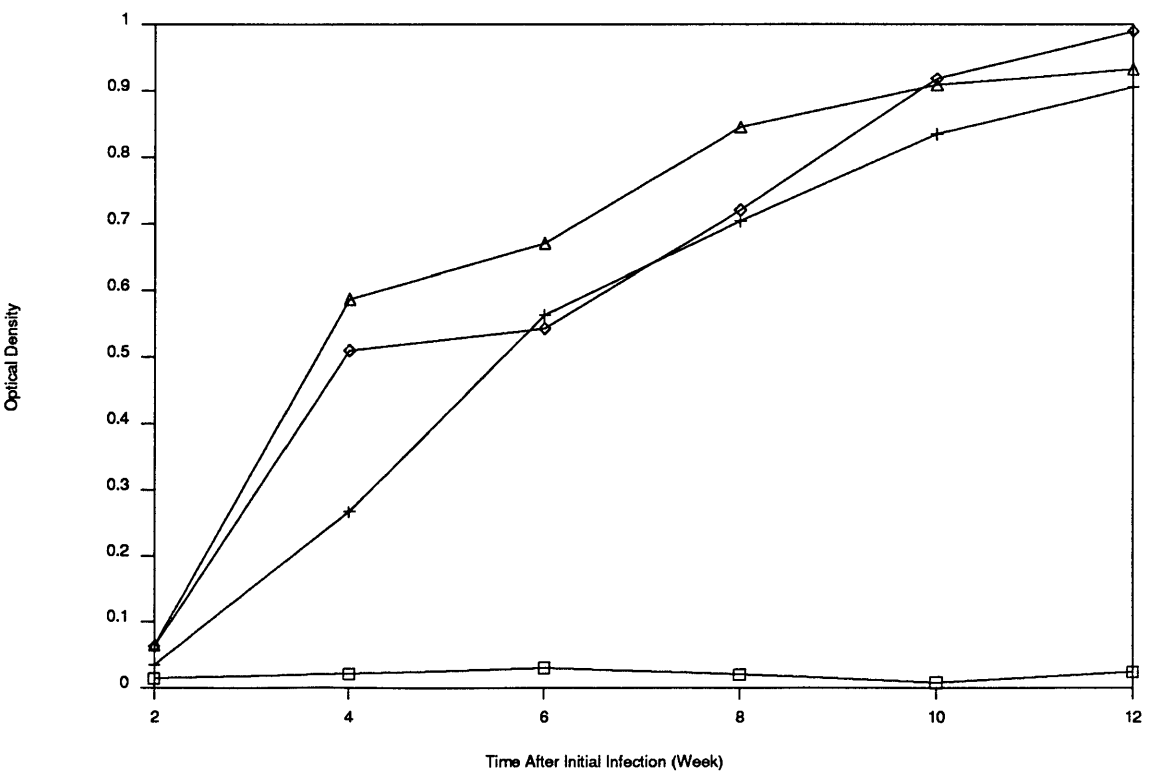


Figure 4.8 b



4.3.3.5b IgG1 Levels

Just as no significant difference could be detected in the amount of total antibody to either of the two homogenates, neither could any difference in the amount of IgG1 antibody to either of the two homogenates be detected (see figure 4.8a & 4.8b for responses to adult and D6L4 homogenate respectively). The difference in IgG1 antibody concentration in the sera of mice receiving different levels of weekly dose followed a slightly different pattern to that of total antibody (for statistical analysis see appendix 2.3b). The level of antibody was significantly lower in the sera of mice which had received 2 L3/week than it was in those mice which had received 10 or 50 L3/week. There was, however, no significant difference in the amount of IgG1 in the sera of mice which had been given either 10 or 50 L3/week. The IgG1 level also showed a significant increase with time but did not plateau at the later times of infection.

4.3.3.6 Proliferation of spleen cells: values expressed as counts per minute

The results of the proliferation of cells from naive and infected mice are given in figure 4.9a-e and the statistical analysis performed is given in appendix 2.4.

4.3.3.6a Non-specific proliferation

The different antigens when cultured with cells from mice which had not experienced the parasite caused different amounts of proliferation. The antigen which had the weakest non-specific effect was the adult homogenate, on culturing with cells from naive NIH mice it caused no more proliferation than that observed on culturing the cells with medium alone. The other antigens all caused more non-specific proliferation than medium alone. Apart from the adult homogenate, all the antigens had the same non-specific proliferative effect. The statistical analysis of the non-specific proliferation is given in appendix 2.4a.

4.3.3.6b Antigen specific proliferation.

The antigens of *H. polygyrus*, when incubated with cells from NIH mice, all caused significantly more proliferation than the amount that occurred when the cells were incubated with medium alone. The antigen which caused the least amount of proliferation was the adult homogenate. Of the remaining antigens, there was only a significant difference between the response induced by the D6L4 ES(MT) and the response that L3 homogenate induced. There was no difference in the amount of proliferation the other antigens induced (for statistical analysis see appendix 2.4b & 2.4c).

4.3.3.6d Change in proliferative ability with length of trickle.

The cells from mice trickled for different lengths of time showed differences in their proliferative ability. The longer the time the mice had received larvae, the greater the amount of proliferation shown to the antigens (for statistical analysis see appendix 2.4d). However, there was a slight decrease in proliferative activity at the last (twelfth) week of assaying. This is unlikely to have been due to a change in *in vivo* immune responsiveness as a general lowering of parasite antigen induced proliferation was noted in other unconnected assays (this may have been due to the use of a new batch of foetal calf serum). It should be noted that at this time of assay the relative order of the response induced by the different antigens remained intact.

Figure 4.9 *The change in the lympho-proliferative response to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate, (f) medium of the spleen cells of NIH mice given repeated low level doses of H. polygyrus. The lines represent the change in proliferation with time for the cells of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.9 a

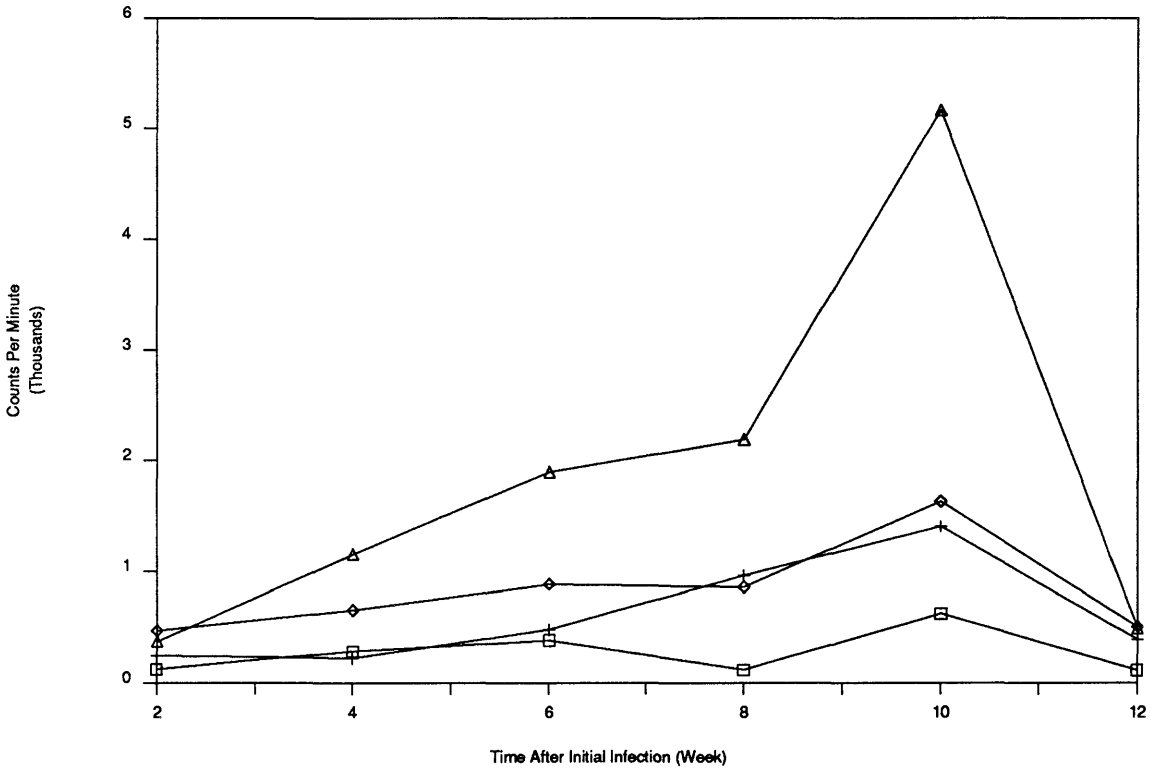


Figure 4.9 b

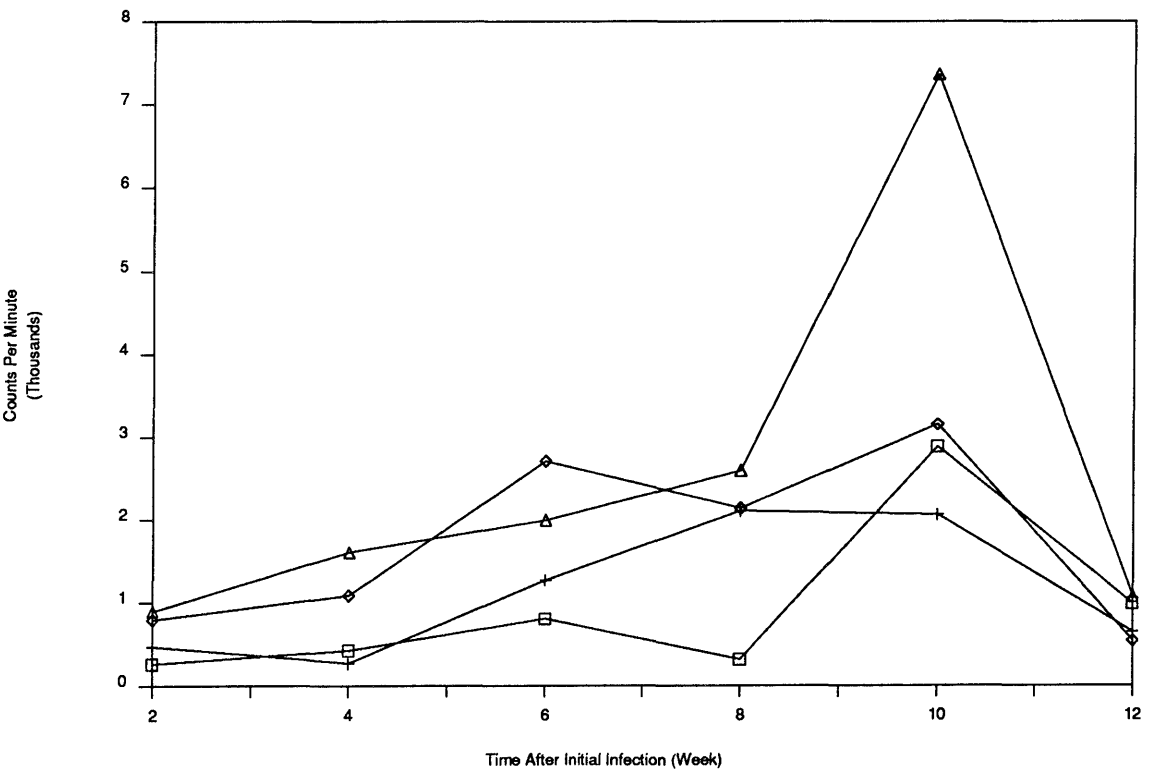


Figure 4.9 c

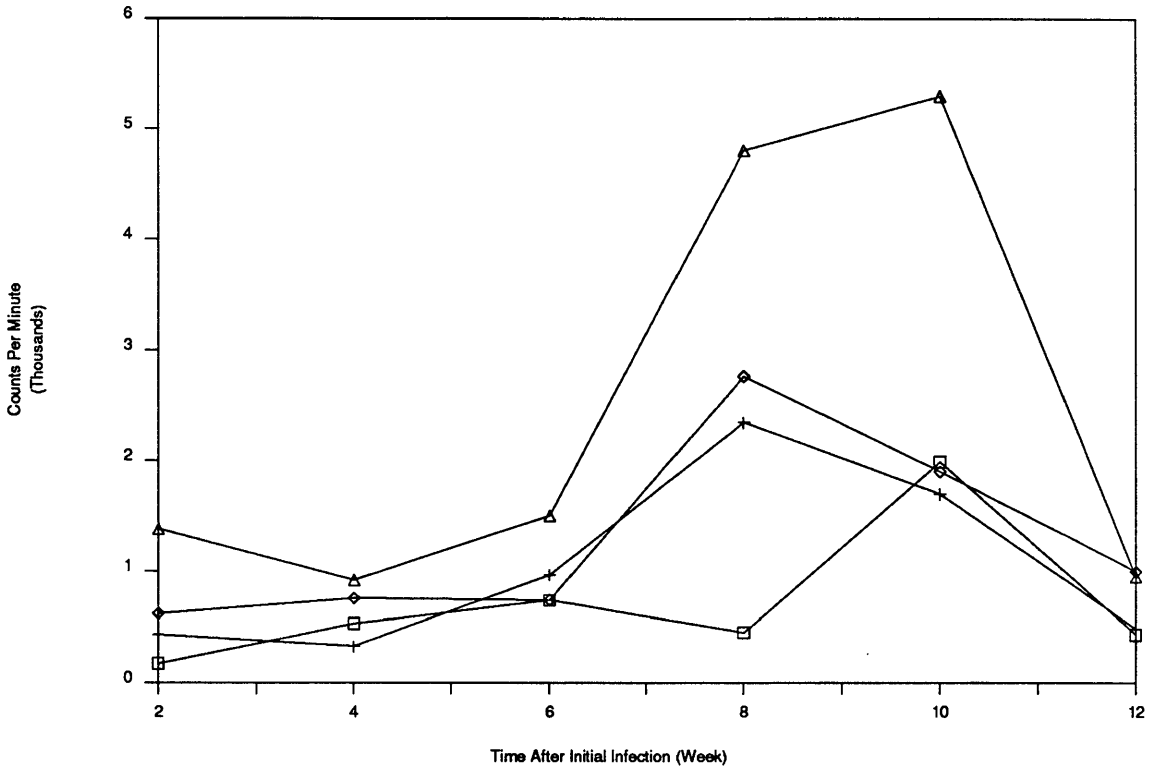


Figure 4.9 d

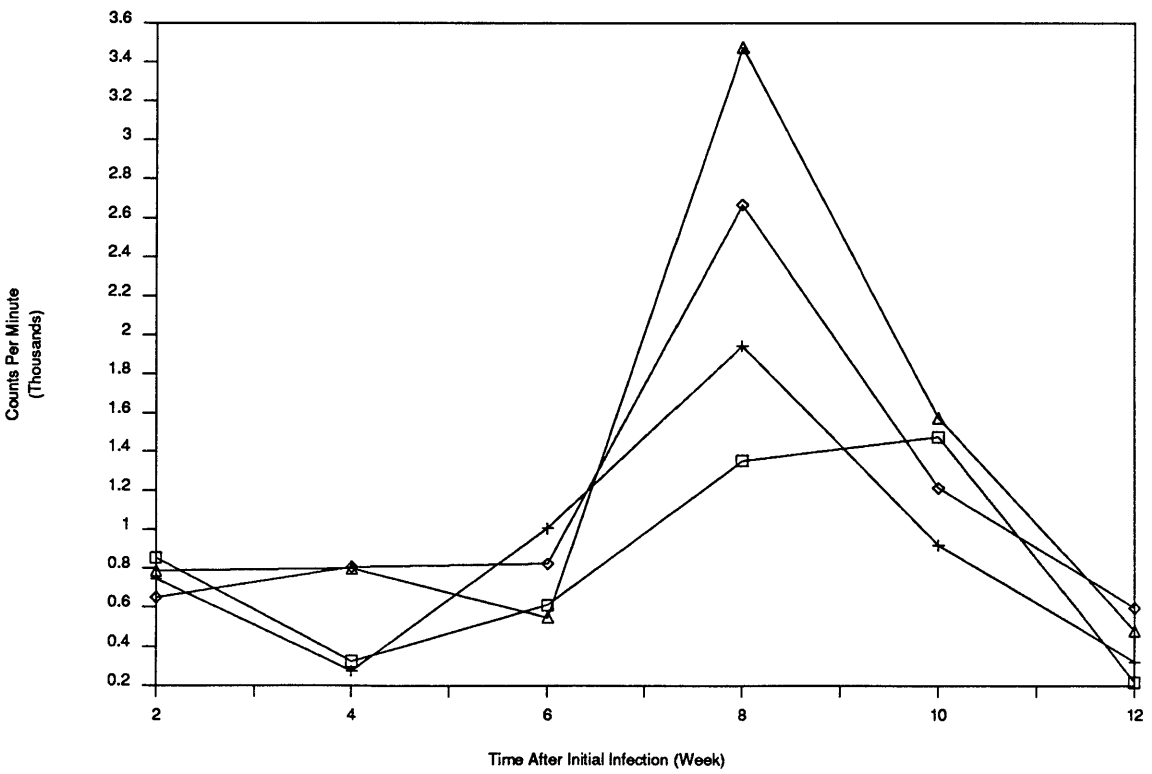


Figure 4.9 e

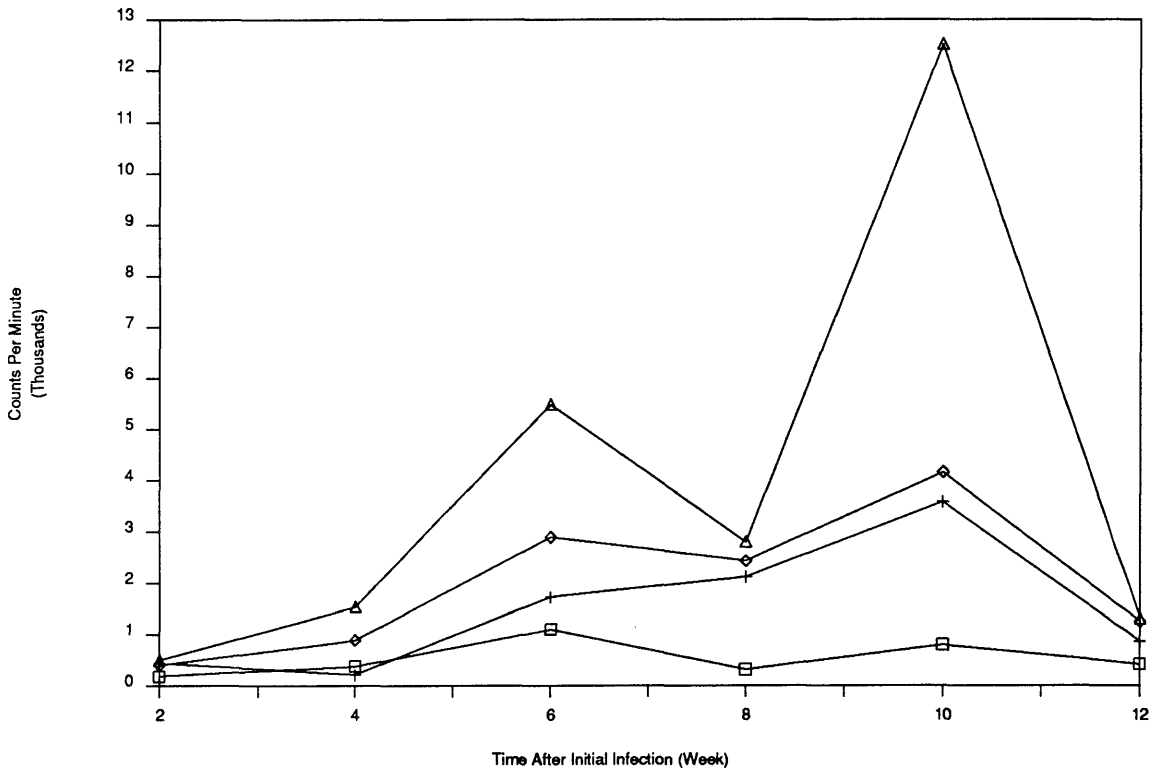
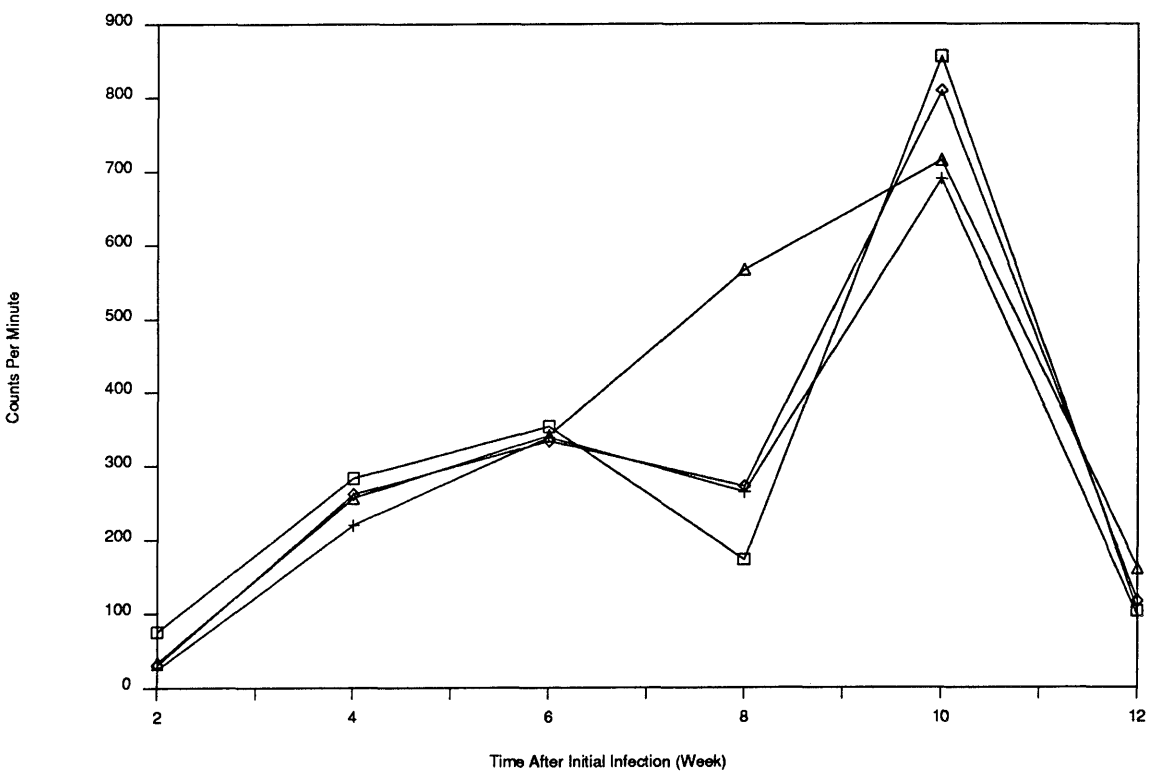


Figure 4.9 f



4.3.3.6e Proliferation considered for each antigen separately.

If the antigen driven proliferation is considered for each antigen separately then a difference in the pattern of response with parasite dose is noted (for analysis see appendix 2.4e).

The L3 homogenate (see figure 4.9e), adult homogenate (see figure 4.9a)and D6L4 homogenate (see figure 4.9c) all induced significantly more proliferation in the cells of infected mice than they did in the cells of naive mice. For all three of the homogenates cells from infected mice proliferated more than did the cells from uninfected mice.

However, it should be noted that although mice may have been given different weekly doses of larvae, this difference was not always reflected in their cell's proliferative ability. When cultured with either L3, adult or D6L4 homogenate, the amount of proliferation shown by the cells from mice given 2 L3/week was not significantly different from the amount shown by cells from mice given 10 L3/week. When incubated with either of the three homogenates the cells from mice given 50 L3/week proliferated significantly more than did the cells from mice given 2 or 10 L3/week.

Cells from mice given 2 L3/week when cultured with either adult ES(MT) (see figure 4.9b) or D6L4 ES(MT) (see figure 4.9d) did not proliferate significantly more than cells from naive mice cultured with the same antigens . When cultured with D6L4 ES(MT), the cells from mice given 10 L3/week did not show more proliferation than did the cells from naive mice. However, on culturing with adult ES(MT) the cells of mice given 10 L3/week did show greater proliferation than did naive mouse cells. When cells from mice given 50 L3/week were cultured with either D6L4 ES(MT) or adult ES(MT), the amount of proliferation shown was greater than that seen when the cells from naive mice were cultured with the same antigens.

4.3.3.7 Proliferation values expressed as stimulation indices.

Analysis of the changes in proliferative ability with larval dose in terms of the stimulation index (Stimulation index (SI)= counts of cells with antigen/counts with cells alone) suggests a different pattern of response than when the changes were analysed in terms of counts per minute. The analysis of these changes is given in appendix 2.5.

The antigens which when cultured with cells from infected mice gave higher SIs than when cultured with cells from naive mice were the L3 homogenate (see figure 4.10e), the adult homogenate (see figure 4.10a) and the adult ES(MT) (see figure 4.10b). Despite this difference not one of the three antigens had a different SI when cultured with cells from mice given different weekly doses.

When cultured with cells from infected mice neither of the two remaining antigens, the D6L4 homogenate (see figure 4.10c) or D6L4 ES(MT) (see figure 4.10d), had SIs greater than when they were cultured with cells from naive mice.

Figure 4.10 *The change in the stimulation indices observed on culturing cells from NIH mice with (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of the spleen cells of mice given repeated low level doses of H. polygyrus. The lines represent the stimulation indices of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.10 a

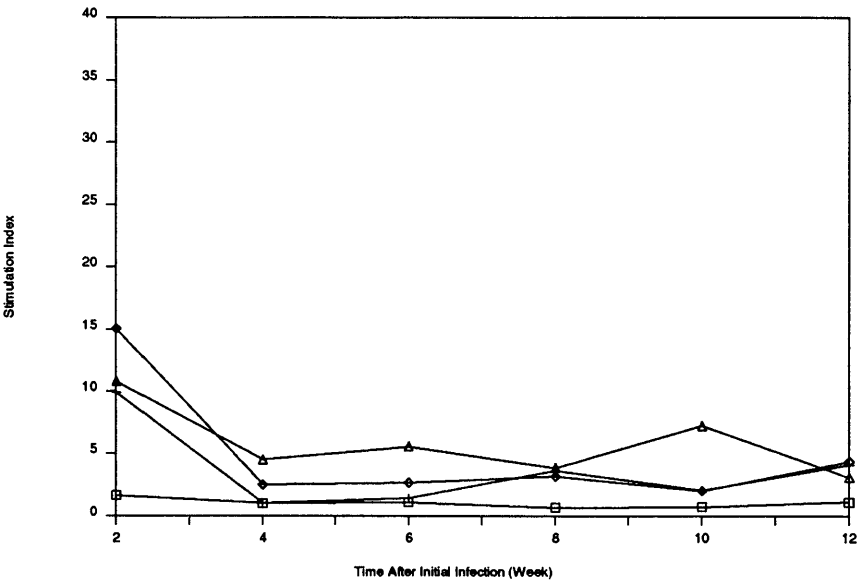


Figure 4.10 b

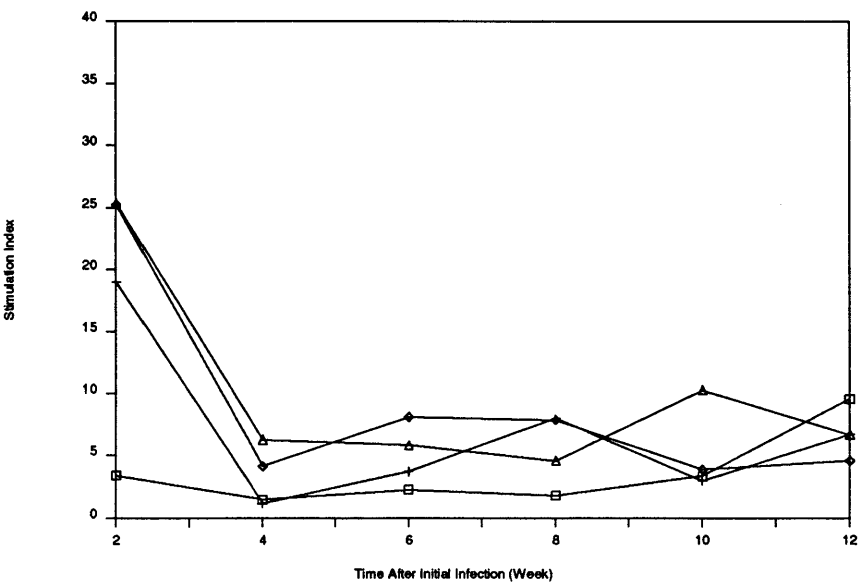


Figure 4.10 c

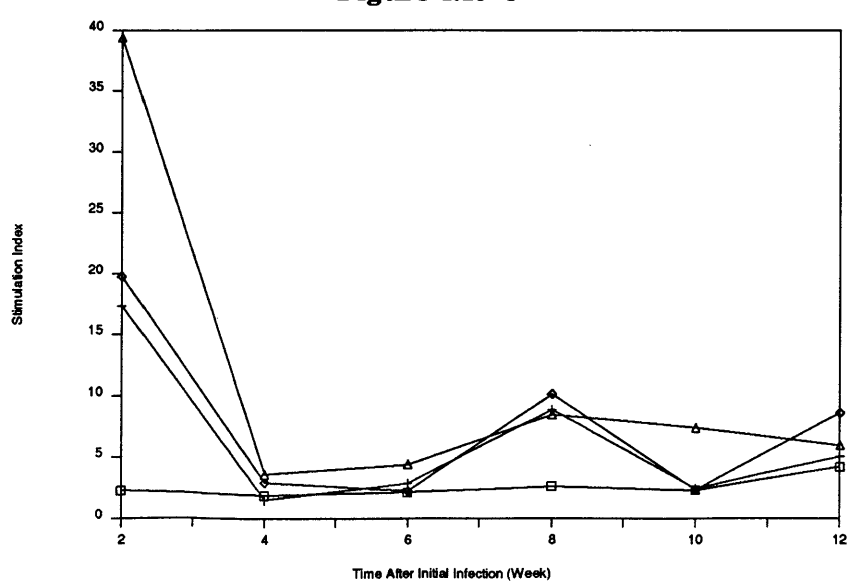


Figure 4.10 d

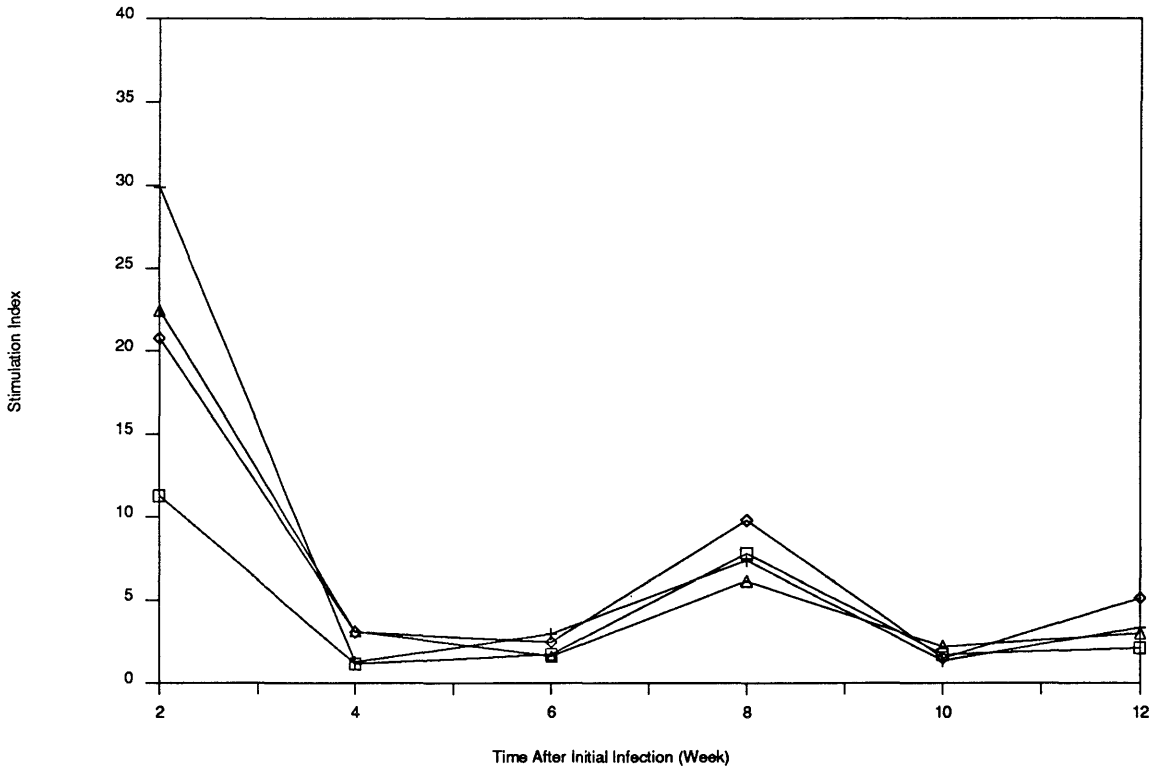
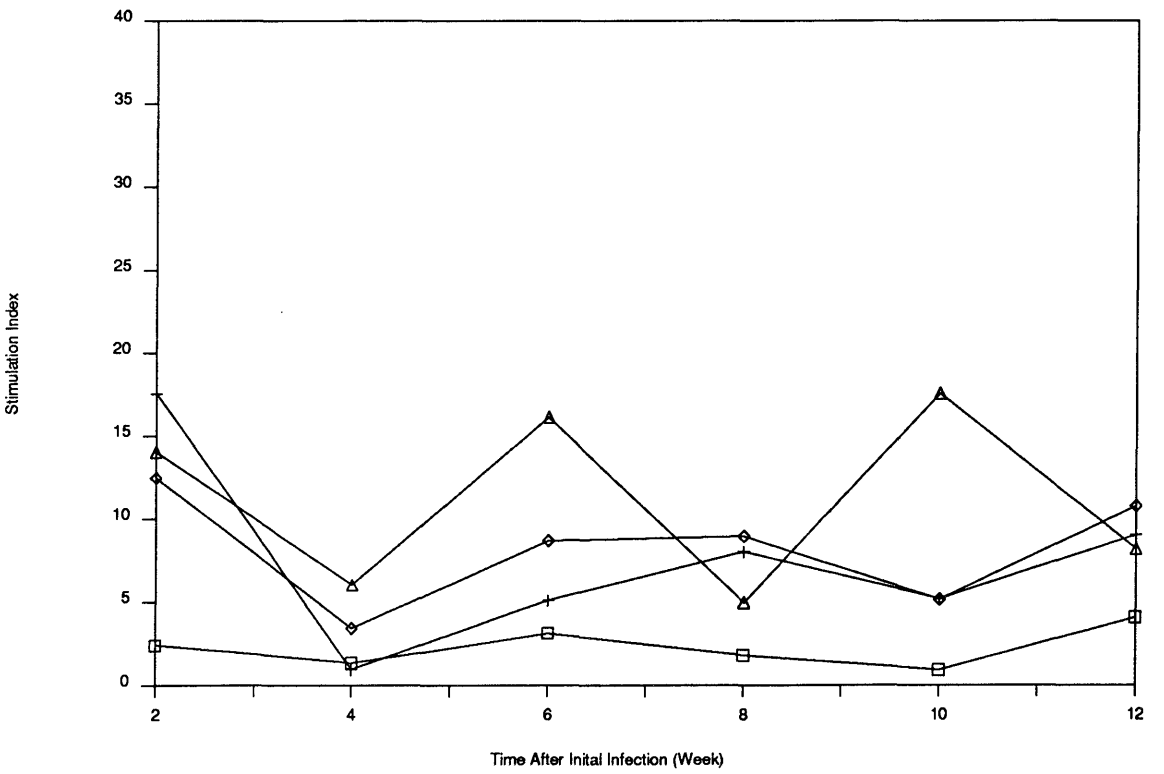


Figure 4.10 e



4.3.4 Repeated low level infections in CBA mice: immunological and epidemiological data

4.3.4.1 Worm burden

The worm burdens of female CBA mice exposed to different trickle infection regimes (shown in figure 4.11) showed similar patterns of change with time. There was an initial increase in worm burden up to the fourth week in mice given 2 or 10 L3/week and up to the sixth week for mice given 50 L3/week. After this initial rise the worm burdens did not increase significantly during the rest of the trickle period. The worm burdens of mice given different weekly doses of the parasite were all significantly different from each other and the larger the weekly dose the larger the resulting parasite burden. The statistical analysis of the worm burdens in CBA mice is given in appendix 2.6a)

4.3.4.2 Sex ratio

The change in sex ratio with time is shown in figure 4.12 and the analysis given in appendix 2.6b. The male to female ratio was not significantly different between any of the doses and neither was the change with time despite a trend towards increasing female bias.

4.3.4.3 Faecal egg output

The observed change in female fecundity (as judged by number of eggs per female per 16 hours) with time was similar to that seen for the worm burden (see figure 4.12 and appendix 2.6c for analysis). The eggs were detected in the faeces of

Figure 4.11 *Worm burden of CBA mice given repeated low level doses of* H. polygyrus. *The lines represent the burdens of mice given: 2 L3/week (square); 10 L3/week (+); 50 L3/week (lozenge).*

Figure 4.12 *The sex ratio (male/(male+female)) of* H. polygyrus *adults in CBA mice given repeated low level doses of the parasite. The lines represent the ratios of mice given the following weekly doses: 2 L3 (square); 10 L3 (+); 50 L3 (lozenge).*

Figure 4.11

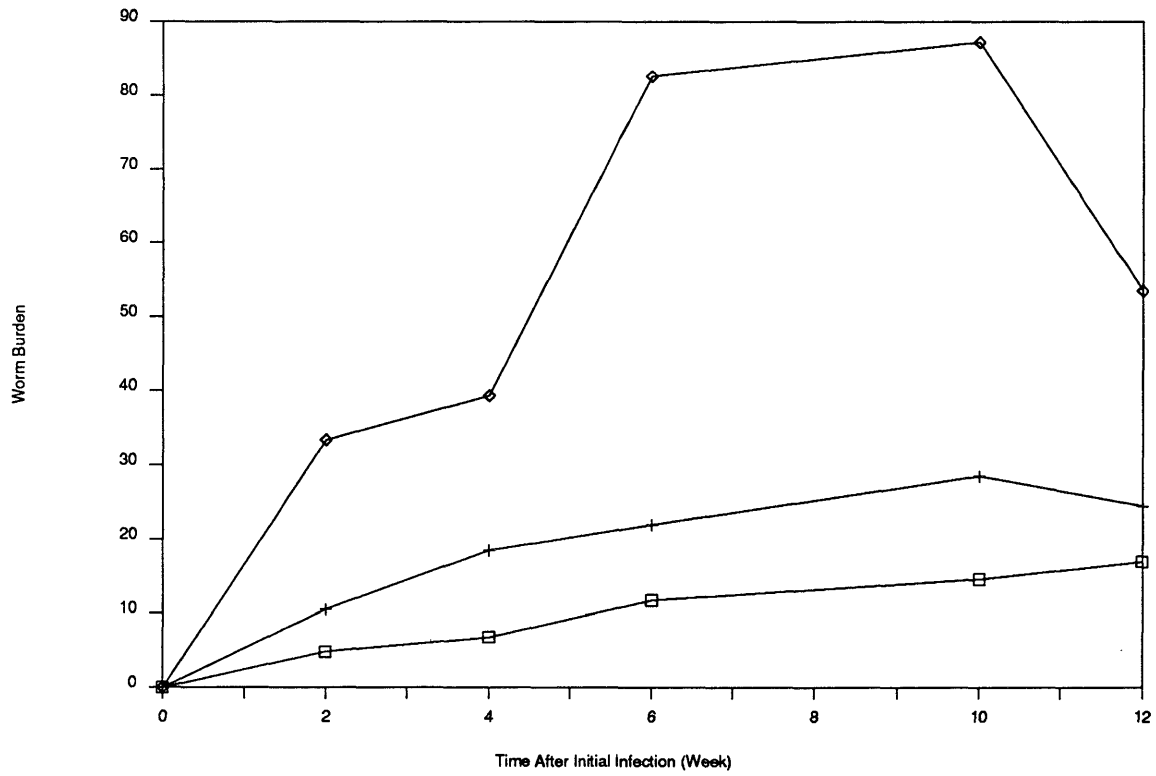
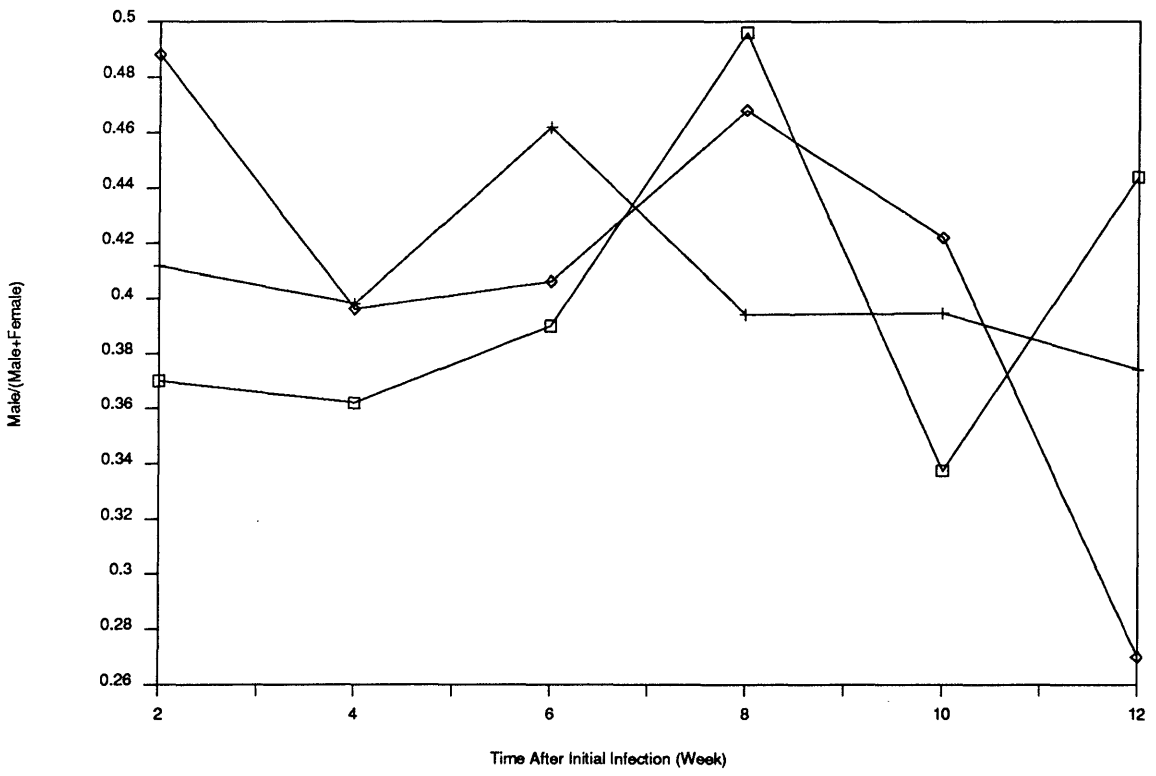


Figure 4.12



the mice on the second week after the initial infection and the numbers of eggs counted rose until the fourth week of infection . After this increase there was no significant alteration in individual female fecundity.

Dosage regimes did have an effect on fecundity as worms from mice given 2 L3/week were more fecund than the worms from mice given 10 L3/week. However, the egg production of worms from mice given either 2 or 10 L3/week was not significantly different from the egg production of female *H. polygyrus* in mice given 50 L3/week.

4.3.4.4 Spleen cell counts

The number of cells in the spleens of uninfected CBA females which acted as age matched controls for the LPA (as judged by the cell counts of single cell suspensions prepared from the organs) did not change during the twelve weeks of the experiment (see figure 4.13). Thus, it is possible to compare the spleen cell counts made at different times of the trickle directly (for analysis see appendix 2.6d).

The mice which had the highest cell counts were those which had received the highest weekly dose of larvae, that is 50 L3/week. The cell counts of the spleens from these mice were all significantly greater than the spleen cell counts of their age matched controls at all times of assaying. The spleen cell counts of mice which had been given the two other weekly doses were not significantly different from

Figure 4.13 *The fecundity of* H. polygyrus *female adults (eggs per female per 16 hr) in CBA mice given repeated low level doses of the parasite. The lines represent the ratios of mice given the following weekly doses: 2 L3 (square); 10 L3 (+); 50 L3 (lozenge).*

Figure 4.14 *The number of cells from the spleens of CBA mice given repeated low level doses of* H. polygyrus. *The lines represent the ratios of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (Triangle).*

Figure 4.13

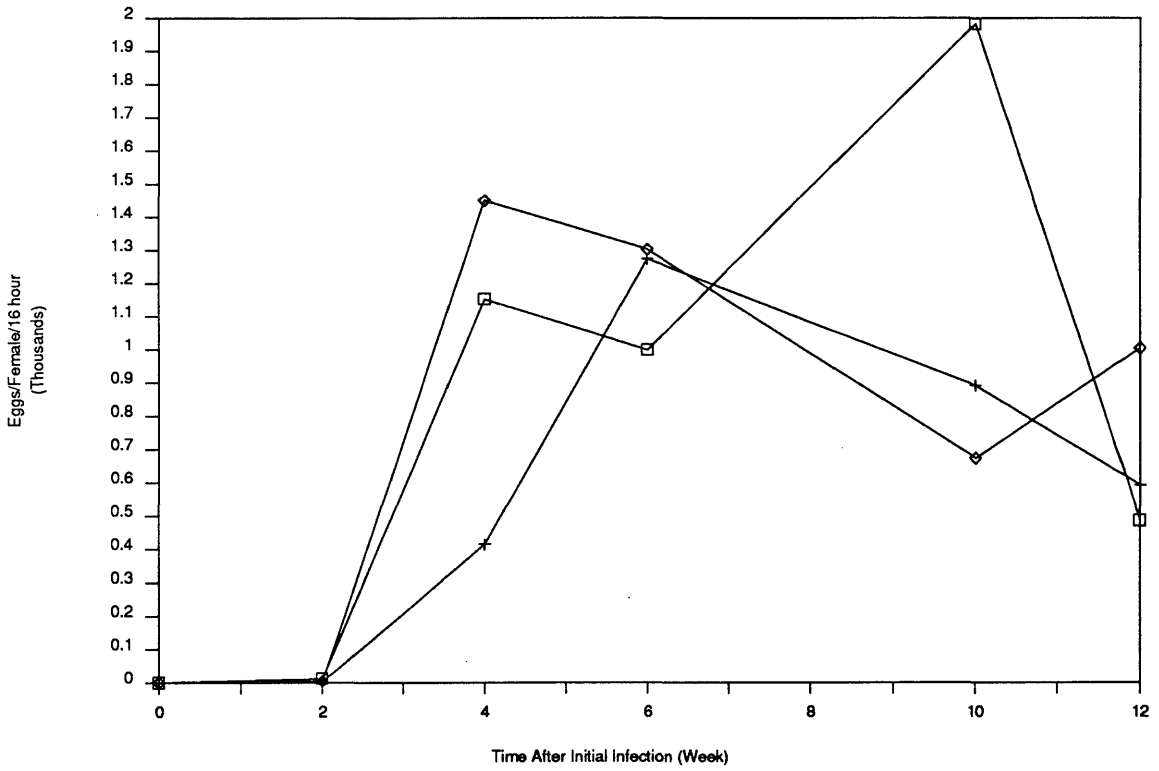
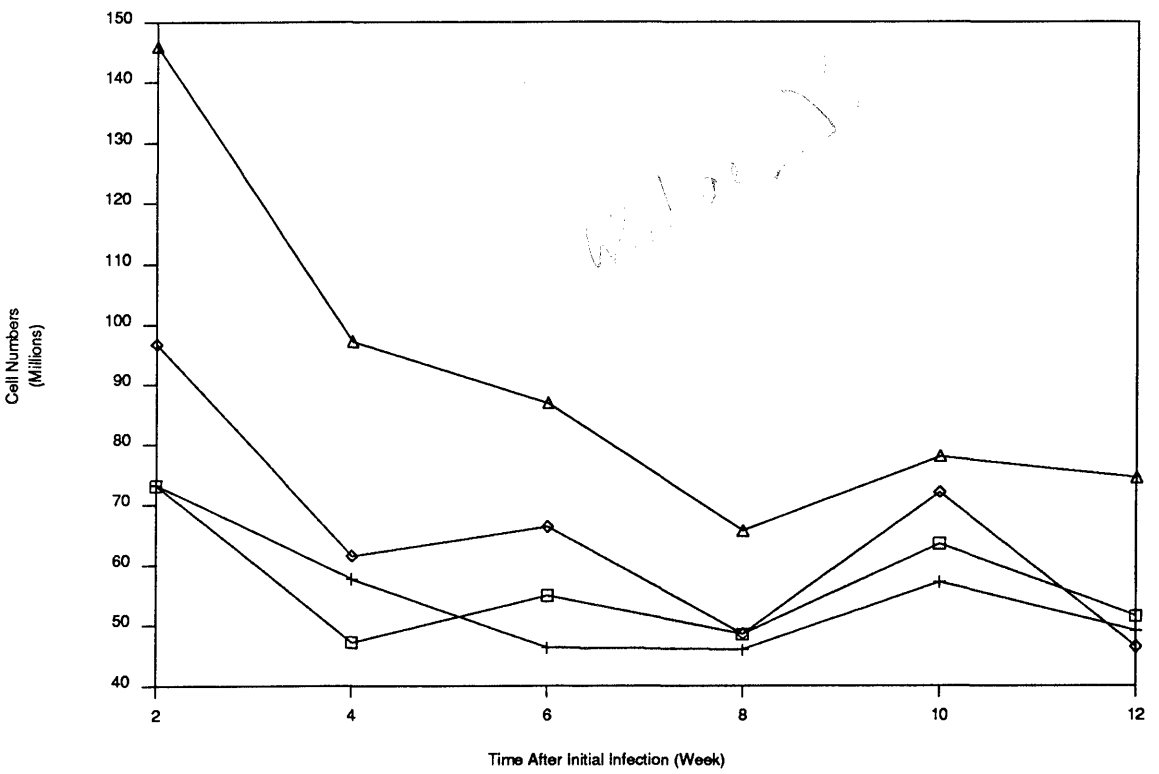


Figure 4.14



the spleen cell counts of the age matched controls. However, there was a trend for the cell counts of mice receiving 10 L3/week to be greater than the counts for mice given 2 L3/week at the earlier times of infection.

For all dose levels, there was a decrease in the spleen cell counts with time. As the cell counts were different from those of the age matched controls only for mice which had been given 50 L3/week then the change is only significant for these mice. However, there is also a decrease in the counts for those mice given 10 L3/week. It is likely that the cell counts for these mice are different from the cell counts of naive mice at the earlier times of assaying but due to the nature of the statistical test used the decrease with time obscures this difference. Unfortunately, it is not possible to test for the difference without increasing the probability of causing a type I error.

4.3.4.5 Immunogobulin levels

4.3.4.5a Total antibody levels

The amount of antibody to the two homogenates, adult and D6L4, was significantly different (see figures 4.13a & 4.13b respectively). However, despite this difference, the changes in antibody with increasing time and different doses were similar. The amount of total antibody was significantly greater in the sera of mice which received 50 L3/week than it was in the sera of mice which received 2 or 10 L3/week. The amount of antibody in the sera of mice which had undergone the two lower dose regimes was not significantly different. For all three doses the

Figure 4.15 *The humoral response of CBA mice. The serum samples from NIH mice were assayed for their total immunoglobulin to (a) adult homogenate and (b) D6L4 homogenate. The lines represent optical densities for the sera of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.16 *The humoral response of CBA mice. The serum samples from NIH mice were assayed for their IgG1 to (a) adult homogenate and (b) D6L4 homogenate. The lines represent optical densities for the mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.15 a

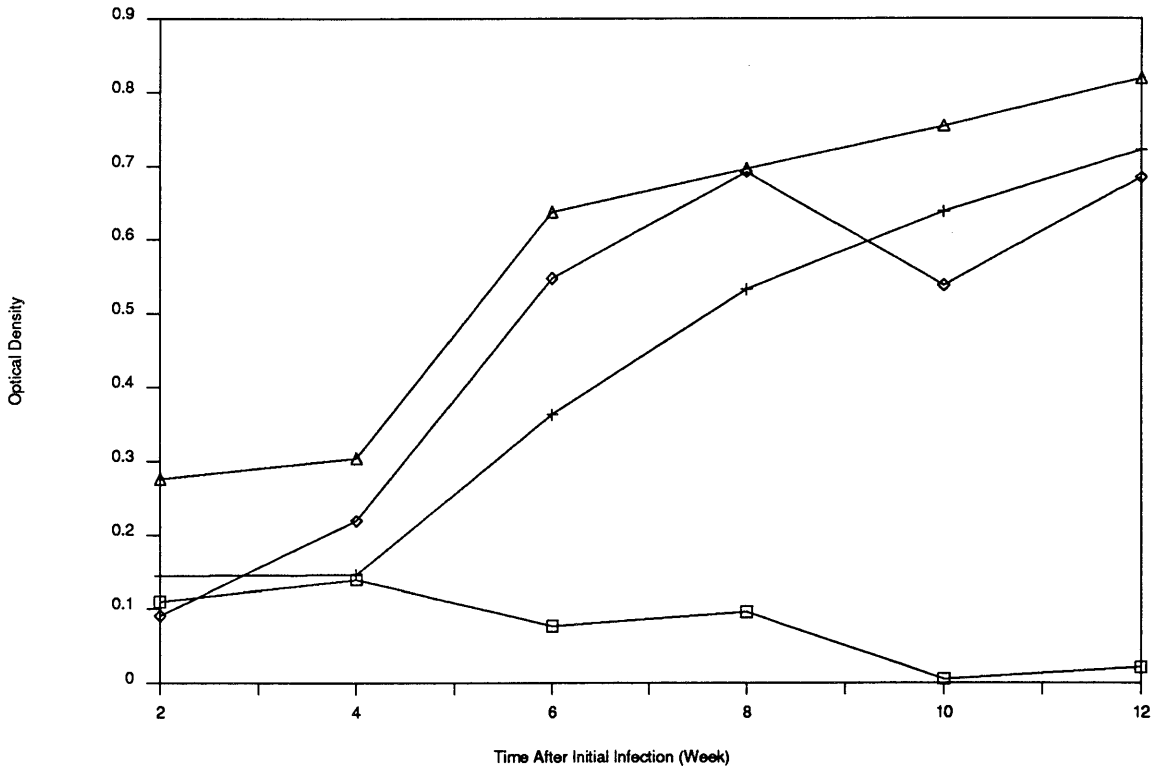


Figure 4.15 b

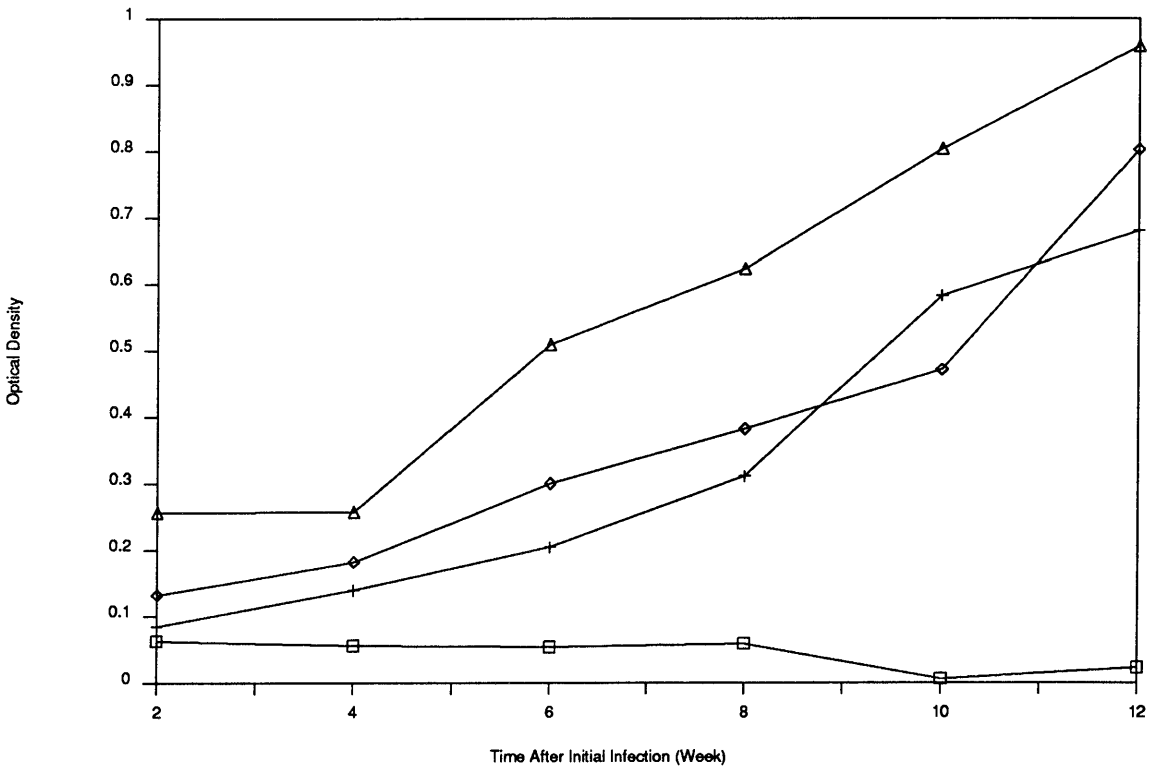


Figure 4.16 a

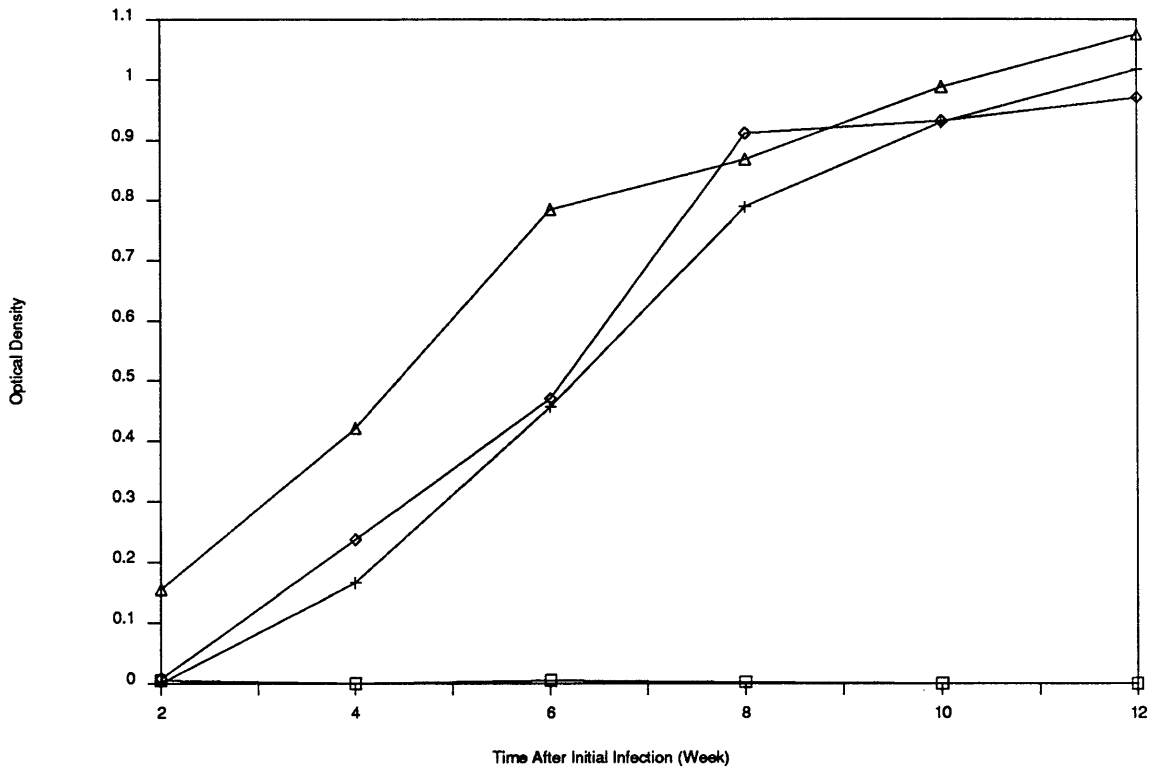
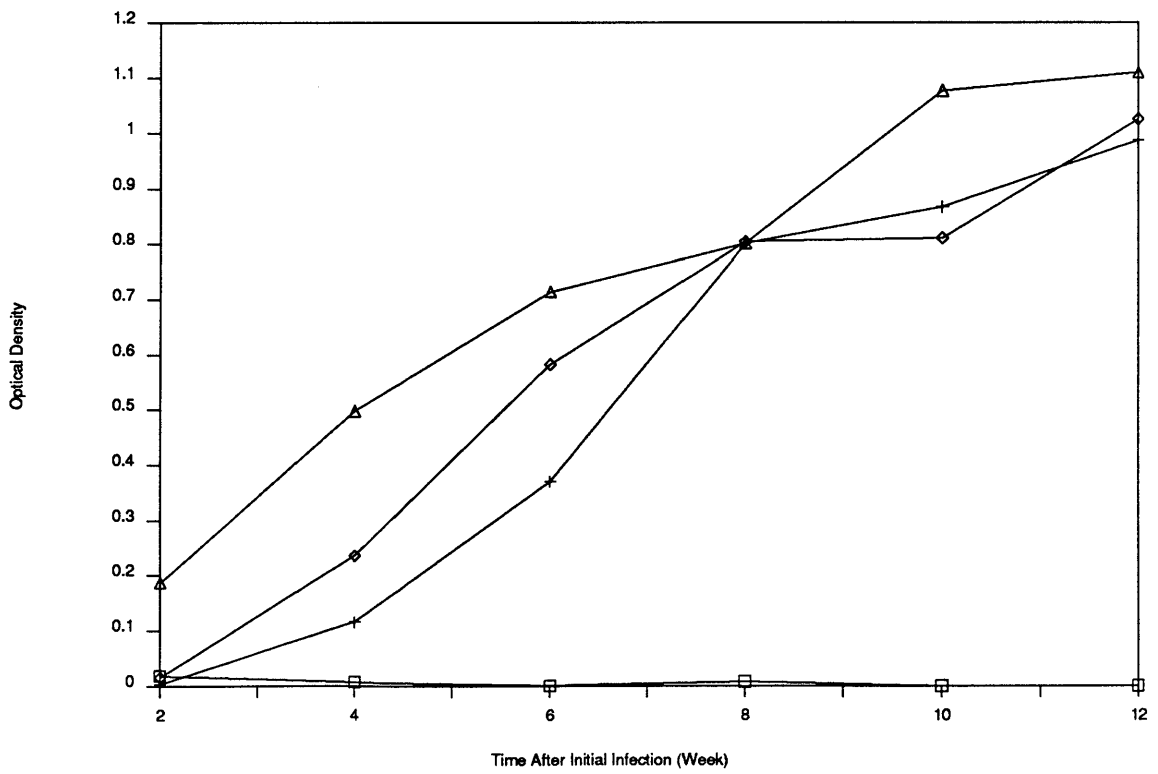


Figure 4.16 b



amount of total *H. polygrus* specific antibody rose with increasing length of trickle. The statistical analysis of the changes is given in appendix 2.7a.

4.3.4.5b IgG1 levels

The amount of IgG1 specific to either the adult or D6L4 homogenate was not significantly different at any combination of parasite dose or time (see figure 4.14a & 4.14b respectively). The amount of IgG1 antibody was significantly greater in those mice which had received 50 L3/week than it was in mice which had been dosed with 2 or 10 L3/week . As for the levels of total antibody, the difference in the amount of IgG1 antibody in the sera from mice given either 2 or 10 L3/week was not significantly different. The statistical analysis of the changes is given in appendix 2.7b.

4.3.4.6 Proliferation of spleen cells: values expressed as counts per minute

The change in the proliferation of cells of infected or naive CBA mice are shown in figure 4.15a-f and the analysis given in appendix 2.8.

4.3.4.6a Non-specific proliferation

When cultured with cells from naive mice all the antigens prepared from *H. polygyrus* caused significantly more proliferation than that observed when the cells were cultured with medium alone (for analysis see appendix 2.8a).

The L3 homogenate had a greater non-specific effect than did the adult homogenate or D6L4 homogenate. There was no difference between the non-specific effect of the D6L4 homogenate or the adult homogenate. There was also no difference in the amount of non-specific proliferation that the two ES(MT) antigens induced. However, the non-specific proliferation the ES(MT) induced was greater than the response their respective homogenates caused.

4.3.4.6b Change in proliferative ability with length of trickle

The change in proliferation with time for cells from trickle infected CBA females was different to the change seen for trickle infected NIH mice. Whereas

with NIH mice the response increased with time, the response of cells from CBA mice increased until the sixth to eighth week of infection before declining.

When cultured with cells from infected mice, each of the antigens caused significantly more proliferation than medium alone. The statistical analysis of these changes is given in appendix 2.8c.

4.3.4.6c Proliferation against each antigen considered separately

The response of the cells from mice to each antigen can be considered separately. Of the five antigens the only one which caused cells from mice given 2 L3/week to proliferate more than when it was cultured with cells from mice given no larvae was the L3 homogenate.

The other antigens only caused significant proliferation (as compared to the response when they were incubated with cells from naive mice) when they were cultured with cells from mice given either 10 or 50 L3/week. When cultured with any of the homogenates the proliferation of cells from mice given 50 L3/week was greater than the proliferation of cells from mice given 10 L3/week. When cells from mice given either 10 or 50 L3/week were cultured with either of the ES(MT)s, no significant difference in the amount of proliferation that each induced was observed. The analysis of these differences is given in appendix 2.6d.

Figure 4.17 *The change in the lympho-proliferative response to (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate , (f) medium only of the spleen cells of CBA mice given repeated low level doses of H. polygyrus. The lines represent the change in proliferation with time for the cells of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.17 a

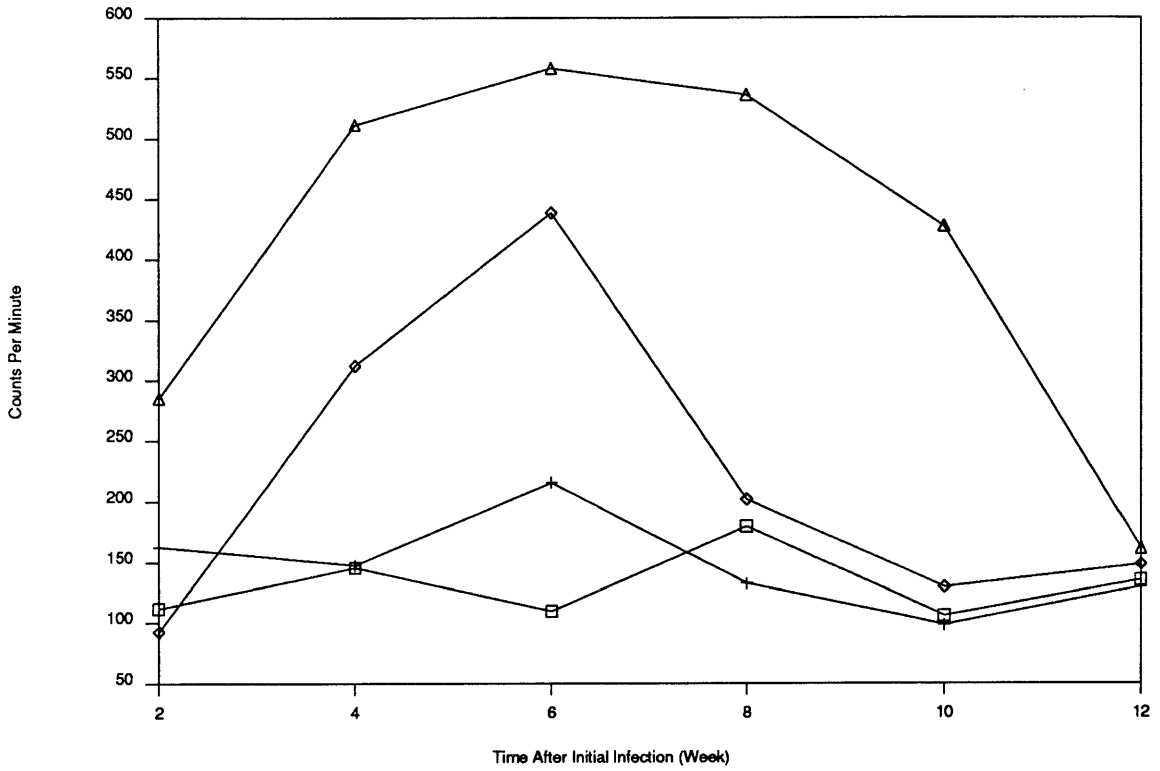


Figure 4.17 b

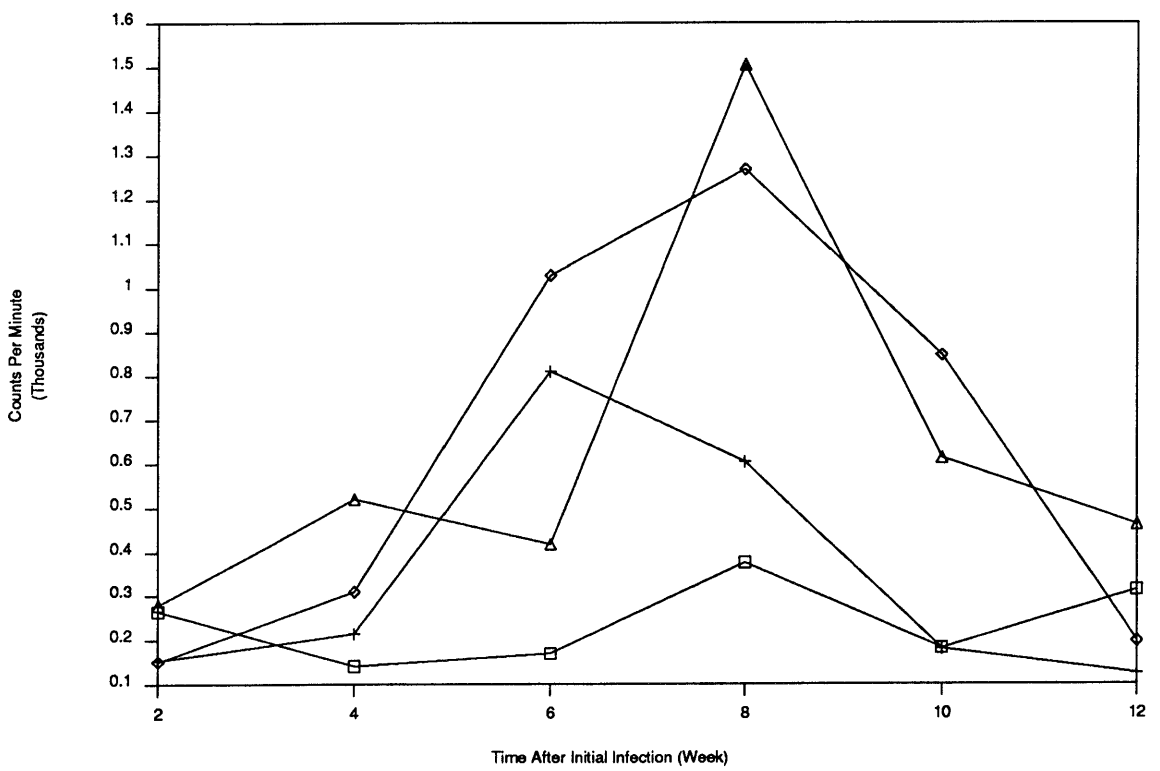


Figure 4.17 c

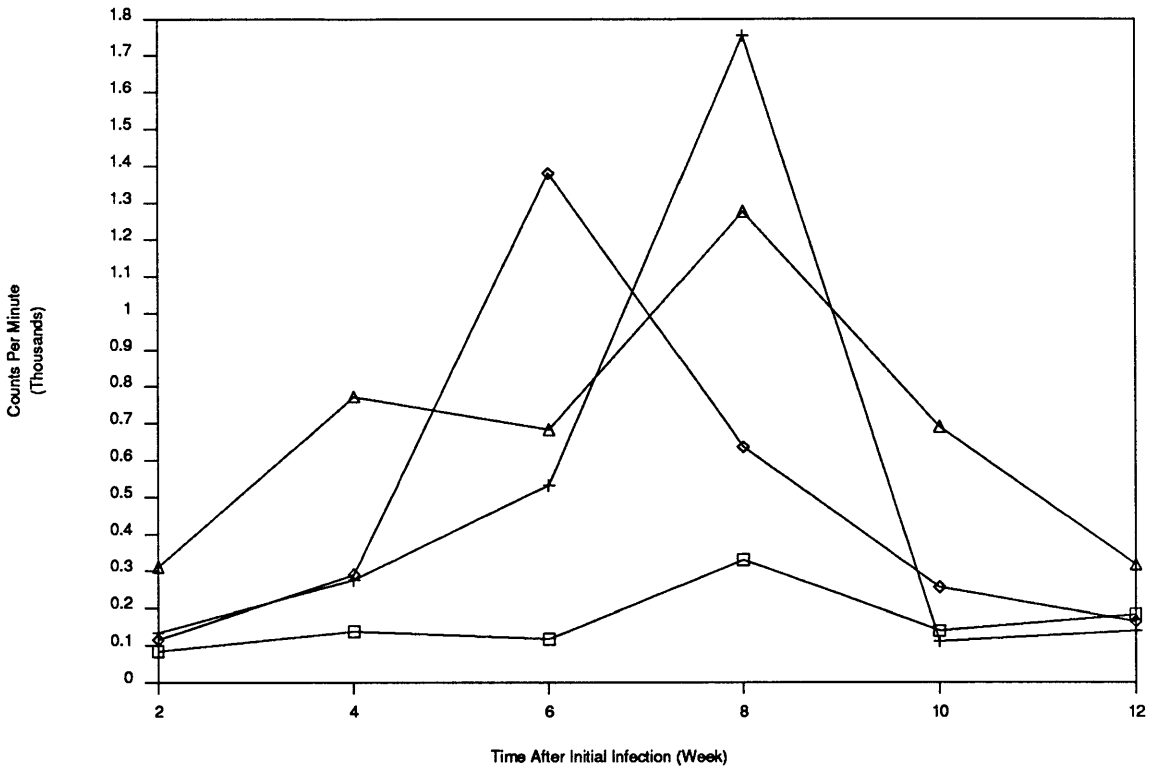


Figure 4.17 d

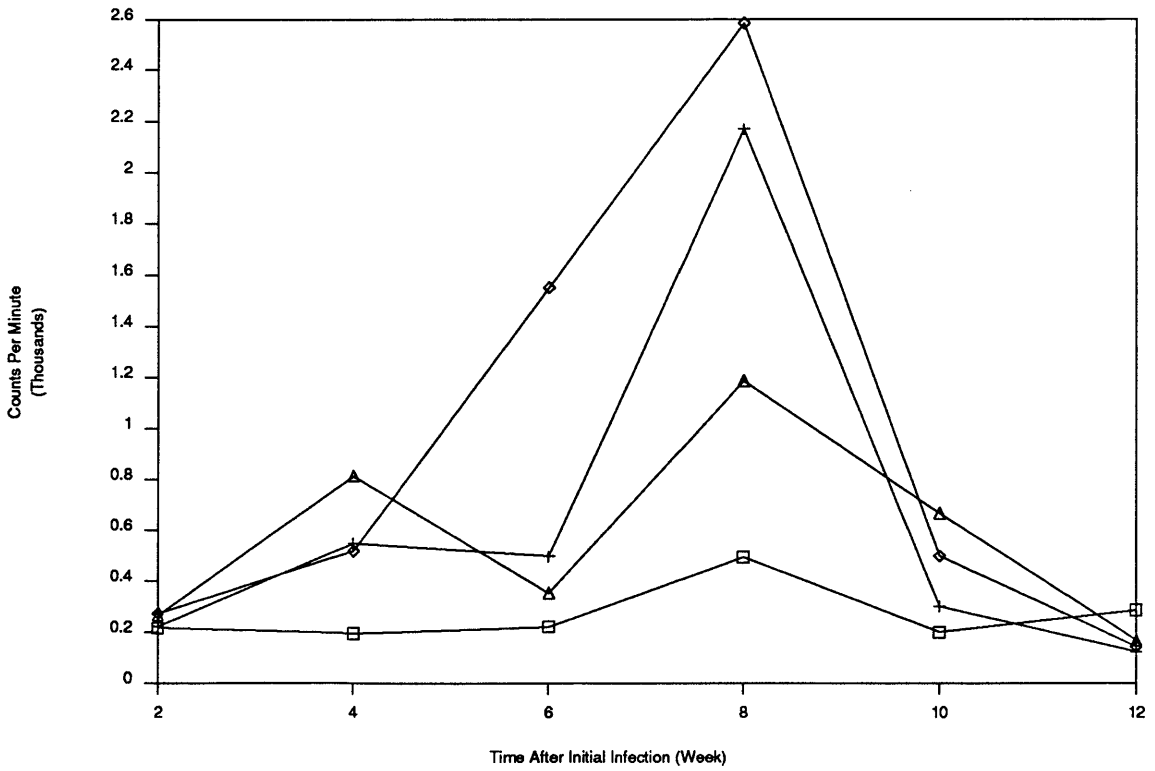
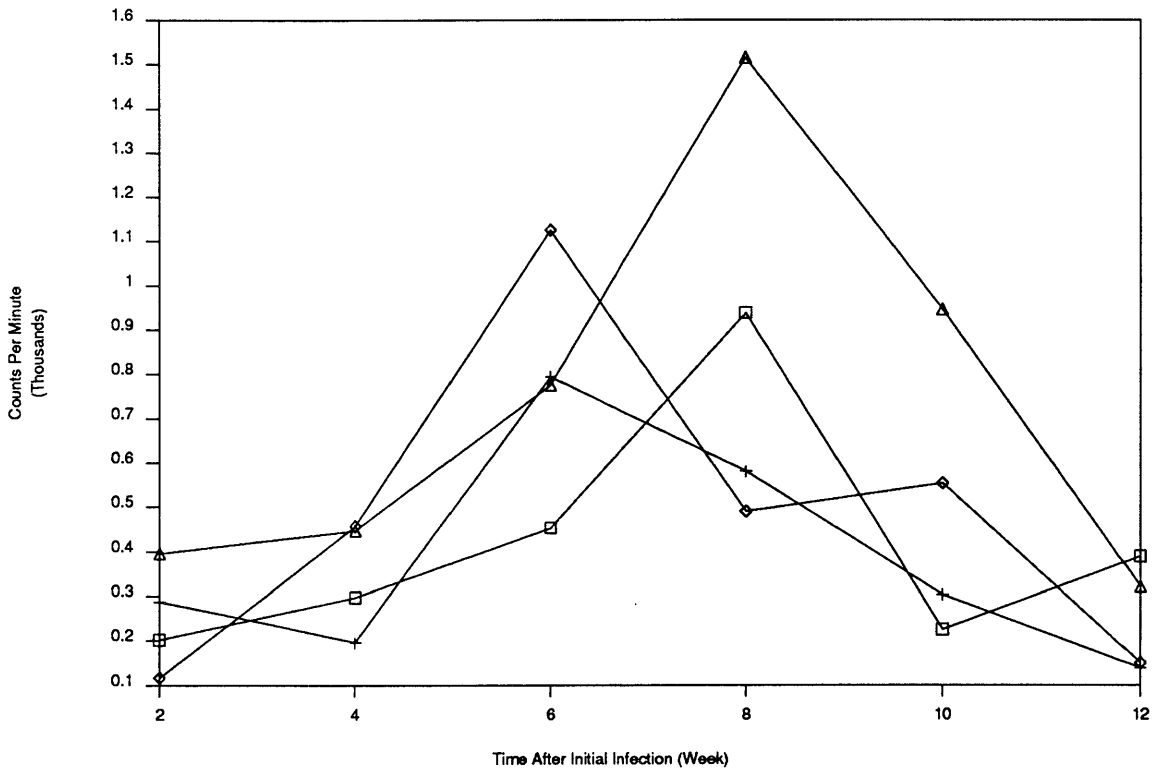
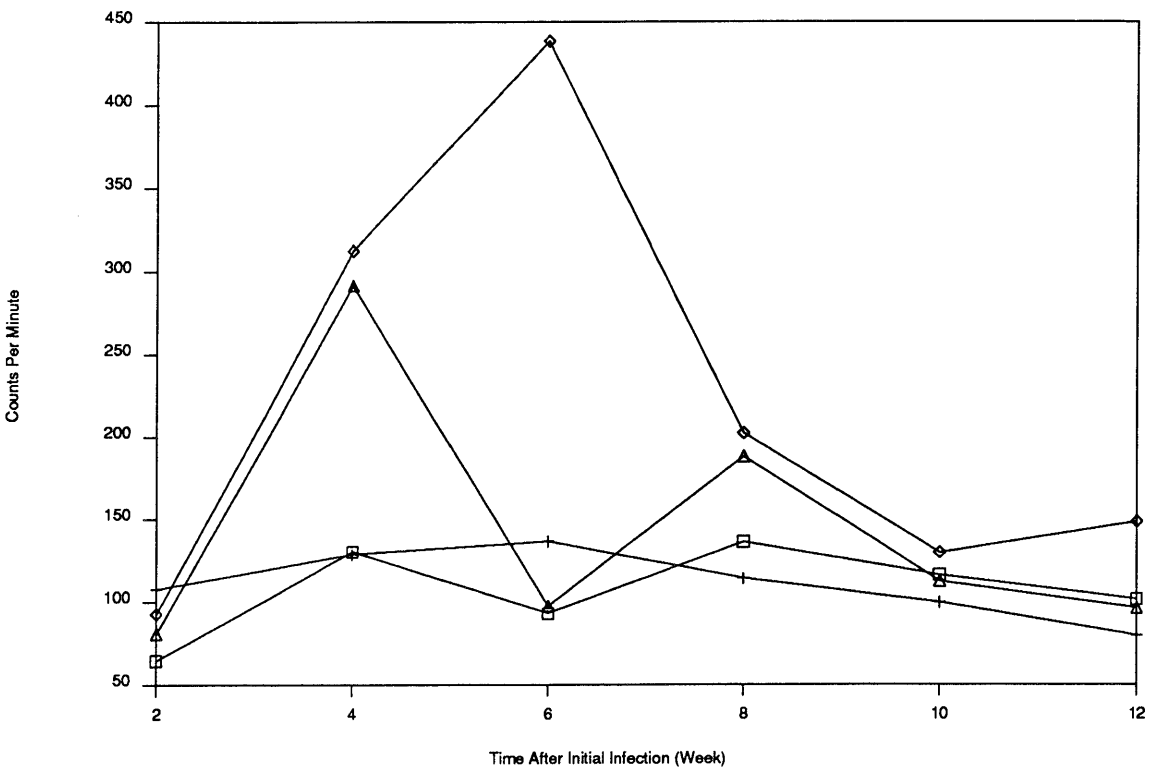


Figure 4.17 e



Figgure 4.17 f



4.3.4.6e Proliferation values expressed as Stimulation Indices

The change in proliferative ability with dose, time and antigen as judged by the stimulation index was different to the pattern observed when the changes are considered in terms of counts per minute.

When cultured with cells from mice given 2 L3/week none of the antigens had an SI greater than when they were cultured with cells from naive mice. It should be noted that when the changes were considered in terms of counts per minute a difference was shown for the L3 homogenate.

A more marked difference between the two methods of data representation was noted when the SI obtained on culturing the different antigens with cells from mice given 10 L3/week were considered. When the counts per minute were analysed, all the antigens caused significantly more proliferation on culturing with cells from mice given 10 L3/week than they did when cultured with cells from naive mice. On considering the changes in terms of stimulation indices (for analysis see appendix 2.9), it was noted that when cultured with cells from mice given 10 L3/week none of the antigens, apart from the D6L4 ES(MT) had SIs greater than they did when cultured with cells from naive mice. It is likely that the observation that the D6L4 ES(MT) had a greater SI when cultured with cells from mice given 10 L3/week than when it was cultured with cells from naive mice

Figure 4.18 *The change in the stimulation indices observed on culturing cells from CBA mice with (a) adult homogenate, (b) adult ES(MT), (c) D6L4 homogenate, (d) D6L4 ES(MT), (e) L3 homogenate of the spleen cells of mice given repeated low level doses of H. polygyrus. The lines represent the stimulation indices of mice given the following weekly doses: 0 L3 (square); 2 L3 (+); 10 L3 (lozenge); 50 L3 (triangle).*

Figure 4.18 a

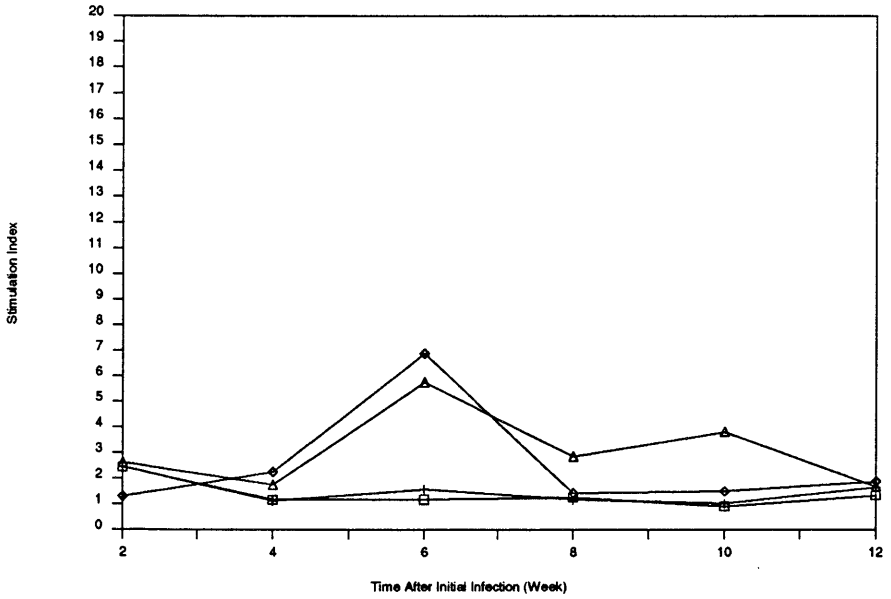


Figure 4.18 b

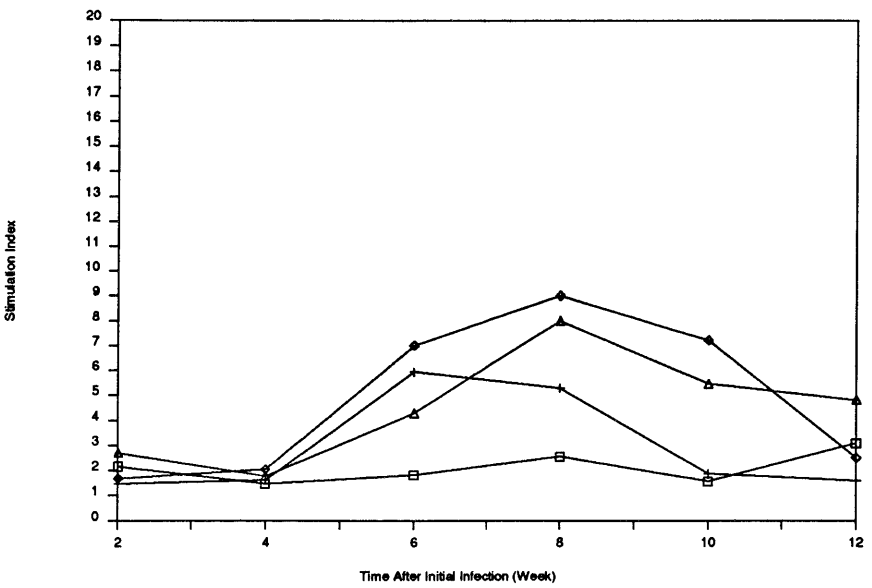


Figure 4.18 c

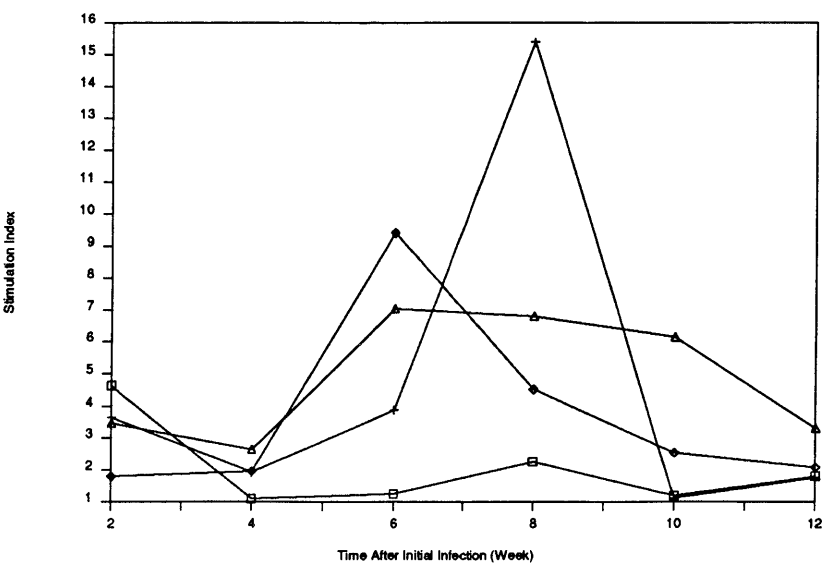


Figure 4.18 d

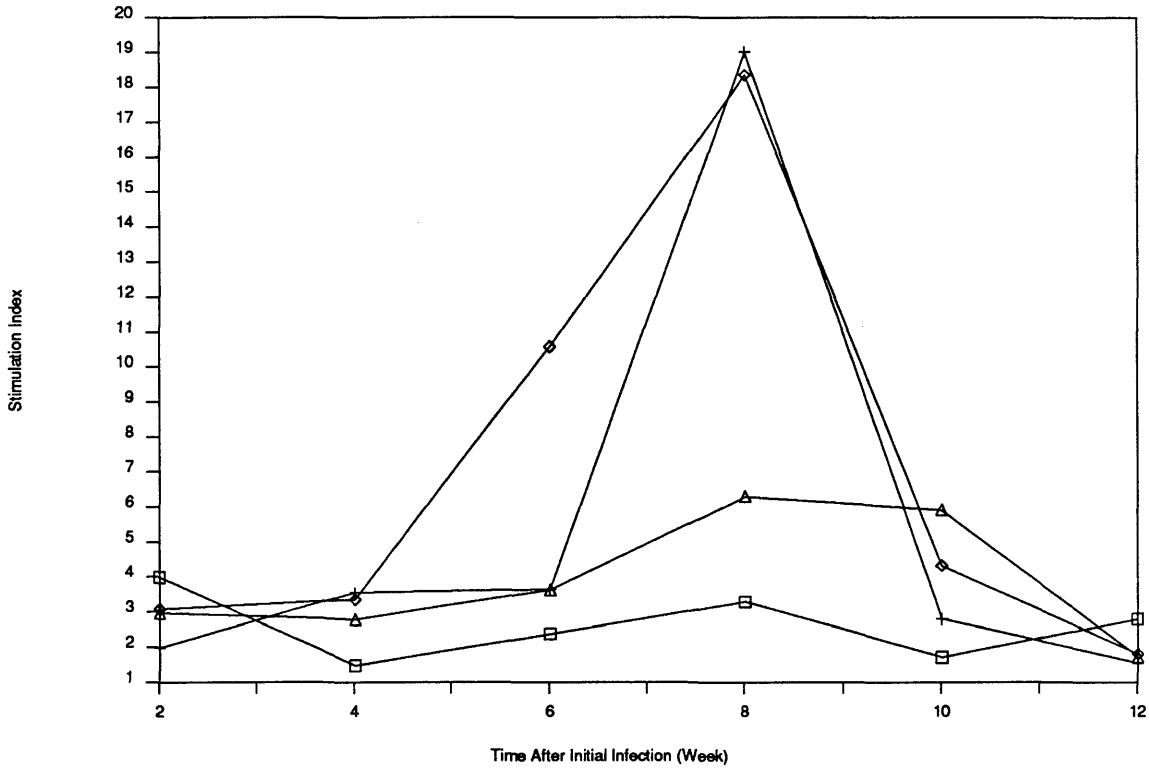
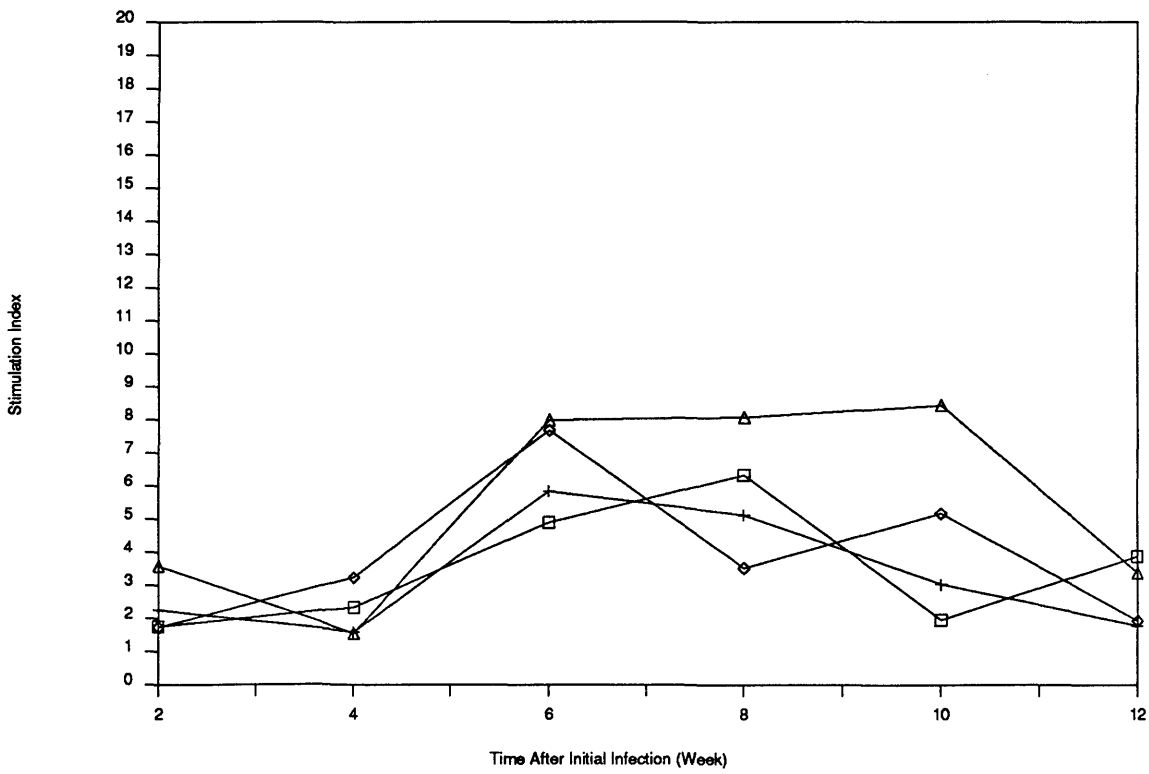


Figure 4.18 e



is an artefact. This is because the SI for the antigen with cells from mice given 50 L3/week was not greater than the response with cells from naive mice.

On culturing the cells from mice given 50 L3/week with either the adult homogenate (see figure 4.16a) or the L3 homogenate (see figure 4.16e) or the adult ES(MT) (see figure 4.16b) the stimulation indices observed were all greater than those observed when the same antigens were cultured with cells from naive mice. Neither the D6L4 homogenate (see figure 4.16c) nor the D6L4 ES(MT) (see figure 4.16d) when cultured with cells from mice given 50 L3/week had SIs greater than when they were cultured with cells from mice given no larvae.

4.5 Discussion

4.5.1 Preliminary assay

The preliminary studies performed to assay changes in cellular proliferation indicated that it was possible to detect antigen specific proliferation of cells from mice given repeated low level doses of *H. polygyrus*. The amount of proliferation was markedly dose dependent. The organ specificity of the reactions and the shape of the antigen dose-response curves was consistent with other experiments in which infections had been abbreviated with anthelmintic. This would suggest that the mechanism of protective immunity (or at least the *in vitro* correlates of it) are similar in mice given repeated doses and in those which have had their infections curtailed by anthelmintic.

The relationship between cellular proliferation and dose was not a linear one. The initial increase in proliferative response with increasing parasite dose indicates that as more larvae are given, more cells respond (recognise) to the antigens of the parasite. This may reflect the requirement for a greater number of effector cells to bring about the rejection/reduction in establishment at the higher doses of the parasite. However, if it was just a simple dose dependent relationship then it would be expected that the cells from mice given 200 L3/week would proliferate most of all. The cells did not and thus an explanation must be sought.

There may have been a higher proportion of antigen specific responding cells in the MLN of mice given the highest dose. Due to the inherent dynamics of antigen-specific cellular responses (Lane *et al.*, 1986) the peak of response may have occurred before the time of assaying in these mice. An alternative explanation to the time dependent antigen dose response curves is that the parasite affects cellular responsiveness. The parasites immunosuppressive capabilities have been reported to be dose dependent (Behnke, 1987) and this immunosuppression may affect the cellular response (Monroy *et al.*, 1989).NB the mice given 200 L3 still showed a significant reduction in worm burden. Thus the

level of response, if it is not an artefact of the assay, is sufficient to have an effect on the parasite.

The amount of proliferation shown to the antigens may reflect their importance as immunogens (although this cannot be stated categorically as the response in the MLN was not considered). It could depend on the inherent immunogenicity of the antigens, which is affected by the proportion of irrelevant proteins (see section 3.7.2; Pritchard *et al.*, 1983; Pritchard *et al.*, 1984c).

4.6 Repeated low level infection: immunological and epidemiological data

4.6.1 Worm burden

The dynamics of the parasite populations are different in the two strains of mice. The immune responses of the two strains also show some differences.

The worm burdens of NIH mice resulting from repeated infection shows a definite peak the size of which is dose dependent as are the rates of parasite loss and gain. The worm burdens of CBA mice also show an initial dose dependent increase but after this increase the number of worms does not increase but remains constant over time. Keymer (1986) gave repeated low level doses of *H. polygyrus* (50 L3 every two weeks) to MF1 mice and obtained a stable worm population which was attributed to the presence of a number of low responder mice in the mouse population.

In the high responder strain, NIH, a single infection of 300 L3 persists for 32 weeks (Behnke and Robinson, 1985), in CBA mice worms survive for eight months before their loss due to senescence (Williams and Behnke, 1983). This suggests that there is little difference in the innate immunity shown by the two stains. Thus the difference between the two strains is likely to be due to differences in the effectiveness of their acquired immune response. Indeed, Enriquez *et al* (1988a)., report that mice differ little in their ability to resist a primary infection but do

show differences on further challenge. The response to the parasite is influenced by both MHC and background genes (CBA mice are H2k and NIH mice are H2q; these phenotypes are common to low and high responders respectively (Behnke and Robinson, 1985); Enriquez *et al.*, 1988b). This difference in resistance may be a combination of the ability of the strain to amount an immune response *per se* as well as the capacity of the strain to overcome the immunosuppressive effects of the parasite (Ali, 1983 in Wakelin, 1986).

The initial rates of parasite establishment in the two strains are similar. This would suggest that the immune response does not affect the parasite at once and that when it does its effects are visible at an earlier time in the NIH strain than the CBA stain. Indeed, Brindley and Dobson (1982) report that on a single infection adaptive immunity does not become effective until about five weeks after infection. It has been suggested that immunity is not stimulated unless certain antigen thresholds are reached. It is also hypothesised that the protective immune response takes time to become manifest as it depends on the quantity and quality of the products of the immune response. The difference between the two strains may be because they possess different thresholds (Cayzer and Dobson, 1983; Monroy *et al.*, 1989)

4.6.2 Sex ratio

It was noted that in both strains the sex ratio was female biased for the parasites and that the ratio did not alter significantly over time. This is accordance with the results obtained by Keymer (1986) who reported a significant female bias and noted that the sex ratio was unaffected by repeated infection. Thus, it is assumed that the effect of acquired immunity on the two sexes is not different.

This observed lack of change on repeated infection is at odds with the work of Dobson and colleagues (Dobson and Cayzer, 1982). They reported that on serum transfer, amongst the many effects of the serum, the male to female ratio was altered. The male worms were more susceptible to immune attrition. Incubation of

L3 in immune serum prior to infection has an effect on the sex ratio, resulting in a female bias (Dobson and Cayzer, 1982). It may be that immunity affects establishment of male and female differently with little effect on their subsequent survival. Adams *et al* (1989) found sex-specific antigens on the surface and in the ES(MT) of *H. polygyrus*. The male parasite has a highly antigenic 66 kilodalton molecule on its surface which is not present on the female. It may function to direct immunity away from the female, allowing it to reproduce and enhance the survival of the species as well as giving rise to the observed bias.

4.6.3 Faecal egg counts

A difference between the two strains is apparent in the fecundity of their female parasites. Not only are there fewer worms in NIH mice but these worms are also less fecund than in CBA mice.

It was noted the effects of acquired immunity were more marked on fecundity than on worm burden. It is unlikely that the difference between the two strains is due to differences in innate immunity. Behnke and Robinson (1985) reported that the faecal egg output of worms in NIH mice was not significantly different from the fecundity of worms in the low responder strain C57BL/10 (Behnke and Robinson, 1985). It is considered, by some, that faecal egg output after a single infection is a good indicator of innate immunity (Brindley and Dobson, 1981).

Thus, not only is acquired immunity affecting the establishment and survival of the parasite, it also has an impact on the parasite's fecundity. This effect is strain dependent and has been reported by others (Brindley and Dobson, 1981; Dobson and Brindley, 1985; Sitepu and Dobson, 1982; Sitepu, Dobson and Brindley, 1985). The more effective acquired immune response seen in the NIH mice causes a decrease in the individual female fecundity just before the last of the worms is expelled.

The effects of acquired immunity on fecundity are probably complex and involve antibody as well as less direct factors such as changes in the host's

physiology. It has been shown that transfer of serum from immune mice to naive mice reduces the fecundity and size of worms in the recipients (Dobson and Cayzer, 1982). Monroy *et al.* (1989) found that on immunization of mice with a 60 k adult *H. polygyrus* antigen worm fecundity but not worm survival was reduced. A similar phenomenon has also been reported by Adams *et al.* (1988). It is hypothesised that this was due to the importance of the molecule in reproduction but not survival *per se*.

The lack of a difference in fecundity of the worms from different doses would suggest that there is no density dependent effect on fecundity. The importance of density dependent effects on fecundity have not been well documented in model systems (Keymer, 1982). Its existence in the field has recently been brought into question by Keymer and Slater (1985). However, Kerboeuf (1980) found that *H. polygyrus* egg production declined with increasing levels of infection. Keymer (1986) provides evidence for density dependent regulation of parasite population growth during repeated low level infection.

4.6.4 Spleen cell counts

The cell counts of the CBA mice given 10 and 50 L3/week were about 2 higher than those of their naive age- and sex-matched controls, decreasing with time to levels similar to those of their controls. This is in notable contrast to the events in the NIH mice. In these mice there was no difference between the size of the spleen of infected and uninfected mice, even though Hagan and Wakelin (1982) have noted that *H. polygyrus* causes a decrease in the cellularity of the MLN.

The changes in the CBA mice are the result of increased cell migration to the spleen and cell division within it. It is probable that B-cells, as well as T-cells, play a part in organ enlargement during *H. polygyrus* infection (Chapman *et al.*, 1979). The involvement in spleen enlargement of both types of cells has been shown in *Babesia microti* infections (Inchley, 1987), *T. spiralis* infections (Ljungstrom, 1980) and *S. mansoni* infections (Chensue and Boros, 1979).

Enlargement of the spleen during *H. polygyrus* infections has been observed by other workers (Liu, 1965; Crandall, Crandall and Franco, 1974; Chapman *et al.*, 1979; Price and Turner, 1986b; Ali and Behnke, 1985). In C57Bl mice the spleen increases in size from 1.7 to $4.4x10^7$ cells after 3 weeks infection with 300 L3 (Price and Turner, 1986b). The strain dependence of the enlargement found here has also been noted by others (Ali and Behnke, 1985). However, Prowse, Mitchell, Ey and Jenkin (1979) noted greater enlargement of the MLN in the higher responder C3H/Hej than in the lower responding CBA/H. This phenomenon has also been shown in *T. spiralis* infections (Wakelin *et al.*, 1986).

The enlargement of the spleen was seen only when the CBA mice were given the two higher weekly doses and it was greater the larger the dose given. This would suggest, in agreement with work by Ali and Behnke (1985), that the spleen only enlarges when it is processing antigen that the MLN cannot as a result of overloading of its processing capacity. The lack of an increase in the size of the NIH's spleen would imply that its MLN is capable of processing the products of infection. However, the lack of enlargement of an organ should not be taken as evidence of its non-participation in the immune response. For example, enlargement was not seen in the spleen but other studies have detected antigen in it (Behnke, 1987), cells that proliferate to *H. polygyrus* antigens (Prowse, 1982; Enriquez *et al.*,1987) and that produce *H. polygyrus* specific antibody can be obtained from it (Chapman *et al.*, 1979) and cells from it can adoptively transfer immunity (Cypess, 1970).

4.6.5 Humoral response

In common with a large number of studies (see section 1.3), it was found that there was a marked increase in the amount of serum IgG1 in CBA and NIH mice infected with *H. polygyrus*. The similarities of the changes in total antibody concentration with those of IgG1 would suggest that the increase in the serum immunoglobulins is largely the result of an increased production of IgG1. This is

especially likely as it has been reported that the concentration of other isotypes do not increase by the same amount (Williams and Behnke, 1983; Chapman *et al.*, 1979).

The increase in *H. polygyrus* specific antibody was the same for both strains and implies that serological status does not necessarily reflect responder status. This result concurs with the work of Brindley and Dobson (1982). They could not differentiate between their high and low responders after a primary infection on the basis of the titre and function of the serum concentration of *H. polygyrus* specific antibody. However, they could detect some differences after repeated infection but these did not correlate with resistance. Sitepu *et al.* (1982) detected higher antibodies in their higher responders than in their lower responders but also suggested that it was the quality of the serum which was important. Prowse *et al.* (1981) also reported that there was no correlation between the levels of IgG1 and the resistance shown by different strains of immunised mice. On multiple infection of a number of strains of mice, Brindley and Sitepu (1982) could not correlate differences in protective immunity with different levels of antibody. A further example of the lack of correlation of antibody level and protection is seen in DBA/2 mice which have high antibody levels yet retain their parasites (Dobson, 1982).

However, Sitepu, Brindley and Dobson (1986) during breeding experiments noticed a relationship between antibody titre and protective immunity and suggested a causal relationship. This relationship did not hold in the later generations of mice and it was suggested that quality became important.

This similarity of the humoral response in different responder strains has also been shown for other parasites (e.g. for *S. mansoni* by James *et al.*, 1988). However, other authors have reported differences in antibody production that correlate with resistance to *H. polygyrus* (Jenkins and Carrington, 1981; Dobson, 1982). A positive correlation between antibody titre and protection has been shown

in Biozzi mice, although the low antibody producing mice did show resistance on repeated infection (Jenkins and Carrington, 1981). Furthermore, Williams and Behnke (1983) found that multiply immunised NIH mice produced less antibody at their last assay period than did the low responders they used. However, at other assay times the NIH mice's production was comparable with or greater than the antibody production of the other mice.

The lack of an observable difference between the two strains may be because of a number of reasons. It may be that the response is an irrelevant one that results from chronic high dose, strong antigenic stimulation, a similar response occurring on exposure to sheep red blood cells. Alternatively, the antigens could act as B-gamma-1 mitogens (Chapman *et al.*, 1979). However, the observation by Pritchard, Williams and Behnke (1982) that IgG1 is host protective indicates that the response is relevant to antigen-specific stimulation not mitogenic stimulation.

It may be that antibody, although important, cannot bring about parasite expulsion alone, a suggestion put forward by Wakelin (1978) and Maddison *et al.* (1979). Another reason may be that only a proportion of the parasite specific antibody is necessary to cause a reduction in worm burden. This supposition is supported by the observation that the antigenic determinants of L3 are quickly saturated when worms are incubated in immune serum. Sera from mice exhibiting different protective patterns reduce the survival of worms which have been incubated in their sera to the same extent (Dobson and Cayzer, 1982).

It is possible that the lack of correspondence between antibody production and resistance is because of the source of antigen used in the assay. Ey (1988) noted that antibody to the internal and cuticular antigens did not retard larval development whereas those to the ES(MT) did. It was suggested that the ES(MT) antigens are essential for survival and that removal of these antigens by antibody is detrimental to the survival of the parasite.

A related point is that only certain antigenic determinants are important (Monroy *et al.*, 1989) but the crudity of the antigens used masks this. Parkhouse and Ortega-Pierres (1984) make a related point: there is turnover of surface molecules which results in their recognition by the host's immune system. The processing of these antigens may reveal epitopes normally hidden in the intact worm. They suggest that this illustrates the problems of the validity of the serological test. Measuring either the quantity or quality of antibody to antigen does not allow rational conclusions to be drawn relating to protection if the assay does not discriminate between antigenic determinants that are hidden or are exposed. One possible way to overcome this problem is to use surface antigens stripped from the nematode epicuticle using the detergent cetyltrimethyl ammonium bromide (Pritchard, Crawford, Duce and Behnke, 1985). It could be that the difference between the two strains lies in their abilities to recognise the full range of antigens, as suggested by Wakelin (1985).

The amount of antibody produced by infected mice differed according to the dose given: the larger the weekly dose, the more antibody was produced. The difference in production may be related to the need for more antibodies to react against the greater number of parasite antigens. It does suggest that the IgG1 production is related to stimulation, although it may not necessarily be related to protection *per se*. Maizels (pers. comm.) suggests that an important function of the antibody is to remove circulating antigen. This notion has also been put forward by Ninneman and Leuker (1974). They hypothesize that one of the functions of antibody is to assist the process of antigen collection and subsequently this leads to the destruction of larvae in the mucosa. Parkhouse and Ortega-Pierres (1984) suggest that antibody to non-cuticular or non-ES(MT) components do not play a part in rejection processes. They must, therefore, have another function.

Neither of the strains produced more antibody to the D6L4 homogenate than to the adult homogenate, which accords with the work of Price and Turner (1986b).

The results would appear to be supported by the work of Pritchard *et al.* (1983). They found that of the IgG1 resulting from an immunising regime, 48% was specific to the adult and the rest expressed immuno-fluorescence against the larval stages.

The similarity in the levels of antibody to the two preparations does not mean that stage specific antigens (shown by Pritchard *et al*, 1984c; Monroy *et al* 1989a, 1989b) are not important immunogens. It may be that the antibodies against one stage are more effective at reducing worm establishment and/or survival than antibodies against another. This may be the case, as sera from immune mice has been shown to react more potently (as judged by immunoprecipitation) against D6L4 surface antigens than against the adult surface antigens (Pritchard *et al.*, 1984c).

The amount of *H. polygyrus* antibody produced increases with time. This increase is greater than that which occurs after a single infection (Pritchard, Behnke and Williams, 1983). This would suggest that it is the continued input of (infective stages of) the parasite that gives rise to the increase. Cross reactivity between the different stages has been reported by Jacobson *et al.* (1982), Pritchard *et al.* (1984), Pitchard (1986), Price and Turner (1986), Monroy *et al.* (1989b). Jacobson *et al.* note that some adult antigens are similar to protective larval antigens in that both are capable of stimulating resistance.

In both strains on trickle infection the amount of adult *H. polygyrus* specific antibody increases in both strains. This happens in NIH mice which have already expelled their worms and in CBA mice from which parasites can still be recovered. Thus, the increase of the anti-adult immunoglobulin is not dependent on the presence of adult worms. This implies that the increase is a result of the cross-reaction between the adult and larval stages or as a result of residual circulating antigen, as reported by Behnke (1987).

4.6.6 Cellular proliferation

The spleen cells of the mice given repeated low level doses of *H. polygyrus* proliferated *in vitro* to the parasite's antigens. The amount of proliferation was dependent on the strain of mice from which the cells were obtained, the antigens with which the cells were incubated and the weekly dose of the parasite given.

The results of the trickle infection are consistent with those of previous experiments which indicate an organ specificity in antigen-responsiveness. It is only the cells from mice given 50 L3/week that show a significant reaction to the D6L4 ES(MT) suggesting that the response is detectable only when the MLN's processing capabilities are overloaded (Ali and Behnke, 1985).

The difference between the two strains can be seen in their responses to all of the preparations of *H. polygyrus* used. The cells from infected NIH mice proliferated more than the cells from infected CBA mice and at lower parasite doses. The difference between the strains is possibly in the threshold of antigen presentation required to induce a (protective) response (Adorini and Doria, 1981; see section 4.6.1) and in this case overcome the immunosuppression (Sitepu, 1985). It may also be that there are fewer or less activated cells in infected CBA mice. As the lower number of reactive T-cells causes a shift in the peak of response to a later time (Sabbe, Meijer and Jansen, 1980), the response of the CBA mice will appear to be lower than that of the NIH mice. Wakelin (1985) noted, as in this study, that mice which responded poorly *in vivo* also responded poorly *in vitro*. For the low responders to achieve levels of responses comparable with those of the higher responder strains more antigen (and a longer period of culture) was needed.

If this is related to what happens *in vivo* then it has been shown that strains of mice most resistant to *T. muris* respond to a lower level of infection than lower responder strains (Wakelin, 1978). It was suggested that the level at which antigen recognition becomes effective may be genetically determined.

The difference in lympho-proliferative ability of the two strains of mice correlates with their ability to expel their *H. polygyrus* infection on repeated

exposure to the parasite. The ability of a mouse to respond to the parasite's antigens is governed by genes of the major histocompatibility complex (MHC) (Wakelin, 1985). Non-MHC genes are thought to influence translation of the lymphocyte mediated response to a myeloid component (Wakelin, 1986; Behnke and Robinson, 1985). There is good evidence for the linkage of a particular HLA haplotype and lymphocyte proliferative responses to the antigens of *S. japonicum* (Wakelin. 1985) and between MHC haplotype and responsiveness to *T. spiralis* (Manson-Smith *et al.*, 1979; Wakelin, 1986). However, it has been suggested that in the case of *T. spiralis* the response status is partly determined by genetic differences in the myeloid compartment (Wakelin, 1986).

Strain dependent differences in the resolution of an infection and T-lymphocyte response have also been noted in experimental leishmaniasis systems (Rossell *et al.*, 1987; Solbach *et al.*, 1987) and in *T. congolense* (Ellis *et al.*, 1987) in a few helminth systems (see Wakelin, 1985; Dineen and Wagland, 1980).

However, it should be noted that strain differences in resistance do not always correlate with *in vitro* measures of immune activity. For example, James, Labine and Sher (1981), Maddison *et al.*, (1979), Lewis and Wilson (1982) reported a lack of any genetically determined differences in parameters of immunity to *S. mansoni*. Using *T. colubriformis* Dawkins *et al.*, (1989) found that they could not differentiate between high and low responders on the basis of their cellular response to egg antigen.

Enriquez *et al.* (1986) found differences in lympho-proliferation to *H. polygyrus* between susceptible and resistant strains of mice. The spleen and MLN cells of the resistant B10.M mice (haplotype $H2^f$) proliferated more than cells from the susceptible B10.BR mice (haplotype $H2^k$). As in this study the fact that the response in infected mice of both strains was augmented relative to naive mice would suggest that the parasite is recognised by both strains. Therefore, the difference in the ability of the two strains to recognise the parasite is probably

quantitative not qualitative. Furthermore, contrary to the suggestion by Pritchard and Behnke (1985), the parasite does not block its recognition by the hosts immune system. The difference in the ability of the two strains to respond indicates that a gene product affects a specific component of immune recognition but differences in non-specific events that the recognition precedes cannot be ruled out.

In this study, as in other studies, there is an apparent association of haplotype and responder status. Generally, high responders to this and other parasites possess the $H2^q$ haplotype (NIH mice are $H2^q$) and the low responders have the $H2^k$ or haplotype (CBA mice are $H2^k$) (Behnke and Robinson, 1985).

How the gene products of the MHC affect responsiveness is not certain. Langton *et al.* (1988) propose that one way in which responder and non-responder strains are different is in the association of antigens with MHC before presentation. This leads to the induction of either suppressor or helper cells. Wakelin (1985) reports that in strains resistant to *T. spiralis* (with the haplotypes b, s, f, g), antigen presenting cells and T-cells express I-A. This would lead to the priming of T-cell help. Expression with I-E, as occurs in low responder mice (haplotypes: k, r, p), would lead to the induction of suppressor cells with the consequent effects on responsiveness (Wassom et al, 1984)

A difference between the two strains was seen in their patterns of responsiveness with time. The amount of proliferation of the cells of NIH mice increased with time for the duration of the experiment whereas the pattern was convex for CBA mice. The continued proliferation of cells from the spleen of NIH mice in the absence of an extant infection suggests that either the cells that react to the parasite are long lived and their population continues to increase in size. Alternatively, and most likely, there is a continued reaction to the larval stages. The increases in response to the adult antigens in the absence of surviving adults suggests a degree of cross-reactivity between their antigens and those of the larval

stages. Cross-reactivity has also been shown in the antibody response (see section 4.6.5) but it does not necessarily mean that the same epitopes are involved (Male, Champion and Cooke, 1987).

The convex pattern of response in CBA mice occurs in the presence of adults and larval stages. This decline in the face of constant antigenic challenge may reflect a change in location of the responding cells. This occurs in *T. spiralis* infected mice, the responding cells are only present in the MLN of the host NIH mice for a short of time (Grencis and Wakelin, 1982). However, unless there is a difference in the cell dynamics of the two strains this is unlikely to be the explanation. Another possible reason for the lowered responsiveness in the CBA mice could be an alteration in the proportion of T-cells in the spleen. Chensue and Boros (1979) have noticed such an alteration during an *S. mansoni* infection, the proportion of T-cells decreasing due to a greater proliferation of B-cells in the spleen. They state that this depression in the proportion of T-cells may have a great bearing on *in vitro* analysis of immune functioning. Such a change in the draining lymph nodes that correlates with susceptibility has been seen in BALB/c mice which become infected with *L. major* (Solbach *et al.*, 1987).

However, Parker and Inchley (1985) have observed that on infection of CBA mice with *H. polygyrus* the proportion of T-cells in the spleen was unaltered. Also in a *T. spiralis* infection where a decreased mitogen responsiveness is seen, there is no alteration in spleen cell numbers. Thus it is likely that the change in responsiveness in CBA mice is due to qualitative differences. Even when changes in subset ratios have been seen they do not always correlate with resistance (e.g in *T. congolense* infection: Ellis *et al.*, 1987).

Pelley *et al.* (1979) suggest that the decrease in T-cell responsiveness during a schistosome infection is because T-cells are prevented from being stimulated by antigen because antibody removes it before it reaches them. However, unless the

antibody affinity for the parasite's antigens is different between the two strains, it is unlikely that this is a relevant explanation for this system.

The suppression of the cellular response in CBA mice with a concomitant increase in humoral response would suggest that immunological tolerance involving T-cells and B-cells is unlikely to be the reason for the chronicity of the parasite infection

It may be that there is an induced hypo-responsiveness to the parasite antigens which is in some way related to the parasite. This lack of responsiveness happens on infection of BALB/c mice with *Leishmania major* (Solbach *et al.*, 1987). There is a cellular response to the parasite only in the initial stages of the infection. This is not the result of a decrease in the number of T-cells as the Con A response is higher in these low responders during this period of hypo-responsiveness than in the high responders (Solbach *et al.*, 1987).

The decrease in response to *H. polygyrus* antigens may be due to immuno-suppression by the adults as Crawford, Behnke and Pritchard (1989) found that adult *H. polygyrus* antigens suppressed an *in vitro* response to heterologous antigens. They demonstrated a population of suppressor cells in the spleen and by using anti-lymphocyte serum with complement showed that the cells involved did not possess the T-cell phenotype. Price and Turner (1986a) report that there is direct evidence which suggests that regulatory T-cells contribute to the depression of homologous proliferative responses in sheep infected with *H. contortus*. However, they note that in other systems the suppression of lymphoproliferative responses is likely to be mediated by adherent suppressor cells and serum factors.

The similarity of the humoral response in the two strains yet their different cellular responses would suggest that there is a dissociation between the genetic control of each response. However, it need not be the cellular response which is different between strains. For example, two strains of Biozzi mice differ markedly

in their antibody response to most antigens, including those of *H. polygyrus* (Jenkins and Carrington, 1981), but differ in their ability to mount T-cell mediated responses (Adorini and Doria, 1981). This difference was attributed to differences in antigen processing by the macrophages of the two strains.

Stavitsky (1987) has suggested a mechanism for the dichotomy of the humoral and cellular responses in a *S. japonicum* infection. The hypothesis may explain the increase in antibody production with the concomitant decrease in lympho-proliferative ability seen in this study for CBA mice. The phenomenon in which one response is down-regulated whilst another is not is known as immune deviation. It is suggested that there are two subsets of T-cells. One is Th-1, upon stimulation by antigen these produce IL2 and gamma interferon which mediates cellular immunity to the antigen. The other type is Th-2 which when challenged with antigen produce IL4 (B-cell growth factor which mediates production of IgG1 and IgE as well as the production of autocrine growth factors on some T-helper cells and the enhancement of Ia expression on B-cells).

Stavitsky postulates that during an infection the function of the Th-1 subset is diminished and that of the other subset continues or is enhanced. The mechanism whereby IL2 production is turned off has not been elucidated but the phenomenon has been shown to occur in other infections (Solbach *et al.*, 1987; Kierszenbaum, Sztein and Beltz, 1989) and a review of the functional heterogeneity of CD4+ T-cells in leishmaniasis has been given by Liew (1989). Explanations as to the mechanism have included decreased production and/or increased binding and utilisation of IL2 receptors; lack of Th-1 cells; defects in the function of the Th-1 subset,; active suppression by suppressor cells; or defects in the antigen presenting cells (Stavitsky, 1987).

There is a dissociation between the humoral and cellular responses, which has been noted for other parasite systems (e.g. *S. mansoni* in rhesus monkeys: Maddison *et al.*, 1979; Pelley *et al.*, 1979), and a correlation between the cellular

response and responder status. However, this does not mean that only the one response is important or that other genetically determined differences (such as differences in the eosinophil response: Wakelin and Donachie, 1983) do not play a part in determining responder status. Indeed, there is evidence that two or more components involving antibody and thymus dependent lymphocytes must act to bring about the expulsion of parasites such as *N. brasiliensis*, *T. spiralis* and *T. muris* (Behnke and Parish, 1979). In the case of *H. polygyrus* the difference between the two strains may be due to the differential efficiency of the T-cell mediated response which acts in conjunction with antibody, a suggestion also put forward by Williams and Behnke (1983) and Dobson (1982)

4.7 Summary

This chapter details the changes in parasite population dynamics and host immune responses on repeated low level infection with *H. polygyrus*.

The results are consistent with those of the previous chapter in that the organ specificity of the response is maintained and the amount of proliferation caused by each antigen is different.

On repeated infection the change in worm burden over time for the two strains is very different. The pattern for NIH mice is convex, whereas for the CBA mice the worm burden increases and then plateaus. For both strains the size of the maximum worm burden is dose dependent. A difference between the two strains is also evident in terms of their worm's fecundities, the CBA's parasites being the most fecund. The sex ratio of both strains is female biased.

As the two strains do not differ in their innate immunity, the observed differences are likely to be due to their abilities to mount an effective immune response against the parasite and its products. The amount of T-cell proliferation shown by the two strains is different, as is its change with time. The level

increases with time for NIH mice whereas the pattern of response of infected CBA's cells is convex. The difference between the two strains is not reflected in their humoral responses. This would suggest that the humoral and cellular responses are dissociated and that high antibody response cannot be used to determine responder status, although it can be used as an indicator of helminthiasis. It should be noted that the antibody produced does play a part in the protective immune response. The correlation between the cellular responsiveness and the persistence of the parasites would suggest that the *in vitro* response could be used as an indicator of response status even though it is unlikely that the T-cells are the ultimate effectors of immunity. It should be stressed that this study does not rule out the possibility of genetically determined differences in the effectiveness of non-specific effector cells.

Both the T-cell and antibody assays indicate that there is cross reactivity between the antigens of *H. polygyrus*.

Chapter 5: Repeated low-level infections abbreviated by anthelmintic

5.1 Introduction

The effect of chemotherapy on *H. polygyrus* acquisition in CD1 mice infected with larval doses of increasing size was investigated by Kerboeuf and Jolivet (1984). Apart from this study, little work has been done on the effect of chemotherapy on the dynamics of *H. polygyrus* in mice subjected to repeated low level infections.

The approach of infecting mice with *H. polygyrus* and then abbreviating the infection with anthelmintic followed by re-exposure of the host to the parasite mimics in part the control strategy of mass chemotherapy in human or farm animal communities. Despite the practical relevance of studies on reinfection there has been surprisingly little systematic field research on the subject (Wilkins, 1989). The pattern of *H. polygyrus* re-acquisition in laboratory mice may give some insight into the pattern of worm re-acquisition in human communities and this may be especially relevant as knowledge of population based drug application to control helminth of veterinary and medical importance is limited (Anderson and Medley, 1985).

The effect of drug treatment on development of resistance and on immune changes can also be investigated. This is especially relevant in the case of *H. polygyrus* infections as chemotherapy removes the immunosuppressive adults (Behnke and Robinson, 1985; Kerboeuf and Jolivet, 1984).The development of resistance to *H. polygyrus* after anthelmintic abbreviation of an infection is strain dependent and is thought to be linked to the haplotype of the mouse (Behnke and Robinson, 1985). It has been shown that CBA/j (and BALB/c) mice develop little resistance after a nine day infection whereas NIH and (LAJ/f1) mice are very good

responders. Thus it may be possible to correlate the different levels of resistance with *in vitro* measures of the immunological response.

This chapter describes the results of the experiments that were used to investigate the changes in parasite acquisition and host responses that occur after drug treatment. The use of two types of responder aids identification of those responses that correlate with resistance.

The chapter describes the results obtained on interrupting a trickle infection with ivermectin and then either re-exposing the host to the parasite's infective stages or ceasing dosing with the parasite. The parameters measured to assess the parasite's population dynamics were the worm burden, parasite sex ratio and egg production. The host's functioning was assessed by measuring immunoglobulin production, the number of cells in the spleen and the *in vitro* proliferative response to preparations of the parasite antigens. These results are then discussed with particular reference to the difference in reacquisition and response of the two mouse strains

5.2 A caveat

Interpretation of the results from these immunological and epidemiological experiments was made difficult as subsequent to the start of the experiment it was discovered that the mice were infected with Mouse Hepatitis Virus (MHV). The mice showed the symptoms of MHV infection (weight loss and paresis: Cunliffe-Beamer and Les (1987)) and on analysis of the sera from some of the infected mice antibodies to the virus were detected (serological analysis was performed by J.F. Needham Ltd. UK).

The mice infected with the virus were obtained from an external supplier of livestock. Mice obtained from the animal breeding unit at Imperial College showed no symptoms of the disease and no antibodies to the virus were detected in their sera. The extent of the infection in the imported mice was not known but its

method of transmission (it is spread orally (J. F. Needham pers. comm.) and in droplets (G. Childs pres. comm.)) coupled with the method of *H. polygyrus* infection (intubation by gavage tube) makes it likely that all the mice given the parasite would also have become infected with the virus within a short space of time.

The virus is a member of the hepatoencephalitis group (Hoag and Meier, 1966) has been known to be a common contaminant of mouse stocks (Carthew, 1978). It is an example of a virus whose clinical effects are influenced by the strain and age of the mouse and the strain of virus itself. Some of the strains may be enterotrophic and neurotrophic. The enterotrophic effects are responsible for diarrhoea and high mortality in pre-weaners (Carthew, 1978) and the neurotrophic effects of the virus are manifested clinically as wasting, depression and paralysis (Cunliffe-Beamer and Les, 1987). Another group of strains are of low virulence and infections are inapparent unless the mice are exposed to factors that modify their responses (Cunliffe-Beamer and Les, 1987). Amongst the factors which enhance the disease caused by the low virulence strains and potentiate the effects of the more virulent strains are irradiation, cyclophosphamide and cortisone (Cunliffe-Beamer and Les, 1987) and, more importantly, the immuno-suppressive parasite *H. polygyrus* (J. F. Needham pres.comm.).

Even in mice with inapparent infections the virus causes altered immunological responses compared to uninfected mice (Kraft (1982) cited in Cunliffe-Beamer and Les (1987)). The altered immune response may be because in the spleen of normal (non-nude) mice the virus can cause degenerative and necrotising lesions. These lesions decrease in size and are eventually resolved in immunologically intact mice (Tamura, Taguchi, Ueda and Fujiwara, 1977). If infection with *H. polygyrus* causes these lesions to persist then as in nude mice this damage could exhaust resistance. It is also interesting that James (1987) suggests, with little supporting evidence, that latent viral infections in mouse colonies may affect immunological resistance. Resistance may also be affected by

the pathological lesions that the virus causes in the intestine, meninges and brain (Cunliffe-Beamer and Les, 1987).

It was interesting to note that although symptoms of the disease occurred in both strains, their severity was greater in the CBA strain than in the NIH strain. This may be because the CBA strain is more affected by the immunosuppressive effects of *H. polygyrus* than the NIH strain. J. F. Needham (pers. comm.) also suggests that the strain of mice may influence the clinical signs.

5.3 Method

The changes in parasite numbers and in the host response on re-exposure to the parasite after an anthelmintic interruption were assessed in high responder NIH mice. One hundred and five (105) six to eight week old NIH mice were divided at random into seven groups of fifteen. Of these groups, two were given 10 L3/week for 4 weeks and another two were given 50 L3/week for 4 weeks. The mice in these groups were then given ivermectin at a dose of 10 mg/kg to clear the worms and a week later one group from each of the dose levels was given larvae at the same weekly level as before. The other group which had received larvae before the anthelmintic were given no more larvae for the remainder of the experiment. After the anthelmintic treatment a further two groups of mice which had not previously been infected with the parasite were trickle infected: one with 10 L3/week, the other with 50 L3/week. The remaining group served as uninfected age-matched controls.

At three weekly intervals after the anthelmintic interruption, faecal egg counts were determined for five mice from each group. The mice were then killed, their sera collected for later analysis, intestines removed for worm burden determination and spleens extracted for use in a lymphocyte proliferation assay. At each interval five age-matched controls were used to determine background levels of proliferation and antibody concentration. The spleens of 6-10 week old female

CBA mice were used in a mitogen induced proliferation assay as inter-experimental controls. At four weekly intervals larval infectivity was assessed by giving five 6-10 week old NIH females 50 L3 and determining their worm burdens two weeks later.

The experiment was repeated using 6-8 week old female CBA mice.

5.4 Repeated low level infections interrupted with anthelmintic in NIH mice: immunological and epidemiological data

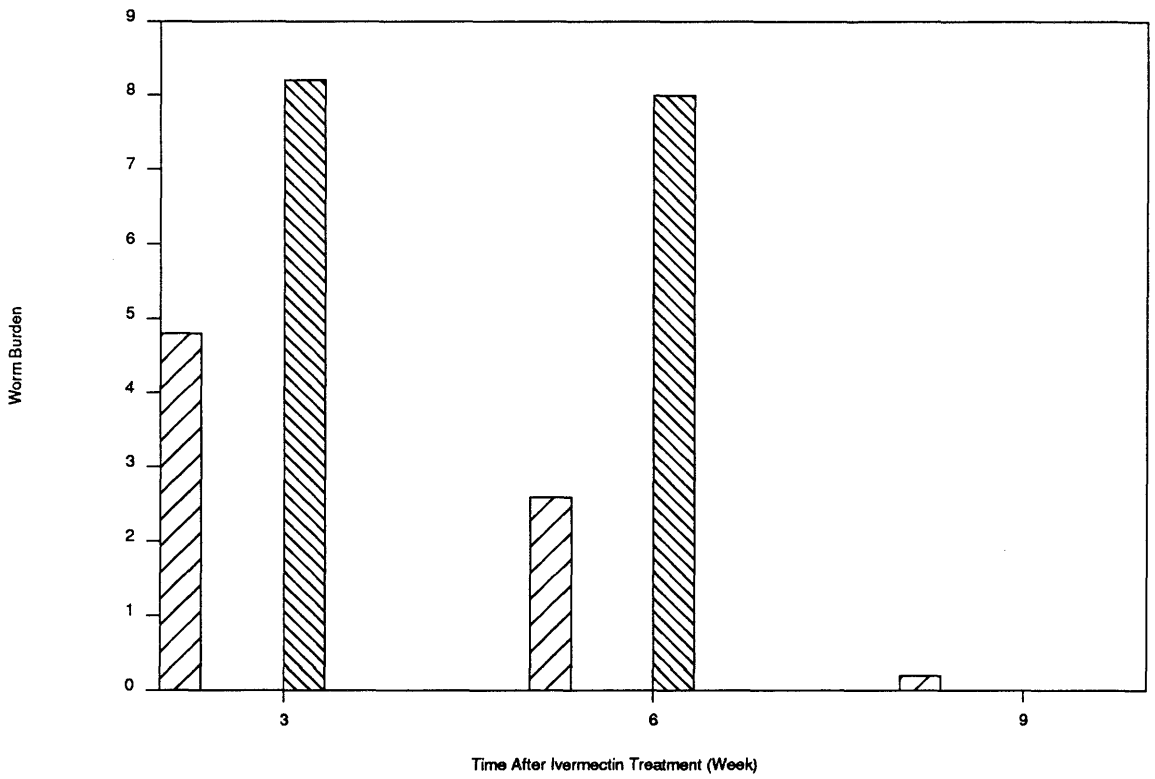
5.4.1 Worm burden

Adult *H. polygyrus* were not recovered from the intestines of mice which had been given low level repeated doses of larvae before ivermectin, regardless of whether the infections were continued or were stopped after the anthelmintic treatment. The only treatment from which worms could be recovered was that in which infection followed the dosing with the drug (the results are shown in figure 5.1 and the analysis given in appendix 3.1a).

The number of worms recovered from mice given either of the two dose levels under this regime was not significantly different. The worm burdens of mice given either of the two dose levels decreased with time. Worms were recovered from the mice given 10 L3/week on the ninth week of infection whereas they were not found at that time in the intestines of mice given 50 L3/week.

Figure 5.1 *Worm burden of NIH mice given larvae of* H. polygyrus *at different times relative to ivermectin dosing. The bars represent the worm burdens of mice given 10 L3/week (1st bar) or 50 L3/week (2nd bar) after ivermectin treatment.*

Figure 5.1



5.4.2 Sex ratio

The sex ratio, (males as a proportion of the total infection) which was always female biased,was not significantly different between the different weeks of determination and neither was there a difference between the doses. The results are shown in figure 5.9 and the analysis given in appendix 3.1b.

Figure 5.2 *The fecundity of female* H. polygyrus *adults (eggs per female per 16 hours) in NIH mice given larvae at different times relative to ivermectin dosing. The bars represent the fecundities of mice given 10 L3/week (1st bar) or 50 L3/week (2nd bar) after ivermectin treatment.*

Figure 5.3 *The ratio of the number of spleen cells of NIH mice to the number of spleen cells in naive mice. The bars represent the ratios for mice given larvae at different times relative to ivermectin treatment i.e. given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.2

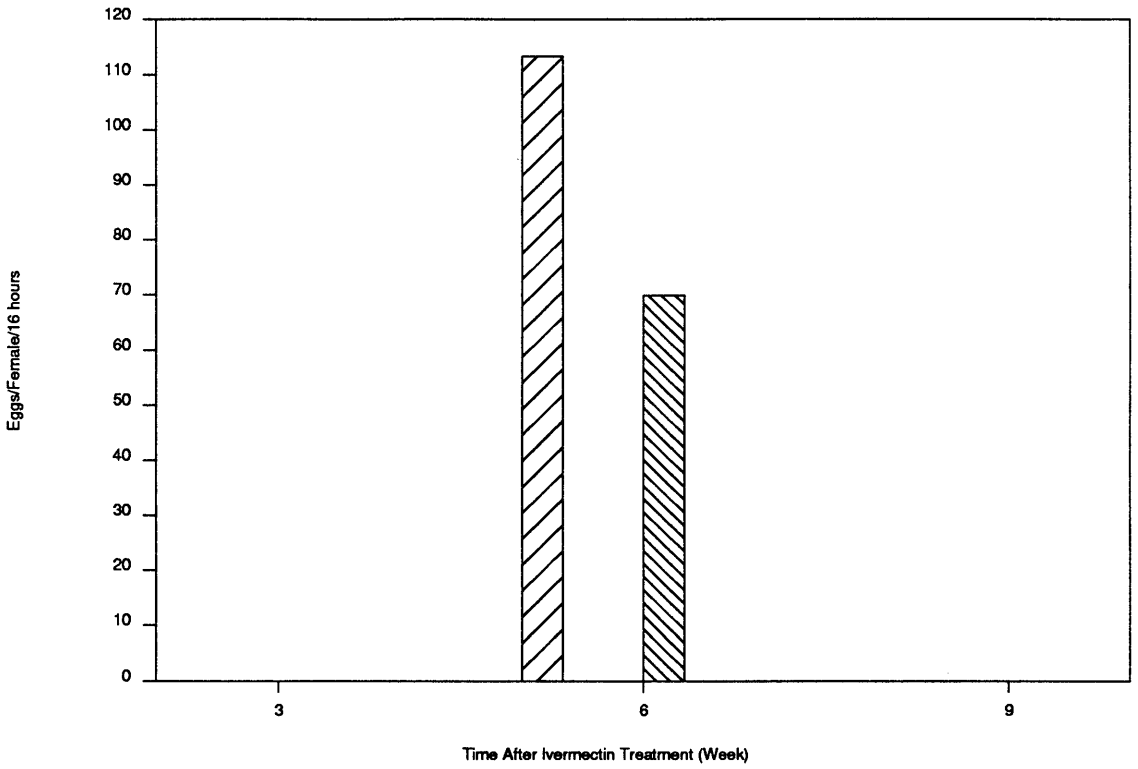
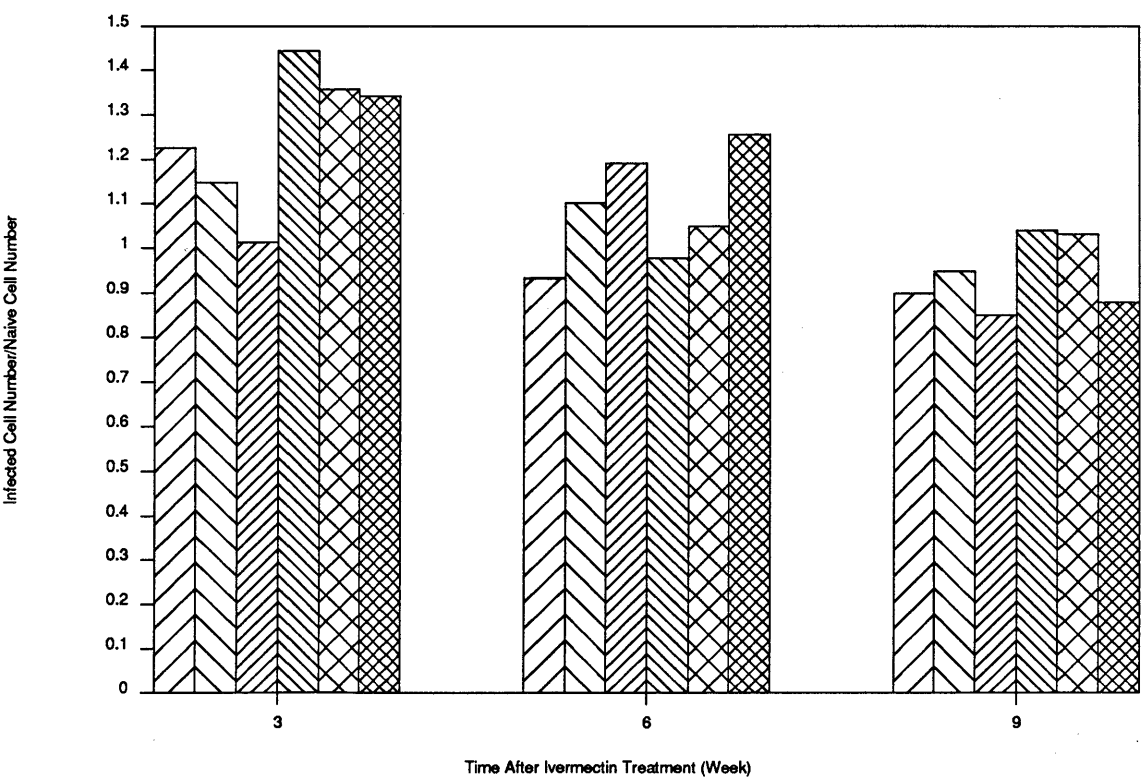


Figure 5.3



5.4.3 Faecal egg output

Eggs could only be detected in the faeces of mice which had been dosed with larvae only after anthelmintic treatment and were detected at weeks six and nine after the initial infection. The fecundity of worms from mice given different weekly doses was not significantly different at these times. The results are shown in figure 5.2 and the analysis given in appendix 3.1c.

5.4.4 Spleen cell counts

On the basis of cell counts alone, it was not possible to distinguish between mice which had been given different doses of larvae, infected for different lengths of time or subjected to different treatments. The results are shown in figure 5.3 and the analysis given in appendix 3.1d.

5.4.5 Immunoglobulin levels

5.4.5a Total immunoglobulin level

5.4.5b Amount of immunoglobulin specific to each homogenate.

On analysis of the sera from infected mice, no difference was detected in the amount of total antibody to either of the two homogenates at any combination of treatment or time.

5.4.5c Amount of antibody in the serum of mice given different doses.

A difference in the amount of total antibody in the sera of mice given different weekly doses was detected, the statistical analysis being given in 3.2a. There was more *H. polygyrus* specific antibody in the sera of mice receiving 50 L3/week than

Figure 5.4 *The humoral response of NIH mice given larvae at different times relative to ivermectin treatment. The serum samples were assayed for their total immunoglobulin to (a) adult homogenate or (b) D6L4 homogenate. The bars represent the optical densities for the sera of mice: given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.4 a

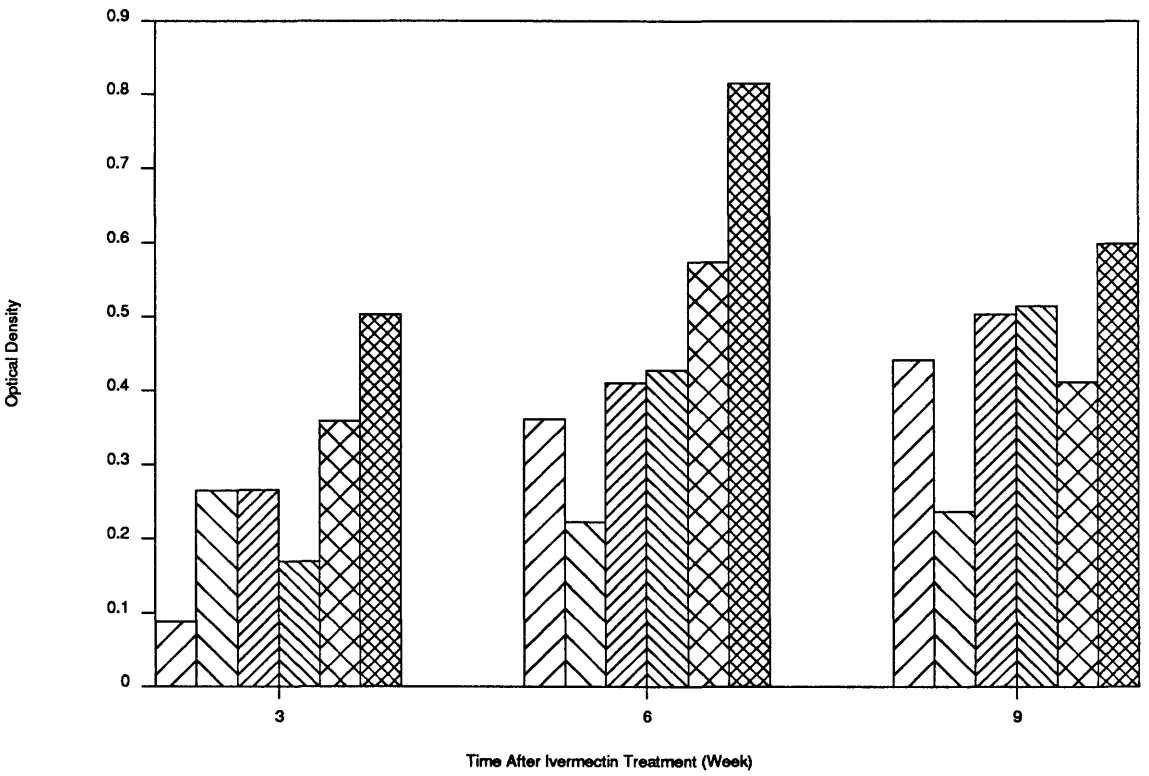
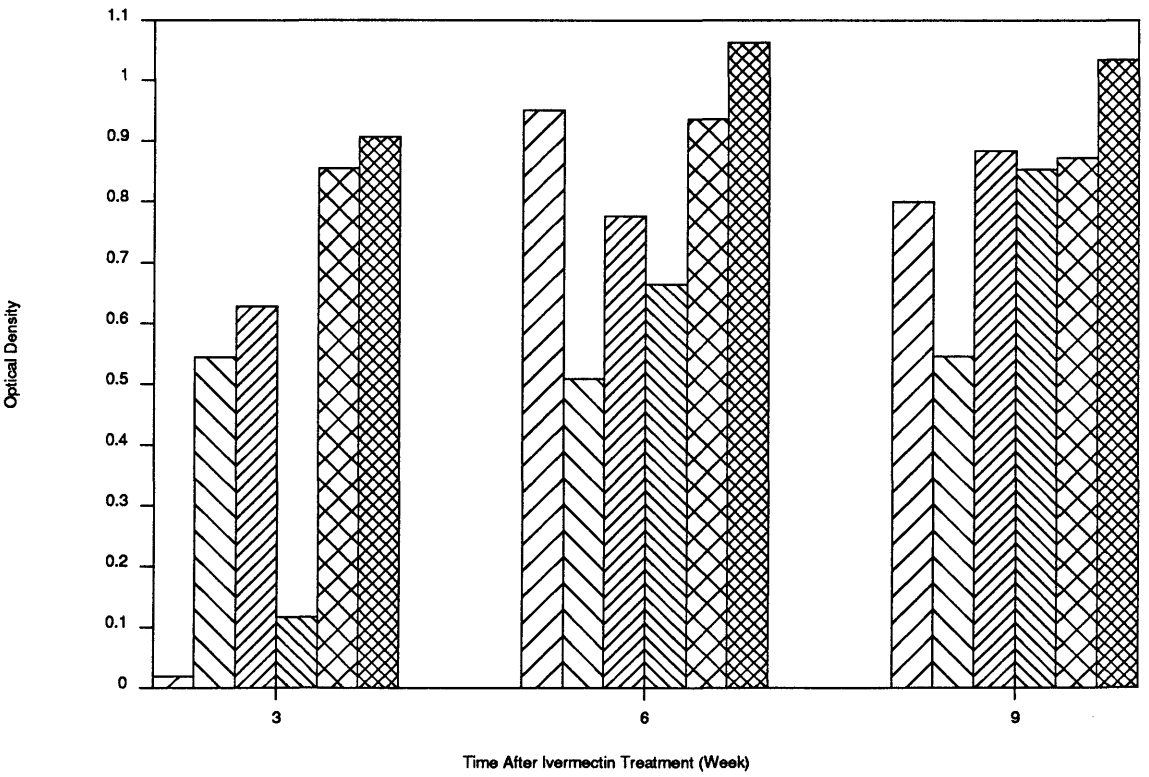


Figure 5.4 b



there was in the sera of mice receiving 10 L3/week (see figure 5.4a & 5.4b for the amount of total antibody to adult and D6L4 homogenate respectively).

5.4.5d Amount of antibody resulting from different treatments.

A difference was noted in the amount of total *H. polygyrus* specific antibody in mice given different treatments. Those mice given larvae before and after ivermectin produced the most *H. polygyrus* specific antibody. No difference in the amount of antibody produced by mice given larvae either before or after ivermectin was observed.

If the change in the amount of total antibody with time is considered for each treatment separately (this can be done, but with an increased probability of committing a type II error) then a difference between the treatments is apparent. The amount of antibody increased with time for those mice given larvae after ivermectin treatment, the ELISA optical density values rose rapidly between the third and sixth week of the trickle.

The amount of antibody increased with time for those mice given larvae before and after ivermectin although the amount did not increase after the sixth week. The increase for the mice given the larvae before and after ivermectin was not as marked for those given the larvae only after ivermectin.

The mice which were given larvae only before ivermectin did not show a significant increase in the amount of total antibody over the length of the experiment despite there being a significant difference between the amounts at the third and sixth weeks.

5.4.6 IgG1 levels

5.4.6a Amount of antibody specific to the two homogenates.

The level of parasite specific IgG1 to either of the two homogenates was not significantly different at any of experimental combinations, the statistical analysis being given in appendix 3.2b. The amounts of IgG1 to the adult homogenate and D6L4 homogenate are shown in figure 5.5a and 5.5b respectively.

5.4.6b Amount of antibody in mice given different weekly doses.

The mice which were given 10 L3/week produced less antibody than those mice given 50 L3/week.

5.4.6c Amount of antibody in mice given different treatments

The mice which produced the most IgG1 antibody were those which were given larvae both before and after ivermectin. The amount of IgG1 antibody that could be detected in the sera of mice given larvae after ivermectin was greater than the amount in the sera of mice given larvae before ivermectin dosing.

The different treatments gave rise to mice which showed different patterns of IgG1 antibody production with time. The mice given larvae only after ivermectin treatment show an increase in the amount of antibody with time, the amount being different at all times of assaying. The amount of antibody produced by mice given larvae before and after ivermectin increased between the third and sixth week after ivermectin dosing but not after the sixth week. Thus, the amount of IgG1 antibody produced increased more rapidly in mice given the larvae only after ivermectin than in those mice given larvae before and after drug treatment. However, less antibody was produced by the mice given larvae only after ivermectin treatment.

The amount of IgG1 produced by the mice given larvae only before ivermectin treatment did not show a significant increase with time.

Figure 5.5 *The humoral response of NIH mice given larvae at different times relative to ivermectin treatment. The serum samples were assayed for their IgG1 content to (a) adult homogenate or (b) D6L4 homogenate. The bars represent the optical densities for the sera of mice: given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.5 a

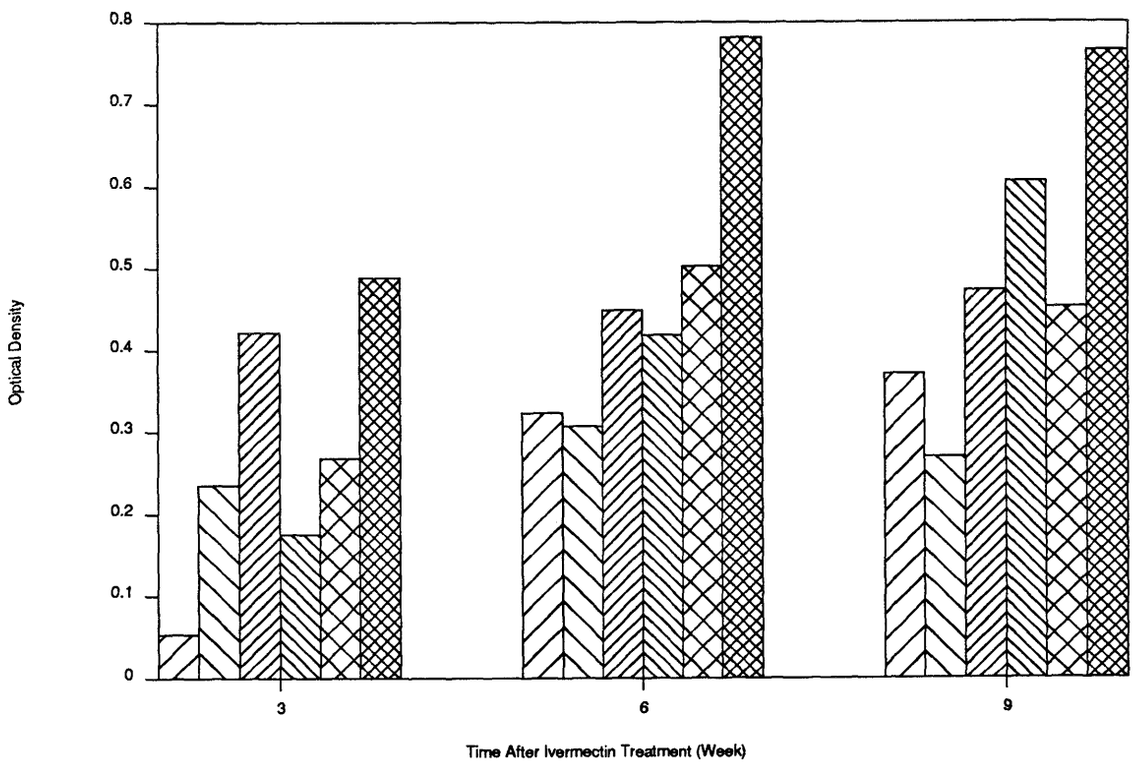
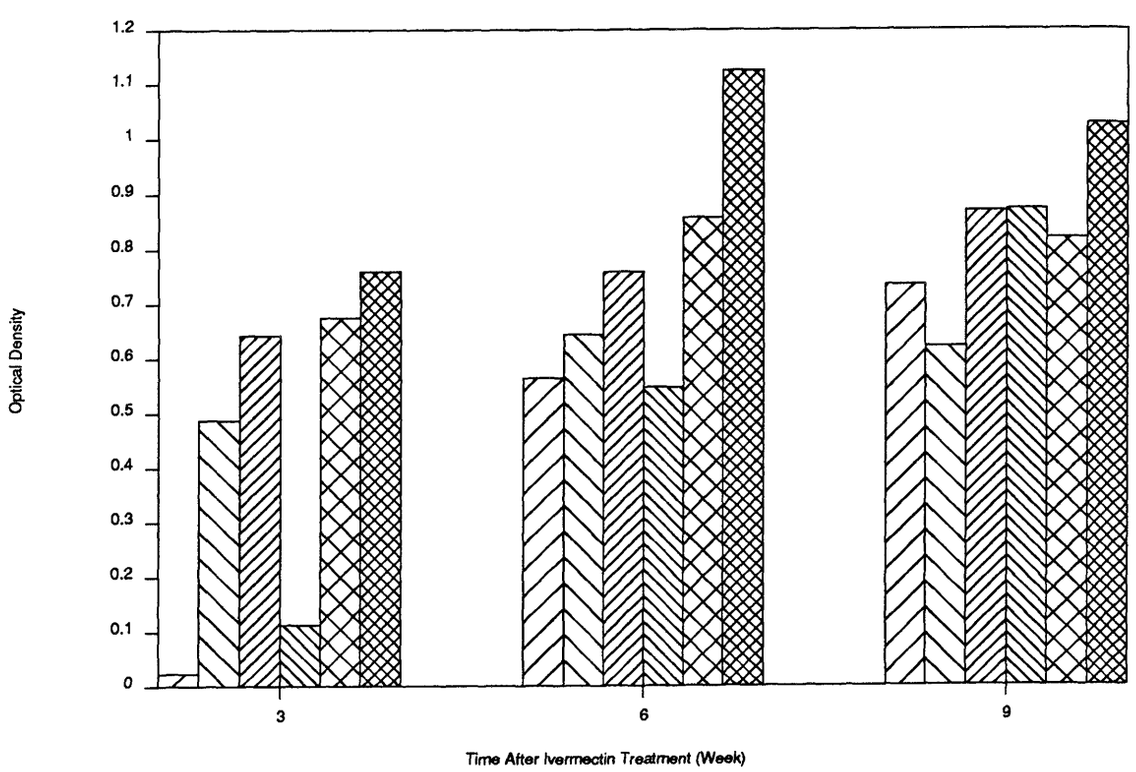


Figure 5.5 b



5.4.7 Proliferation of spleen cells from NIH mice

The changes in proliferation with time of cells from mice given different treatments are shown in figure 5.6a-g and figurre 5.7 a-e. The analysis is given in appendix 3.3

5.4.7a Response expressed as counts per minute.

The antigens against which the cells from infected mice proliferated more than did the cells from naive mice were the adult homogenate (see figure 5.6a, appendix 3.3a) and the D6L4 ES(MT) (see figure 5.6d, appendix 3.3b).

The adult homogenate caused significant proliferation (as judged by analysis of counts per minute) only when cultured with cells from mice given 50 L3/week. The adult homogenate did cause a significant increase in proliferative response with time. Cells from mice which had undergone different dosing regimes did not show a difference in their ability to proliferate against adult homogenate.

The D6L4 ES(MT) when cultured with cells from mice given 10 or 50 L3/week caused significantly more proliferation than when it was cultured with cells from uninfected mice. There was a significant increase in proliferative ability with time when the cells were cultured with this antigen. There was also a significant difference between the response to the D6L4 ES(MT) for mice given different treatments, cells from mice given larvae only after ivermectin proliferated least of all.

Figure 5.6 *The proliferation of cells from NIH mice given larvae at different times relative to ivermectin treatment. The figures are the responses of mice given (a) 10 L3/week after ivermectin, (b) 10L3/weekbefore ivermectin, (c) 10 L3/week before and after ivermectin, (d) 50 L3/week after ivermectin, (e) 50 L3/week before ivermectin, (f) 50 L3/week before and after ivermectin, (g) no larvae. The bars represent the proliferation to: L3 homogenate (1st bar); medium (2nd bar); adult homogenate (3rd bar); adult ES(MT) (4th bar); D6L4 homogenate (5th bar); D6L4 ES(MT) (6th bar).*

Figure 5.6 a

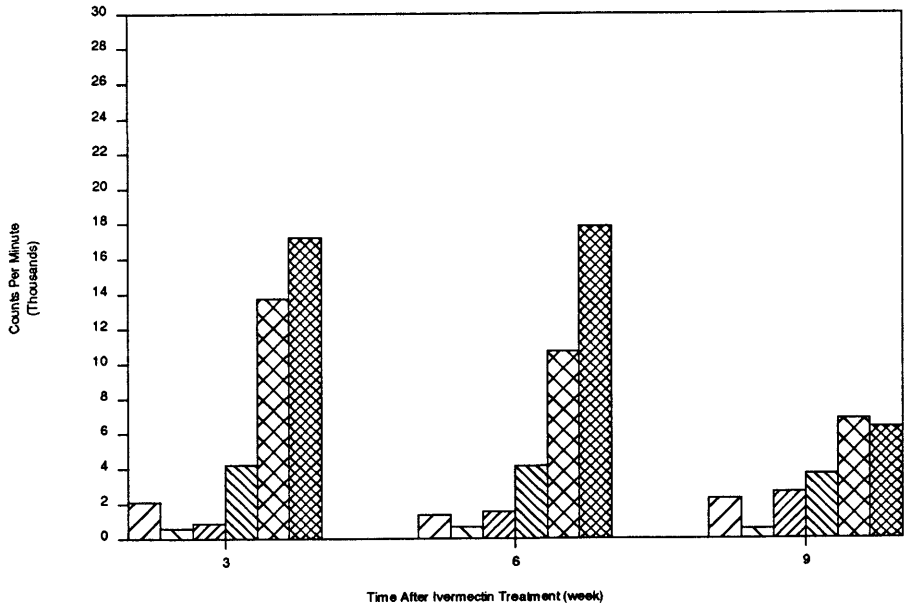


Figure 5.6 b

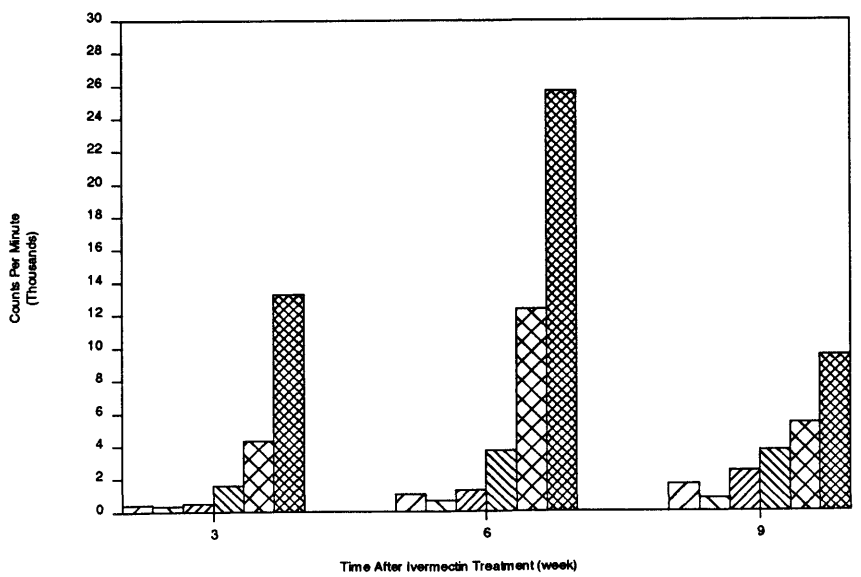


Figure 5.6 c

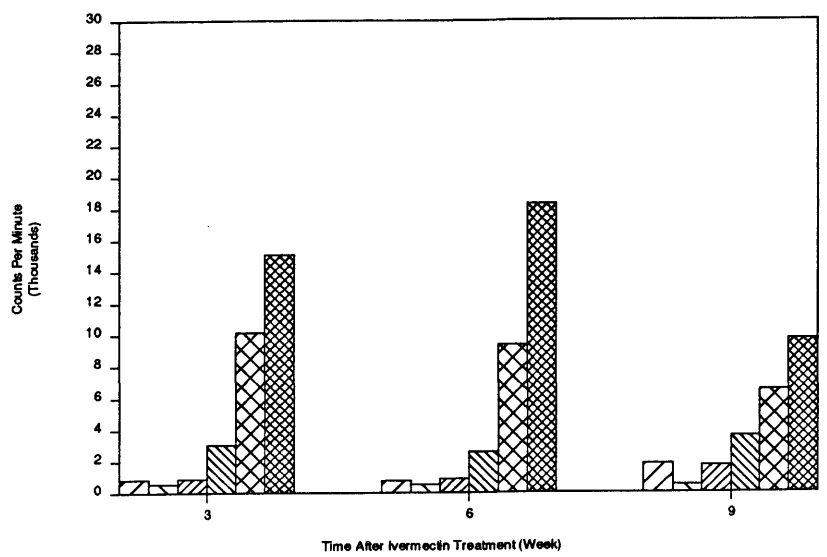


Figure 5.6 d

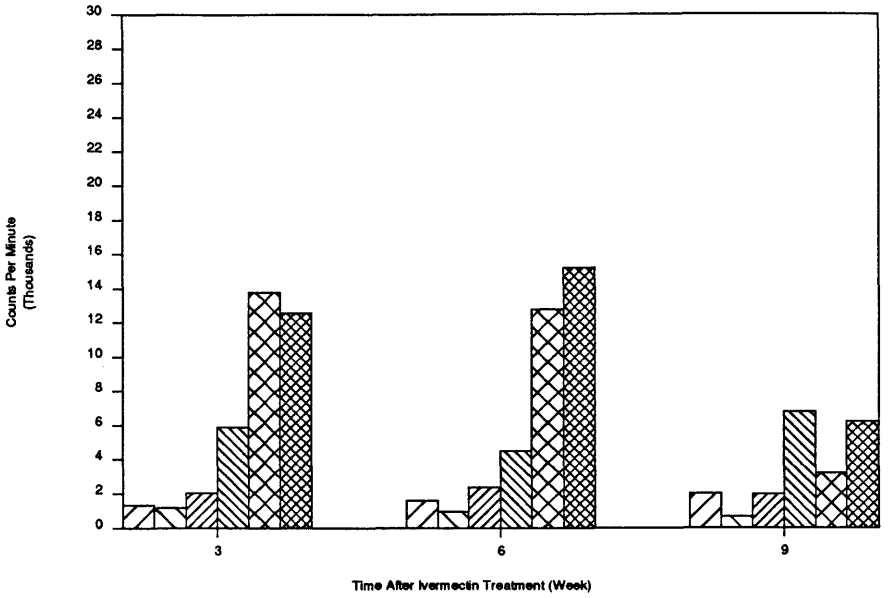


Figure 5.6 e

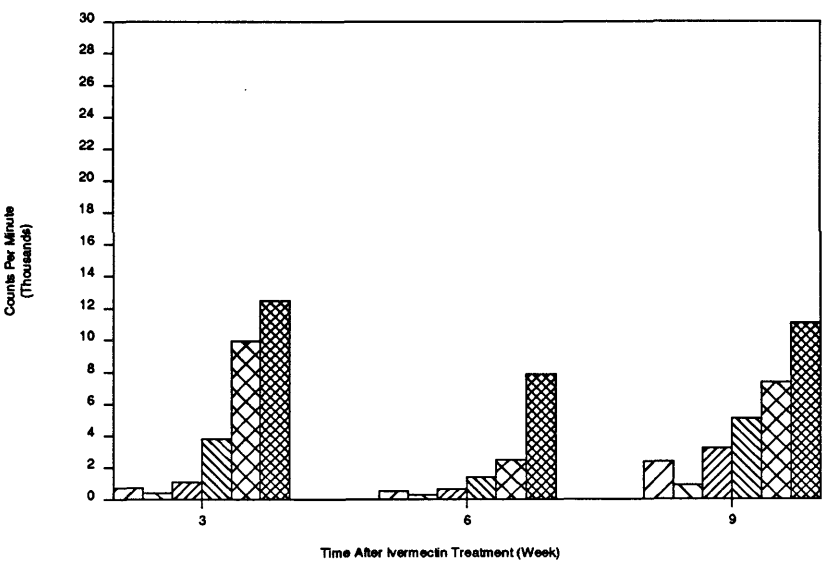


Figure 5.6 f

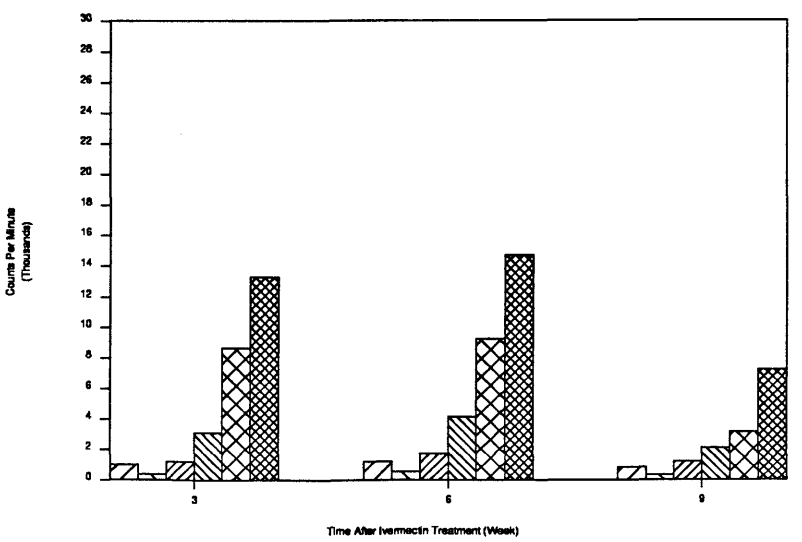
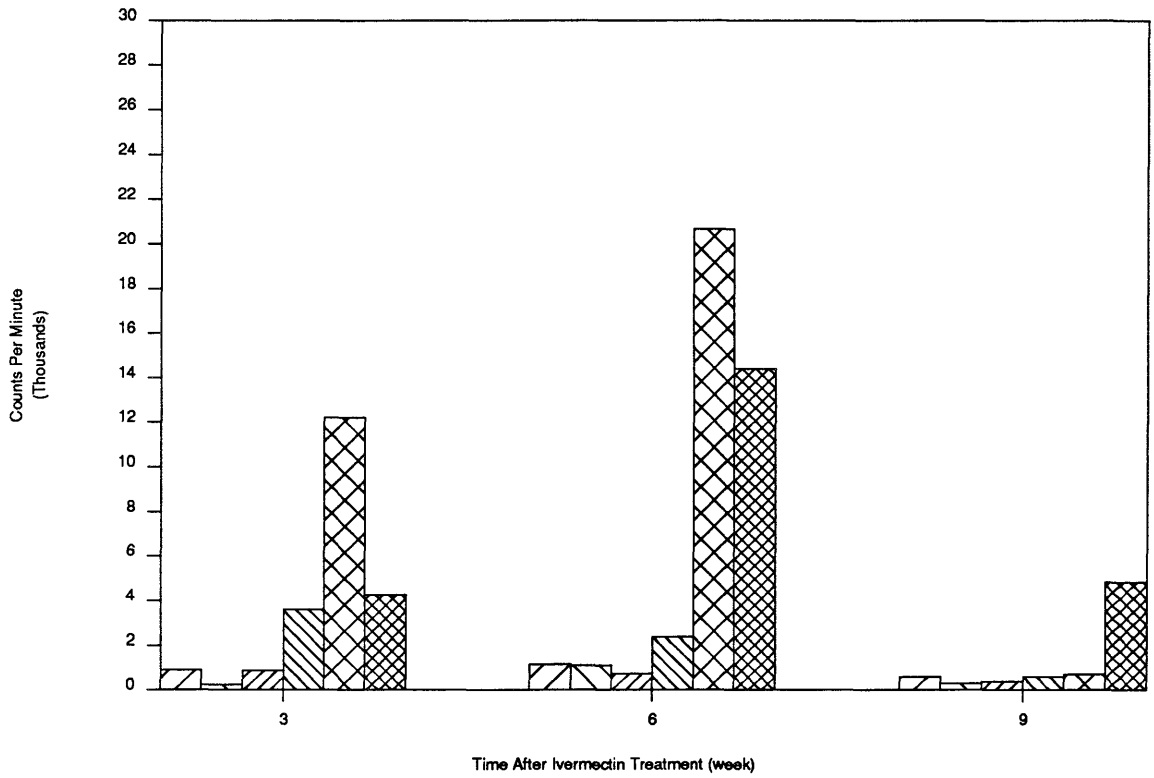


Figure 5.6 g



5.4.7b Results expressed as stimulation indices

When the changes in cellular responsiveness were considered in terms of stimulation indices the pattern of change was similar to that seen when the changes were analysed in terms of the counts per minute.

Only two of the antigens caused more proliferation when cultured with cells from infected mice than when cultured with cells from naive mice. These antigens

Figure 5.7 *The stimulation indices of cells from NIH mice given larvae at different times relative to ivermectin treatment. The figures are the responses of mice given (a) 10 L3/week after ivermectin, (b) 10/week L3 before ivermectin, (c) 10 L3/week before and after ivermectin, (d) 50 L3/week after ivermectin, (e) 50 L3/week before ivermectin, (f) 50 L3/week before and after ivermectin, (g) no larvae. The bars represent the proliferation to: L3 homogenate (1st bar); medium (2nd bar); adult homogenate (3rd bar); adult ES(MT) (4th bar); D6L4 homogenate (5th bar); D6L4 ES(MT) (6th bar).*

Figure 5.7 a

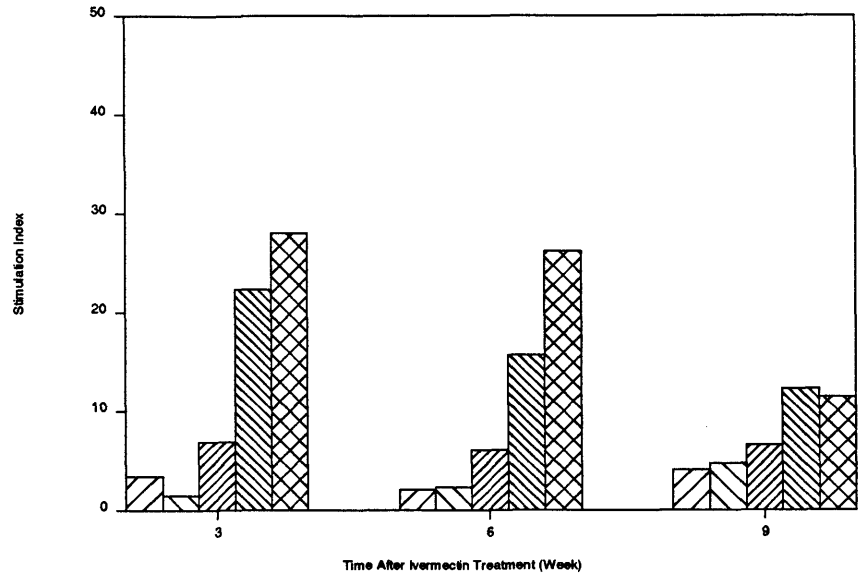


Figure 5.7 b

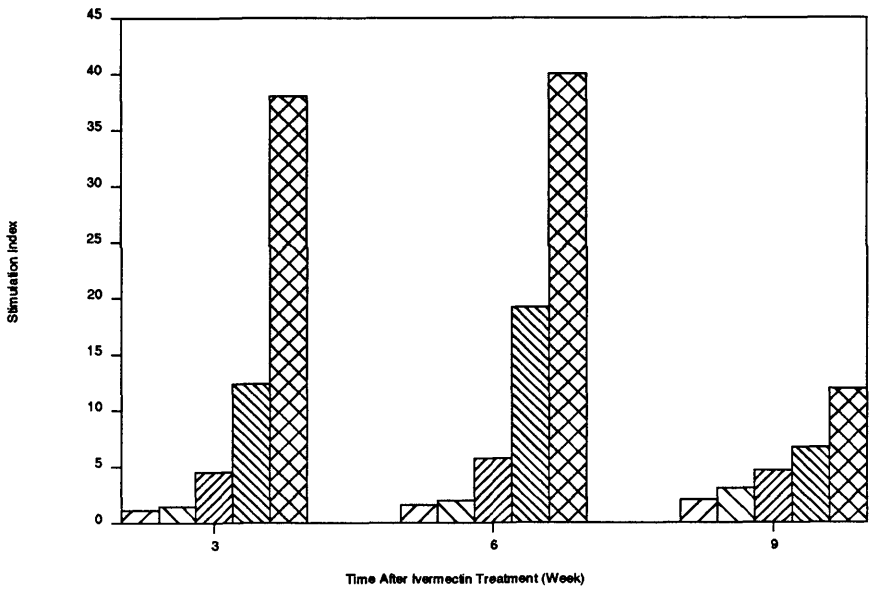


Figure 5.7 c

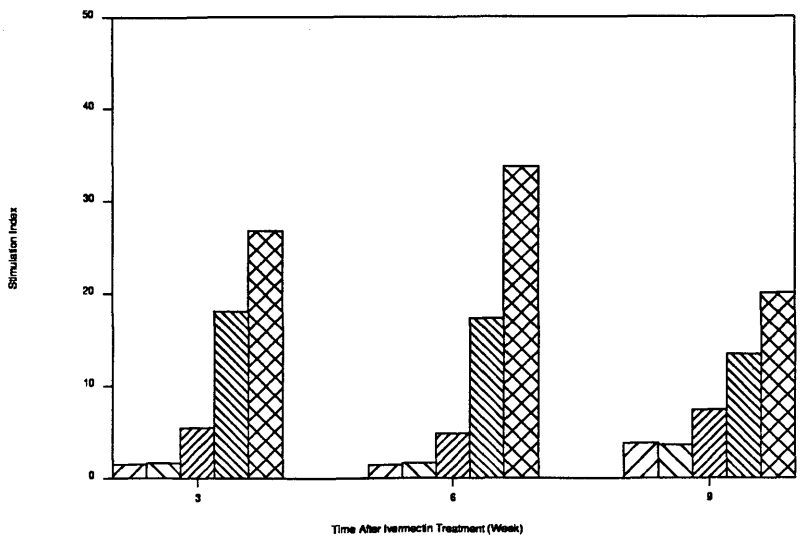


Figure 5.7 d

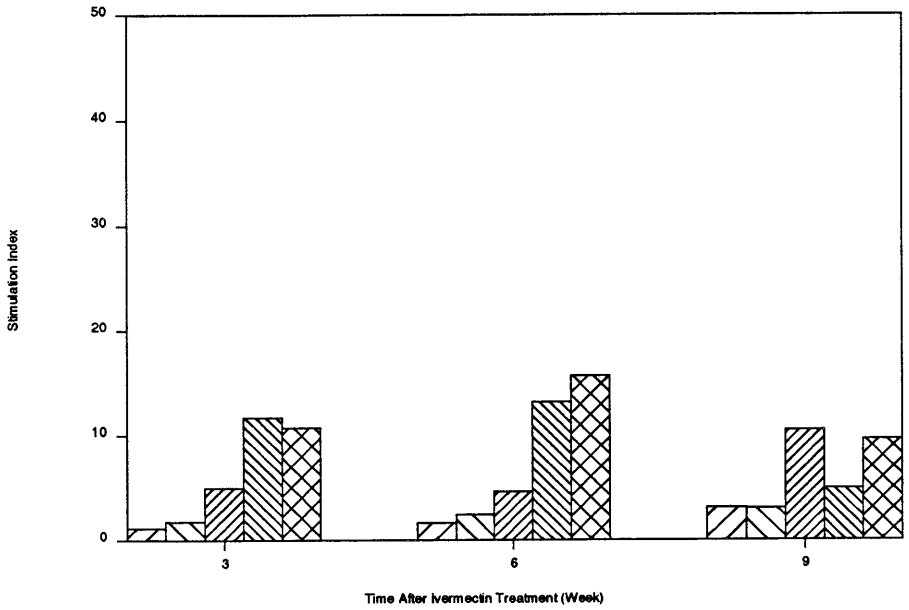


Figure 5.7 e

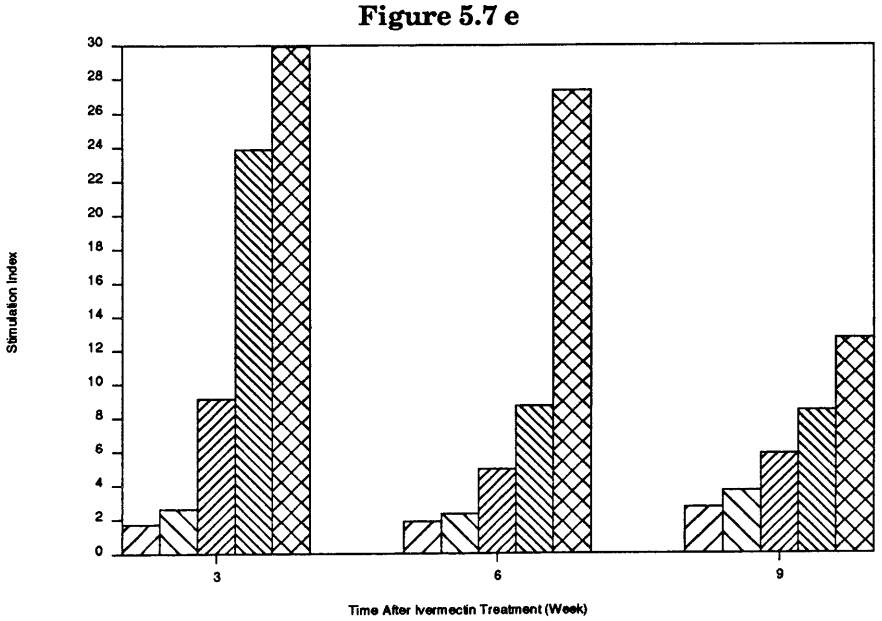


Figure 5.7 f

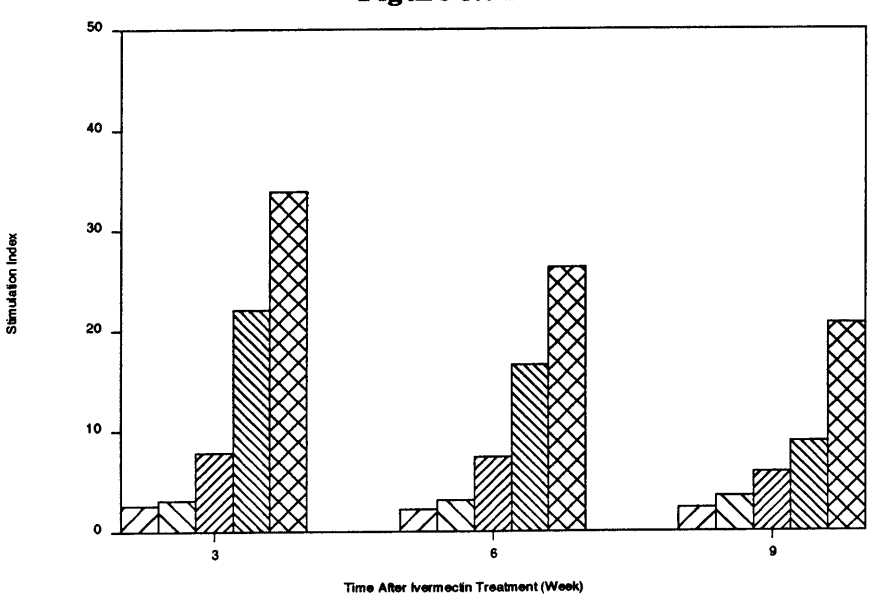
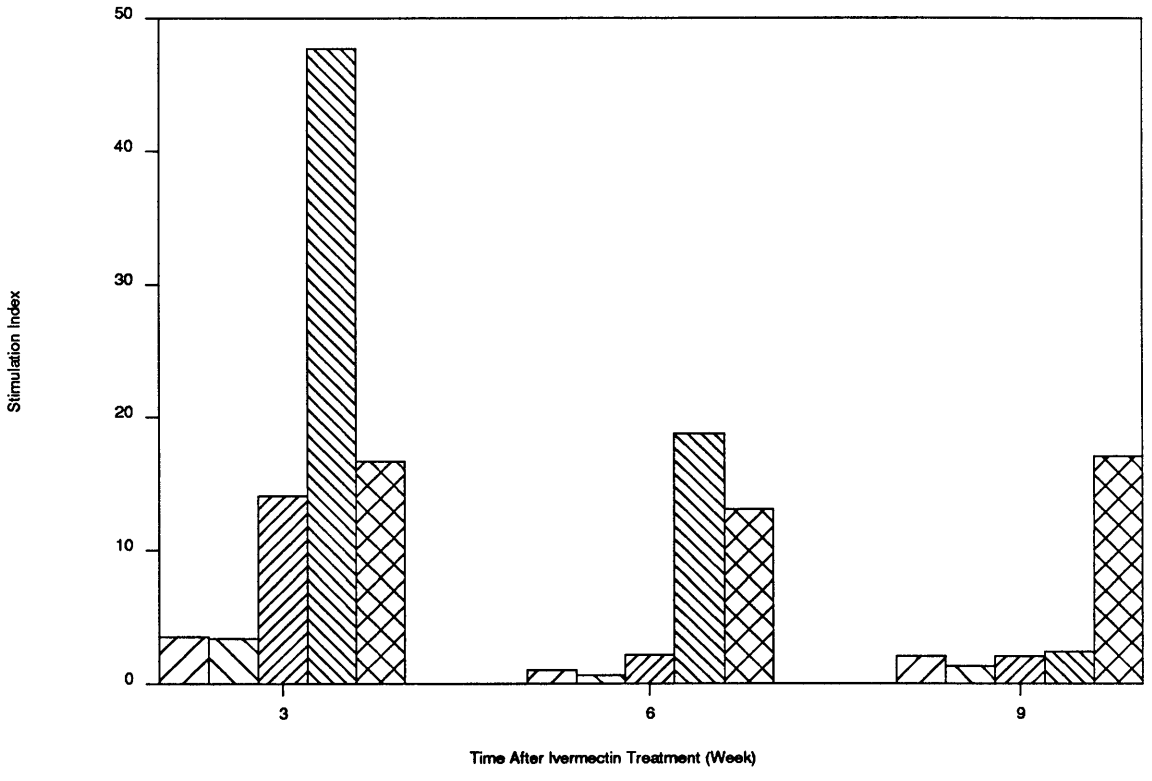


Figure 5.7 g



were the adult homogenate (see figure 5.7a, appendix 3.4a) and the D6L4 ES(MT) (see figure 5.7d, appendix 3.4b)..

Only with the D6L4 ES(MT) was a difference in the SI as a result of culturing with cells from different treatments noted. On culturing with the D6L4 ES(MT), the cells from mice given larvae only after ivermectin proliferated the most of all.

5.5 Repeated low level infections interrupted with anthelmintic in CBA mice: immunological and epidemiological data

5.5.1 Worm burden

Worms were not recovered from mice which were given larvae only before ivermectin treatment. Thus, the treatment of mice with ivermectin proved to be effective at clearing the worms of mice given either of the two doses. The mice given larvae after anthelmintic accumulated worms. The worm burden in the mice given larvae only after ivermectin was significantly greater than the burden in those mice which were given larvae both before and after ivermectin. The results are shown in figure 5.8, for the analysis of these changes see appendix 3.5a.

The worm burden in CBA mice given larvae only after ivermectin treatment increased with time. The worm burden was significantly greater in those mice which were given 50 L3/week than it was in those mice given 10 L3/week .

The worm burden in those mice given larvae before and after ivermectin did not increase with time. No significant difference was detected between the worm burdens of the mice given different weekly doses.

Figure 5.8 *Worm burden of CBA mice given larvae of* H. polygyrus *at different times relative to ivermectin dosing. The bars represent the worm burdens of mice given 10 L3 / week (1st bar) or 50 L3 / week (2nd bar) after ivermectin treatment and the burdens of mice given 10 L3 / week (3rd bar) or 50 L3 / week (4th bar)before and after ivermectin*

Figure 5.9 *The sex ratio (male / (male+female)) of worms in NIH and CBA mice given larvae at different times relative to ivermectin treatment. The bars represent the ratio in NIH mice given 10 L3 / week (1st bar) or 50 L3 / week (2nd bar) after ivermectin and the ratios of the parasites in CBA mice given: 10 L3 / week (3rd bar) or 50 L3 / week (5th bar) after ivermectin; 10 L3 / week (4th bar) or 50 L3 / week (6th bar) before and after ivermectin.*

Figure 5.8

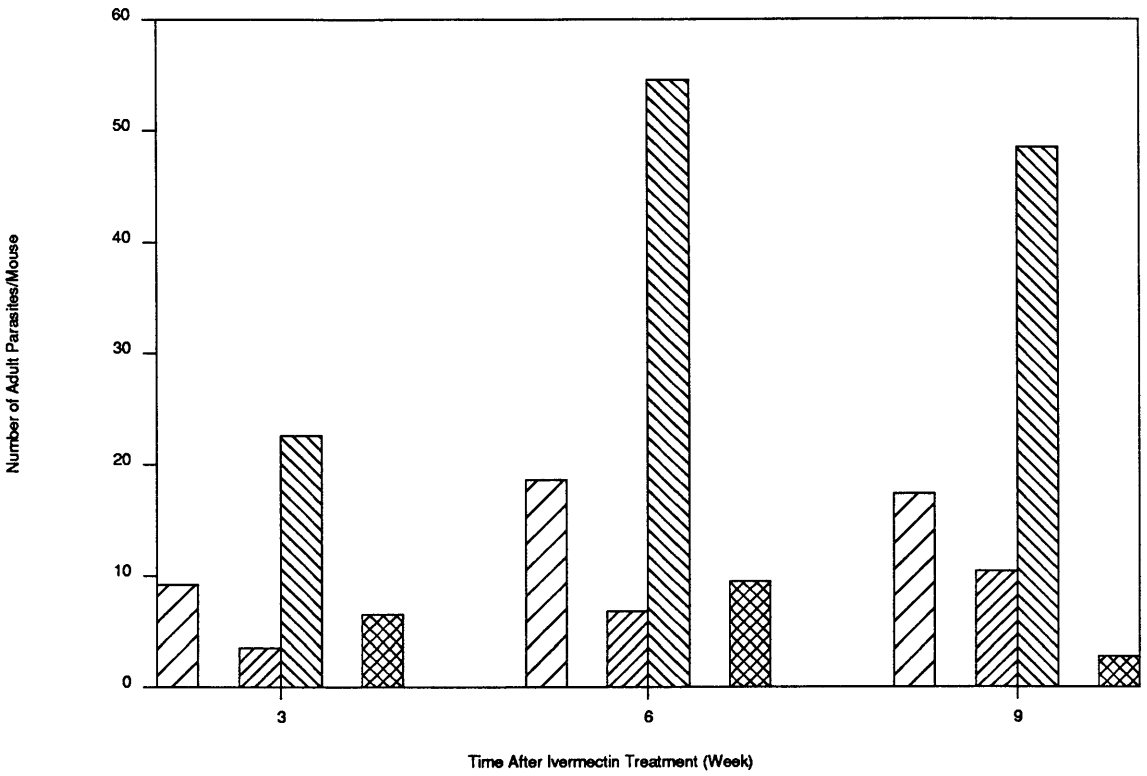
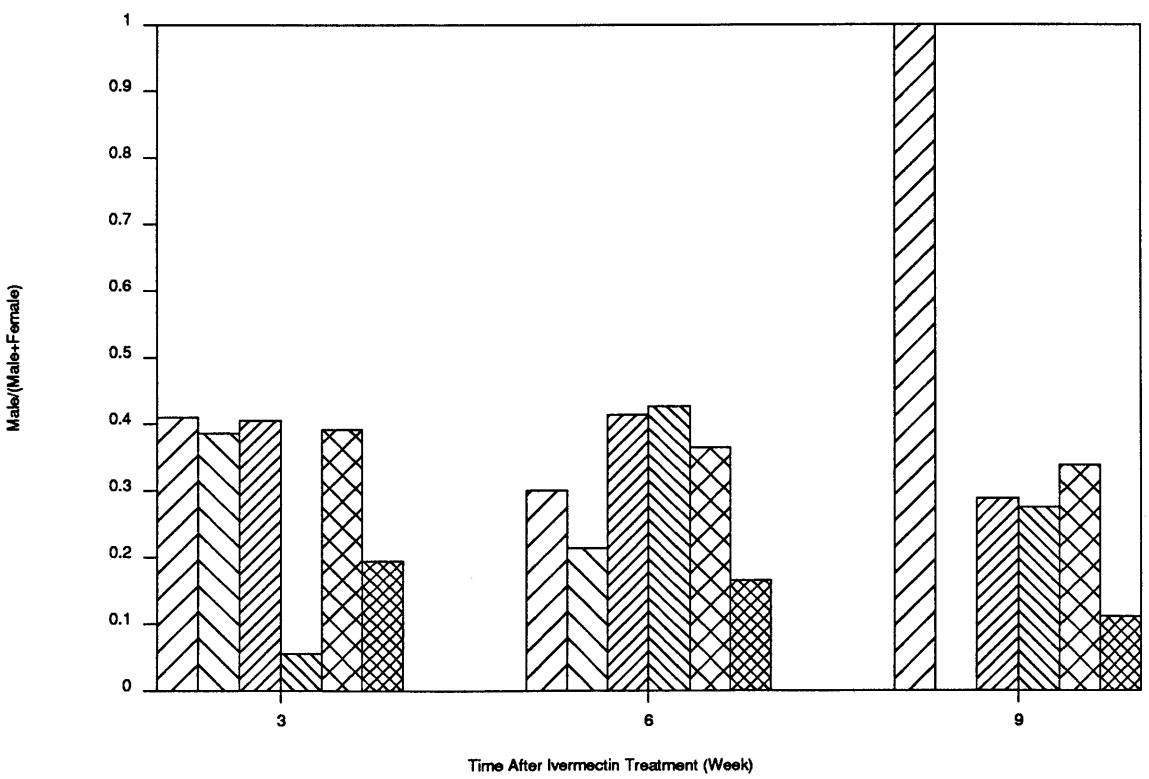


Figure 5.9



5.5.2 Sex ratio

The number of males at all times during the trickle was less than the number of females. The female bias in mice given different doses was not significantly different nor was it different at the different times of the trickle. There was a significant difference between the ratios resulting from the different infection regimes, the ratio being more male biased in the mice which had received larvae only after ivermectin treatment as compared to those given larvae both before and after ivermectin. The results are shown in figure 5.9, the statistical analysis is given in appendix 3.4b.

5.5.3 Faecal egg output

The fecundity of female worms was extremely variable during the experiment and no significant change in fecundity with time, dose or treatment was observed between the groups with patent infections. The results are shown in figure 5.10, the statistical analysis is given in appendix 3.4c.

5.5.4 Spleen cell counts

It was not possible to distinguish between mice which had been infected for different lengths of time, received different treatments or had been given different doses of the parasite on the basis of cell counts alone. The results are shown in figure 5.11, the statistical analysis is given in appendix 3.4d.

Figure 5.10 *The fecundity of female* H. polygyrus *adults (eggs per female per 16 hours) in CBA mice given larvae at different times relative to ivermectin dosing. The bars represent the fecundities of mice given 10 L3/week (1st bar) or 50 L3/week (2nd bar) after ivermectin treatment and the fecundities of the worms in mice given 10 L3/week (3rd bar) or 50 L3/week (4th bar) before and after ivermectin*

Figure 5.11 *The ratio of the number of spleen cells of infected CBA mice to the number of spleen cells in naive mice. The bars represent the ratios for mice given larvae at different times relative to ivermectin treatment i.e. given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.10

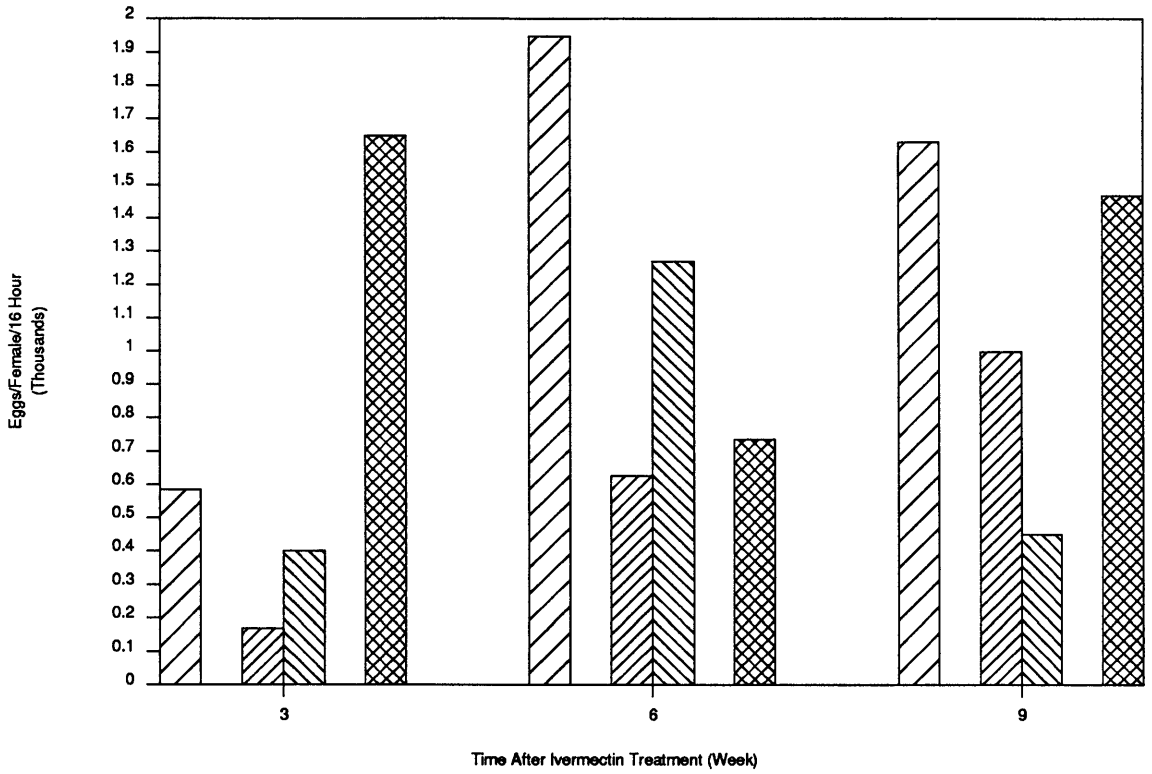
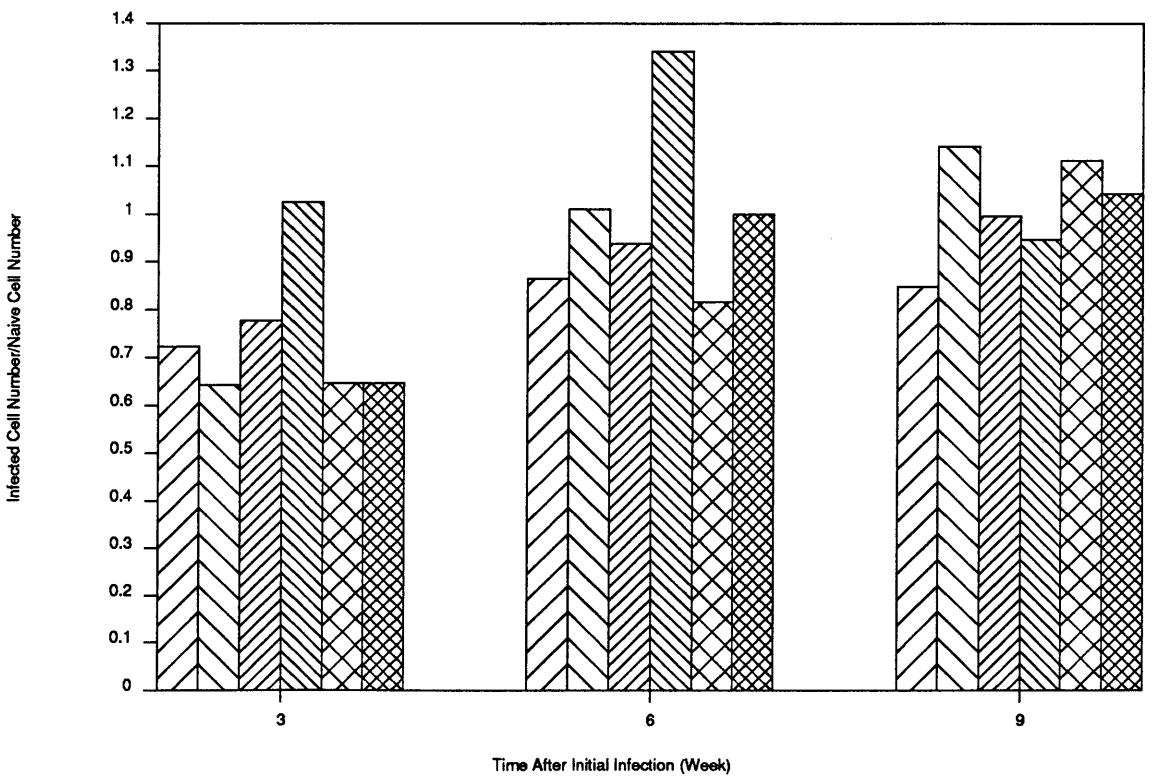


Figure 5.11



5.5.5 Total immunoglobulin levels

5.5.5a Amount of immunoglobulin specific to either homogenate.

The amount of total immunoglobulin detected in the sera of infected mice against the two homogenates was not significantly different at any combination of treatment, time or dose. Figure 5.12a & 5.12b shows the amount of total antibody to the adult homogenate and D6L4 homogenate respectively. The analysis of the differences is given in appendix 3.6a.

5.5.5b Amount of immunoglobulin in mice given different weekly doses.

There was significantly more immunoglobulin in the sera of mice which had received 50 L3/week than there was in the sera of mice which were given 10 L3/week.

5.5.5c Amount of antibody in mice given different treatments.

A difference in the amount of antibody resulting from different treatments was noted. The least amount of antibody was produced by mice given larvae only before ivermectin. Of the other two treatments, more antibody was produced in those mice given larvae before and after ivermectin than it was in mice given larvae only after ivermectin treatment.

If the change in antibody concentration with time is considered for each treatment then a difference between the treatments can be observed. The amount of total antibody produced increased with time only for those mice given larvae after anthelmintic treatment although the amount of antibody did not alter after the sixth week. The mice given larvae before and after ivermectin did not show a

Figure 5.12 *The humoral response of CBA mice given larvae at different times relative to ivermectin treatment. The serum samples were assayed for their total immunoglobulin to (a) adult homogenate or (b) D6L4 homogenate. The bars represent the optical densities for the sera of mice: given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.12 a

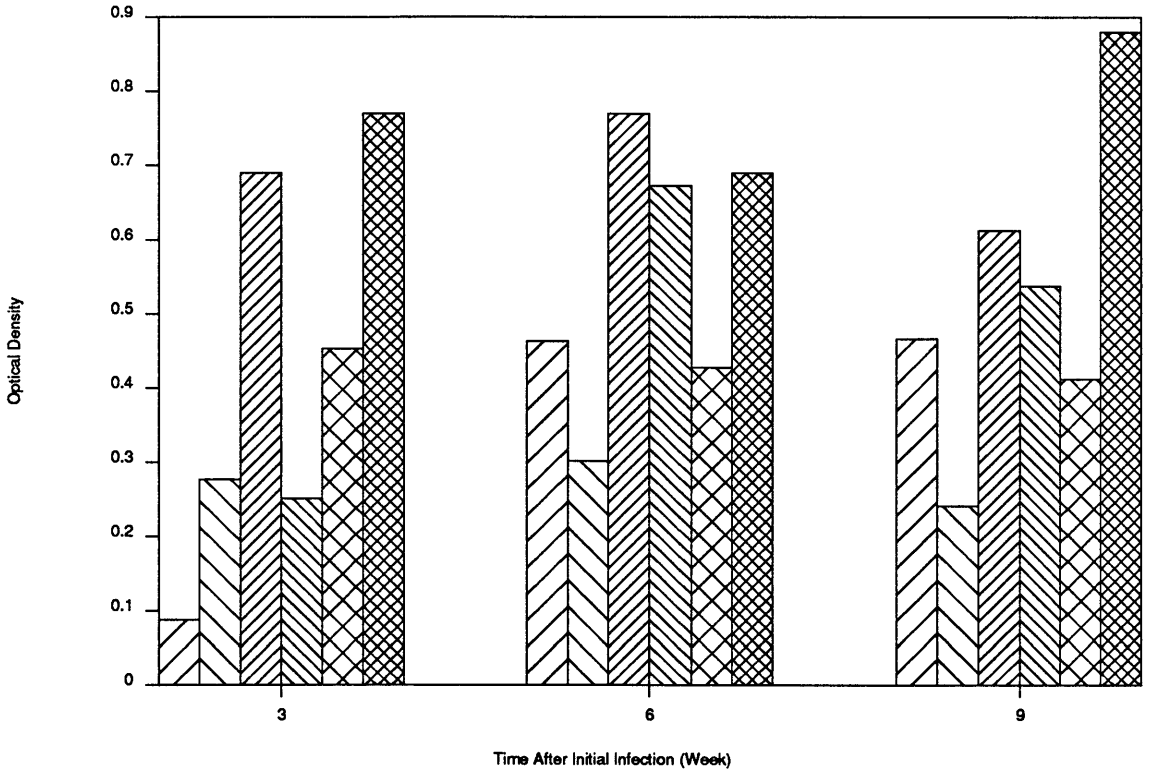
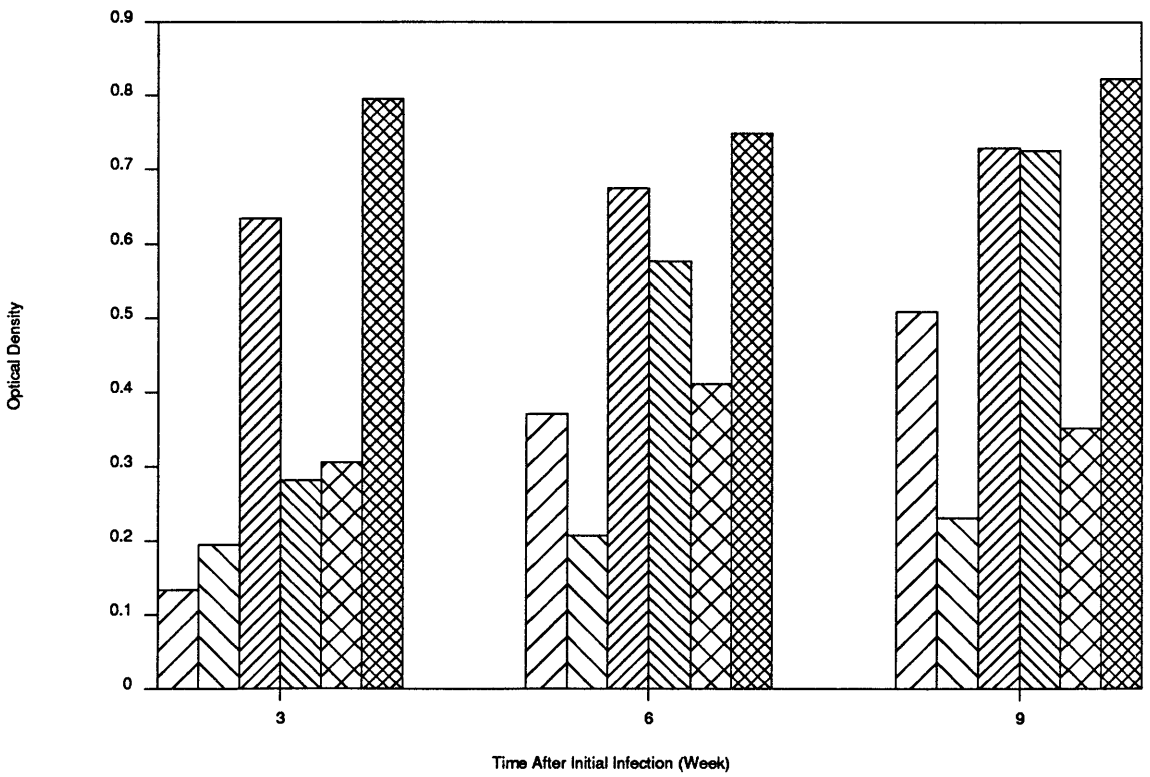


Figure 5.12 b



significant increase in the production of antibody with time and neither did those mice given larvae only before ivermectin treatment.

5.5.6 IgG1 levels

5.5.6a Amount of antibody specific to either homogenate.

The amount of IgG1 antibody against the D6L4 or adult homogenate was not significantly different at any combination of dose, treatment or time. Figure 5.13a & 5.13b shows the amount of total antibody to the adult homogenate and D6L4 homogenate respectively. The analysis of the differences is given in appendix 3.6b.

5.5.6b Amount of antibody in mice given different weekly doses.

More antibody was detected in the sera of mice given 50 L3/week than in the sera of mice given 10 L3/week.

5.5.6c Amount of antibody in mice undergoing different treatments.

The amount of IgG1 antibody in the sera of mice undergoing different treatment regimes was significantly different. Those mice given larvae before and after ivermectin produced the most antibody. The mice given larvae after ivermectin produced more antibody than those given larvae before ivermectin.

The change in the amount of IgG1 with time was considered for each treatment separately and a difference between the treatments was noted. For mice given larvae only after ivermectin the amount of antibody increased throughout the trickle. For the mice given larvae before and after ivermectin treatment the amount of antibody increased with time although the increase was not as marked as that for mice receiving larvae only after ivermectin.

Figure 5.13 *The humoral response of CBA mice given larvae at different times relative to ivermectin treatment. The serum samples were assayed for their IgG1 content to (a) adult homogenate or (b) D6L4 homogenate. The bars represent the optical densities for the sera of mice: given 10 L3/week (1st bar) or 50 L3/week (4th bar) after ivermectin treatment; given 10 L3/week (2nd bar) or 50 L3/week (5th bar) before ivermectin; 10 L3/week (3rd bar) or 50 L3/week (6th bar) before and after ivermectin.*

Figure 5.13 a

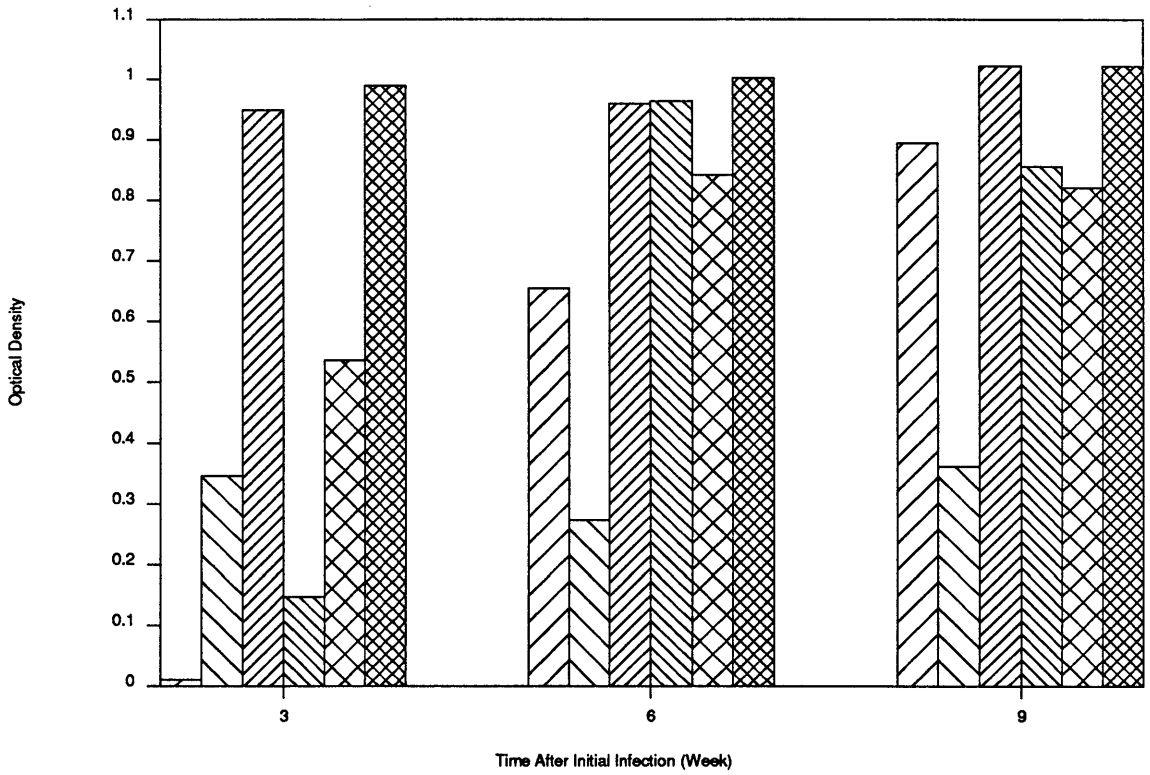
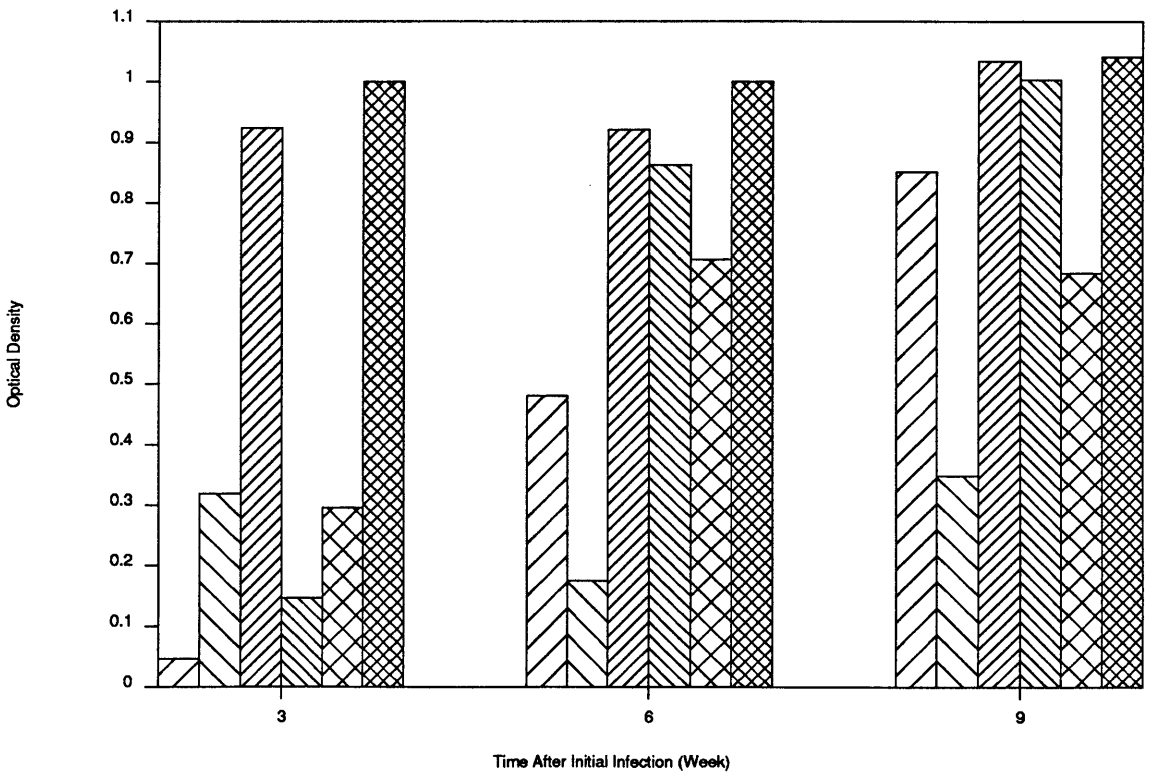


Figure 5.13 b



5.5.7 Proliferation of spleen cells from CBA mice

The statistical analysis of these changes is given in appendix 3.7a and the results are shown in figure 5.14a-e.

5.5.7a Response expressed as counts per minute

Cells from infected mice proliferated more than the cells from uninfected mice only when they were cultured with adult homogenate (see figure 5.14a, appendix 3.7a & 3.7b).

The cells from mice given 50 L3/week were the only cells to proliferate to the adult homogenate more than cells from uninfected mice. There was a significant increase in the amount of proliferation with time when the cells were cultured with the adult homogenate, but no significant difference in the amount of proliferation shown by cells from mice given different treatments could be detected.

5.5.7b Response expressed as stimulation indices

When cultured with cells from infected mice the adult homogenate was the only antigen whose SI was greater than when it was cultured with cells from uninfected mice. The results of the analysis are shown in figure 5.15a-e and the analysis is given in appendix 3.8.

It was only on culturing with cells from mice given 50 L3/week that the adult homogenate's SI was greater than the SI observed on culturing with cells from naive mice. There was no significant difference in the adult homogenate's SI when it was cultured with cells from mice given either 10 L3/week or no larvae. The antigen's SI increased with time. The SI of the adult homogenate was not significantly different when it was cultured with cells from different mice which had undergone different treatments.

Figure 5.14 *The proliferation of cells from CBA mice given larvae at different times relative to ivermectin treatment. The figures are the responses of mice given (a) 10 L3/week after ivermectin, (b) 10/week L3 before ivermectin, (c) 10 L3/week before and after ivermectin, (d) 50 L3/week after ivermectin, (e) 50 L3/week before ivermectin, (f) 50 L3/week before and after ivermectin, (g) no larvae. The bars represent the proliferation to: L3 homogenate (1st bar); medium (2nd bar); adult homogenate (3rd bar); adult ES(MT) (4th bar); D6L4 homogenate (5th bar); D6L4 ES(MT) (6th bar).*

Figure 5.14 a

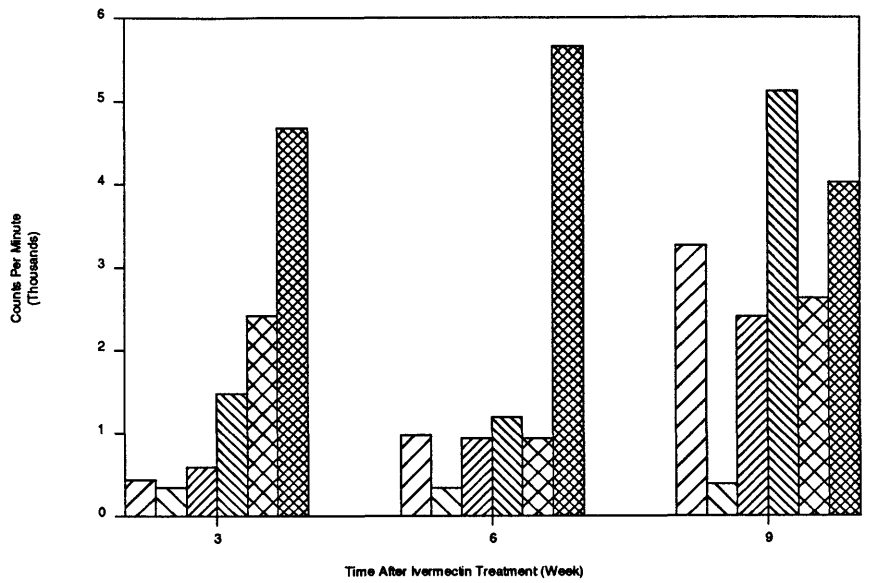


Figure 5.14 b

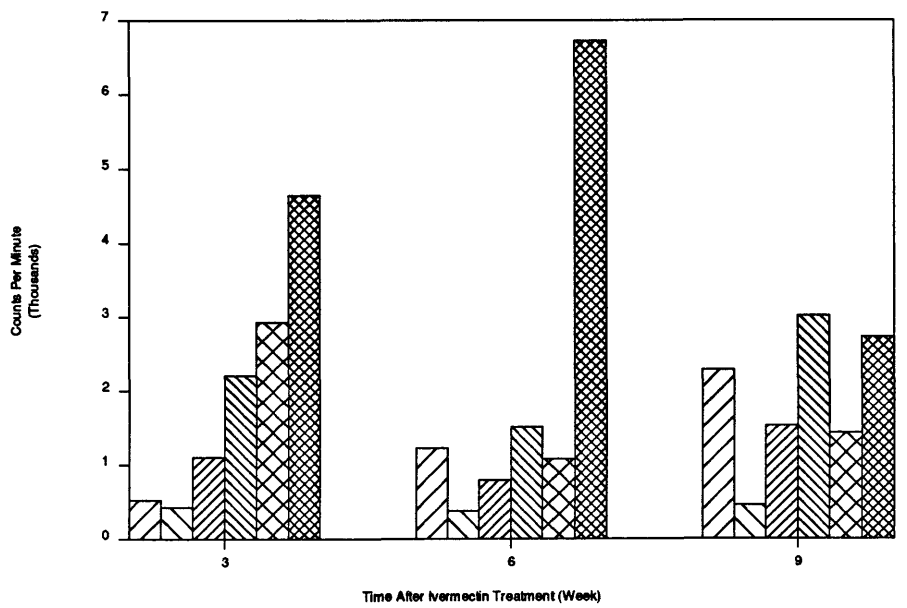


Figure 5.14 c

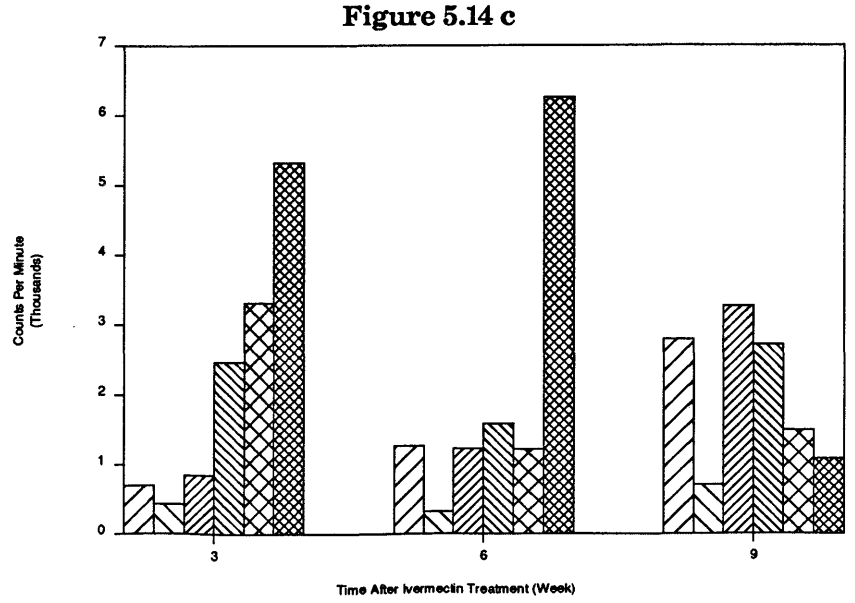


Figure 5.14 d

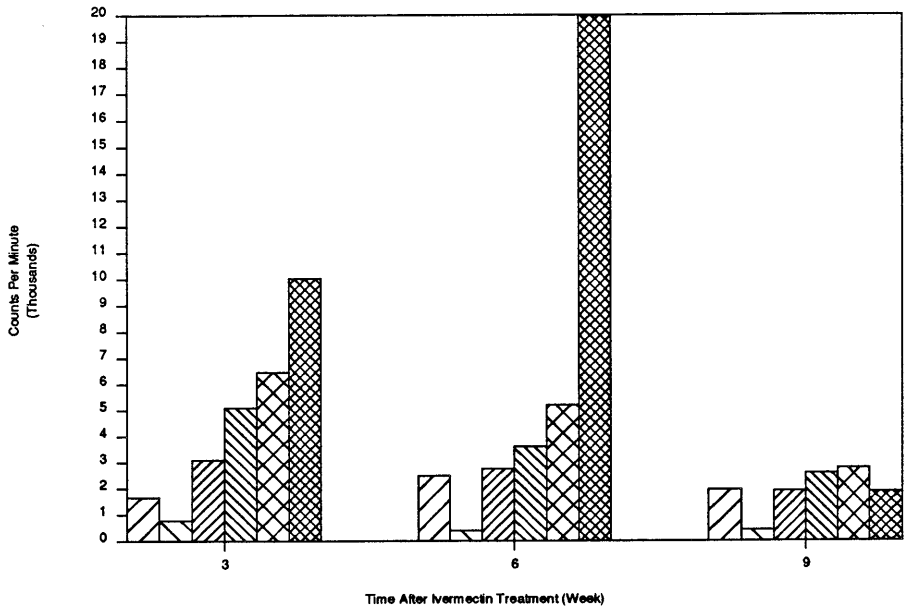


Figure 5.14 e

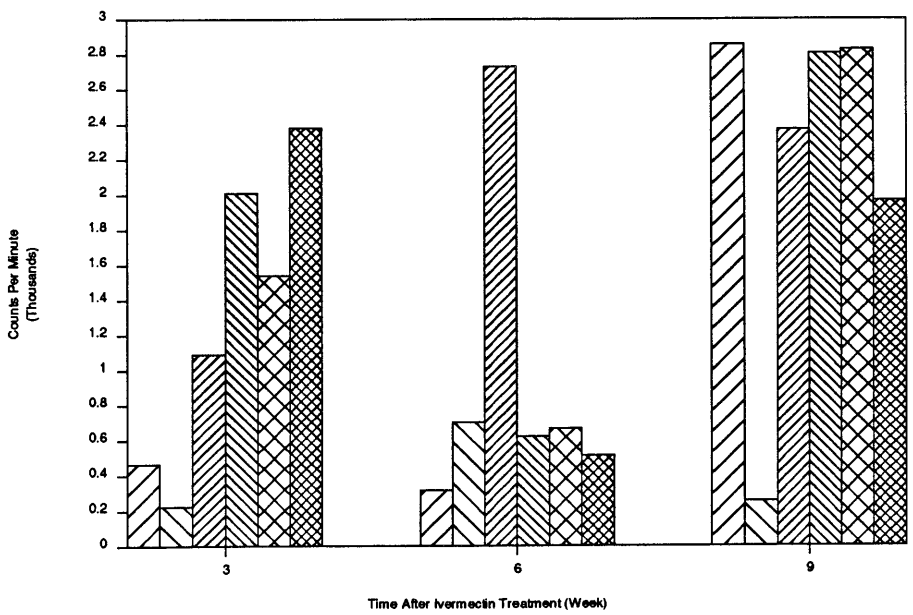
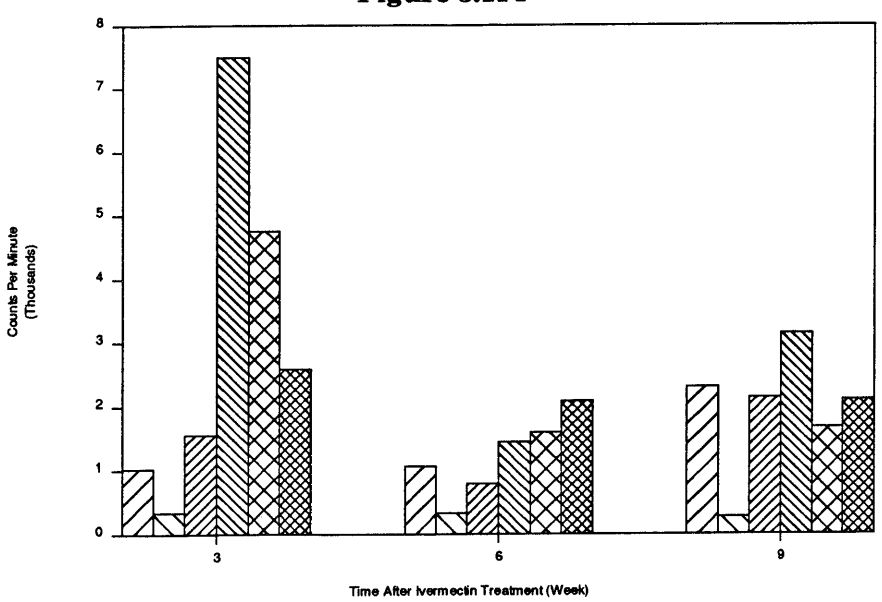


Figure 5.14 f



Figire 4.14 g

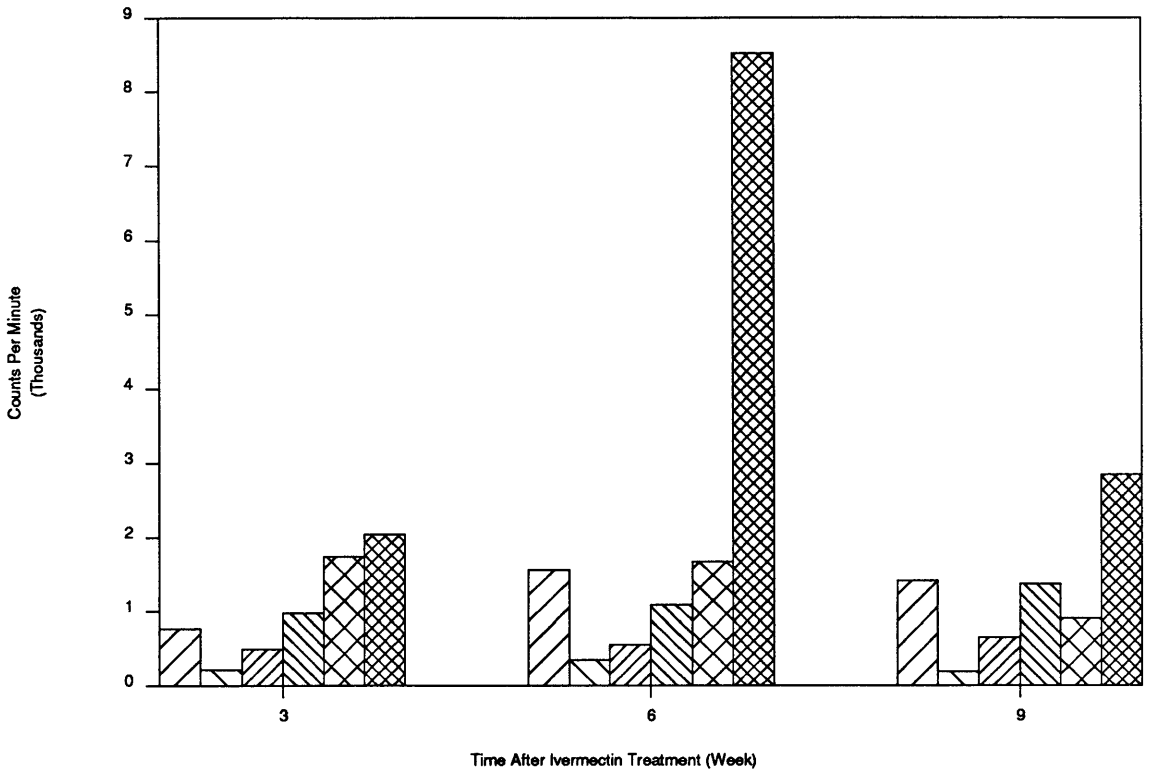


Figure 5.15 *The stimulation indices of cells from CBA mice given larvae at different times relative to ivermectin treatment. The figures are the responses of mice given (a) 10 L3 / week after ivermectin, (b) 10 / week L3 before ivermectin, (c) 10 L3 / week before and after ivermectin, (d) 50 L3 / week after ivermectin, (e) 50 L3 / week before ivermectin, (f) 50 L3 / week before and after ivermectin, (g) no larvae. The bars represent the proliferation to: L3 homogenate (1st bar); medium (2nd bar); adult homogenate (3rd bar); adult ES(MT) (4th bar); D6L4 homogenate (5th bar); D6L4 ES(MT) (6th bar).*

Figure 5.15 a

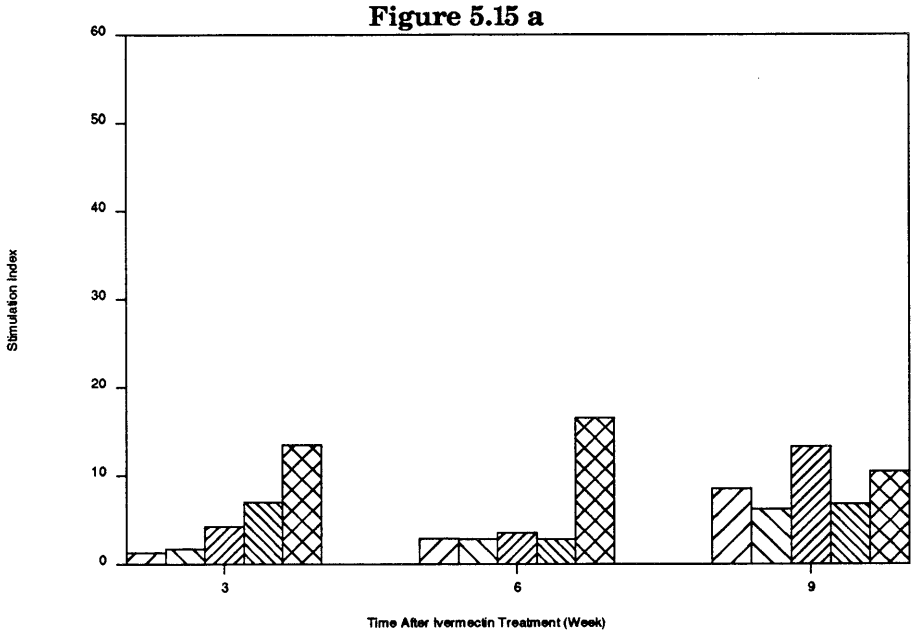


Figure 5.15 b

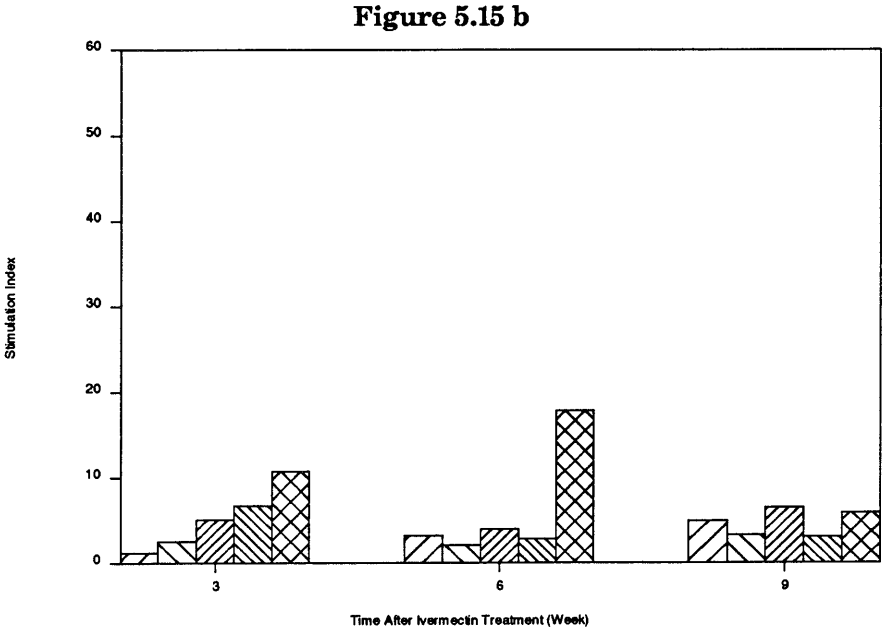


Figure 5.15 c

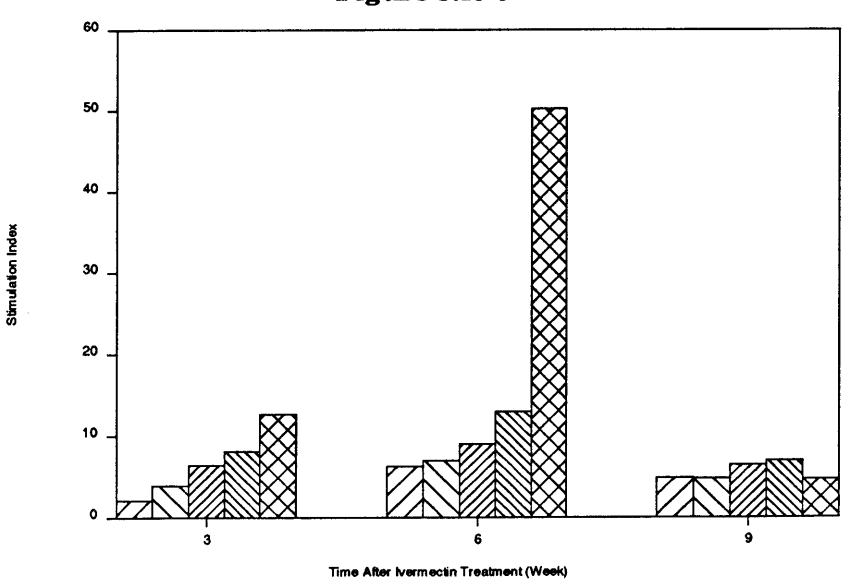


Figure 5.15 d

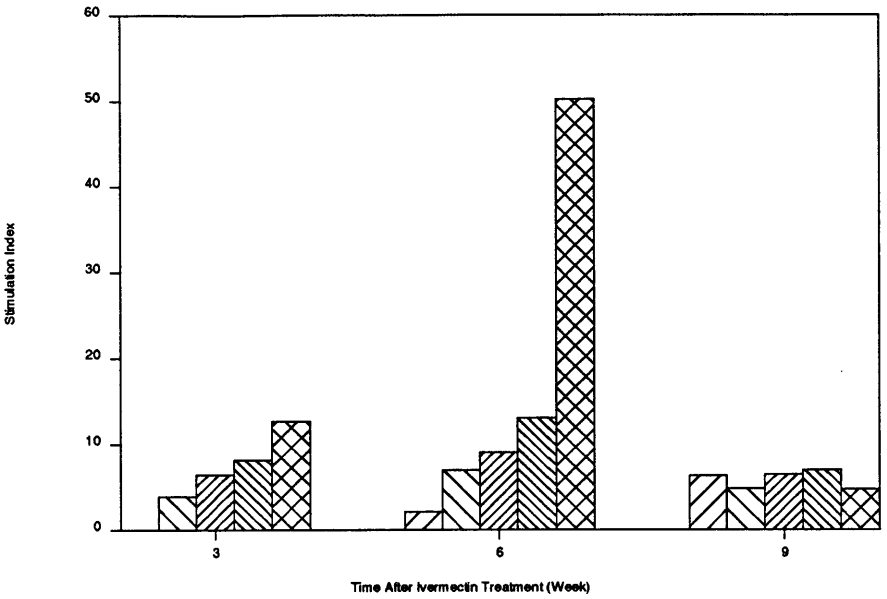


Figure 5.15 e

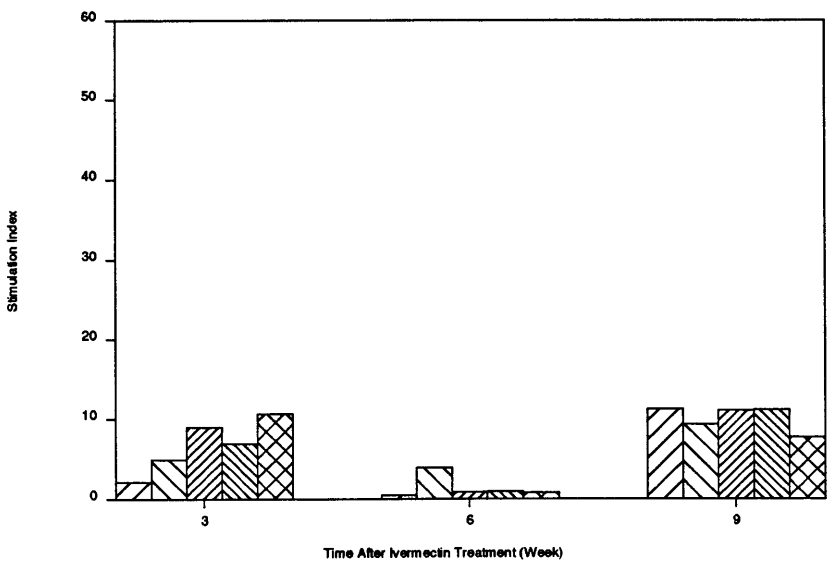


Figure 5.15 f

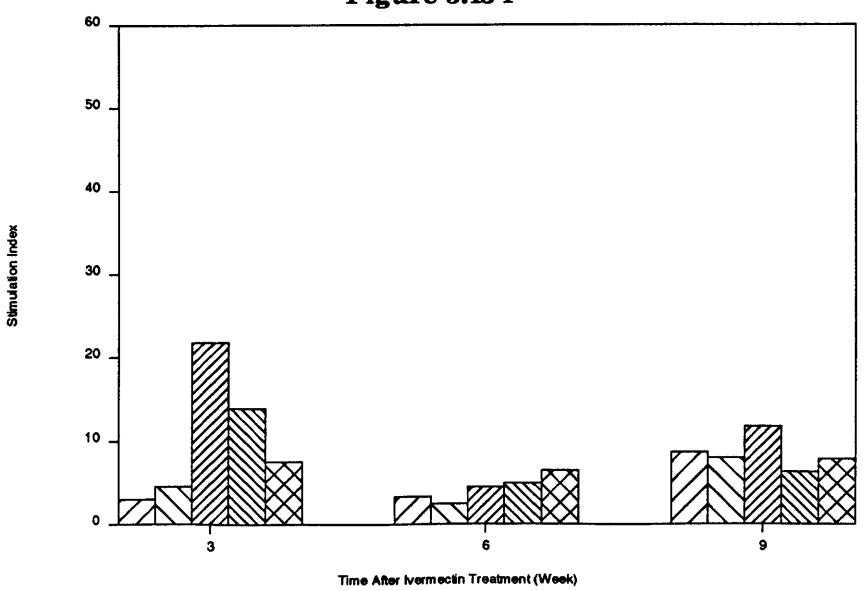
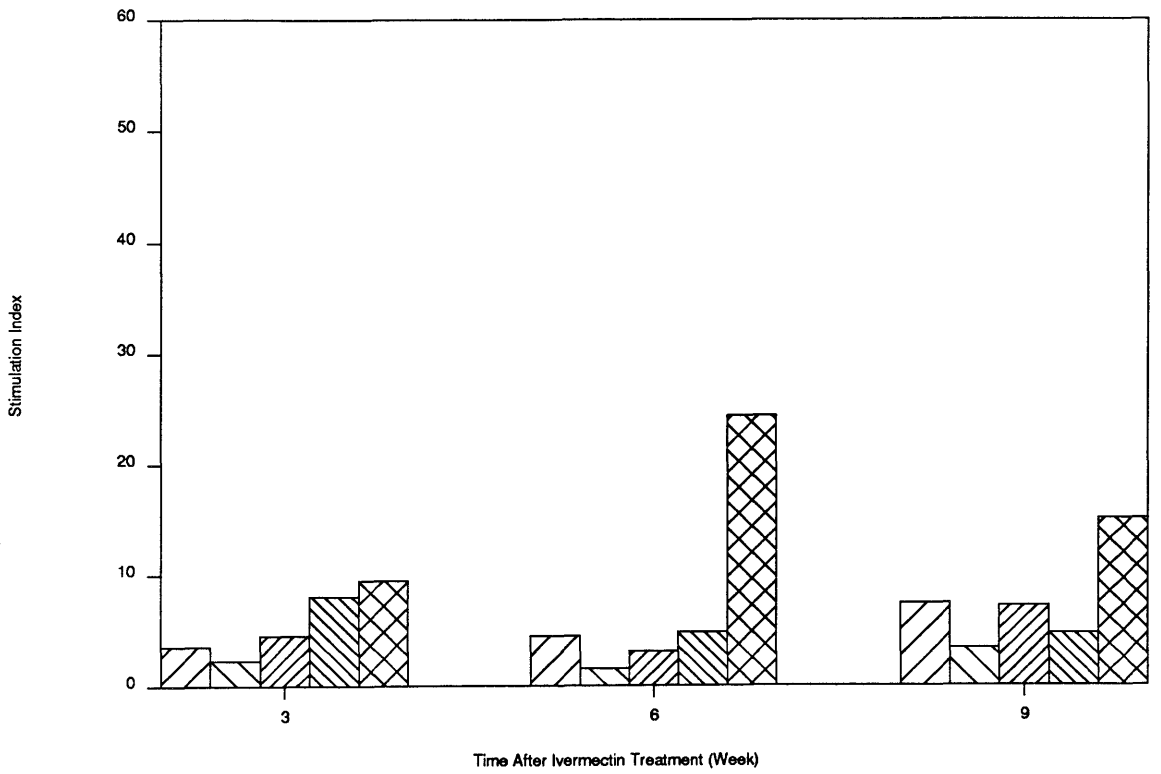


Figure 5.15 g


60
50
40
30
20
10
0
Stimulation Index
3
6
9
Time After Ivermectin Treatment (Week)

5.6 Discussion

5.6.1 Worm burden

The effect of drug treatment on reacquisition of worms was different for the two mouse strains. The drug, ivermectin, was effective at clearing the worms from the intestines of both strains. This result agrees with the high efficiency of the drug against *H. polygyrus* as reported by Sayles and Jacobson (1983).

On reinfection of the hosts parasites were recovered only from the CBA mice, a situation which could be equated to the reacquisition of gastro-intestinal helminths in human populations. This points to the inability to develop fully protective immunity (Ogilvie *et al.*, 1978; Behnke, 1987). Although adult parasites established in CBA mice after anthelmintic treatment, the number that established was lower than in mice which had not previously experienced an infection. This would suggest that a degree of resistance, and therefore parasite recognition, had developed. This development of resistance is almost certainly related to the removal of the adults and the abrogation of their immunosuppressive effect (Pritchard and Behnke, 1985; Dobson *et al.*, 1985).

Parasite establishment probably occurs in the CBA strain as it is more affected by the immunosuppressive capabilities of the parasite than is the NIH strain. Strain dependent susceptibility to immunosuppression has been reported (Behnke, 1987).

The immunosuppressive effects of the parasite last approximately seven weeks after the termination of a primary infection (Dobson, Sitepu and Brindley, 1985). If a longer interval had elapsed between drug treatment and re-exposure to the parasite then greater resistance may have developed.

It is most probable that drug clearance of the adult worms hastened the development of resistance in NIH mice. However, it has previously been shown (see section 4.4) that the mice already show signs of resistance at the time that the

drug was given and thus the anthelmintic was not necessary for the development of resistance.

The lack of a significant increase in the worm burden of mice given larvae before and after ivermectin may have been the result of a normal host functioning. However, it could also be an artefact due to infection of the mice with MHV which resulted in a large number of the mice dying. If the MHV infection caused the heavily infected mice to die then the observed burden would be artificially low. Consequently, no difference between doses would have been detected. Alternatively, low numbers of mice in each cohort may have affected the statistical analysis.

5.6.2 Faecal egg output

In the two strains of mice, the individual female fecundity was not affected by the size of the weekly dose of the parasite given. This is consistent with earlier experiments (see section 4.3.3.4 and 4.3.4.4). The lower fecundity of worms from NIH mice supports earlier suggestions that acquired immunity affects both worm numbers and fecundity.

However, the fact that the worms from CBA mice which show some resistance to *H. polygyrus* (those mice given larvae before and after ivermectin) are no less fecund than those from mice which had not previously experienced the parasite is at odds with this suggestion. Immunity, as measured by a reduction in worm burden, may not affect fecundity (despite observations to the contrary (see section 4.6.1). Indeed, it is possible to separate expulsion effects from anti-fecundity effects (Bell, McGregor and Despommier, 1979; Wakelin and Wilson, 1980; Monroy *et al.*, 1989b). It has been suggested that for *T. spiralis*, anti-fecundity and expulsive responses are under separate genetic control (Rothwell, 1989). Another hypothesis is that the immunosuppression by the adult parasite in some way allows the effects on fecundity to be overcome whilst not affecting the host's resistance to parasite establishment.

5.6.3 Humoral changes

The changes in IgG1 and total immunoglobulin concentration with different doses, treatments and at different times of assaying were similar for both strains of mice. The amount of antibody specific to either of the two homogenates was not different and the production of antibody was dose dependent. These results are consistent with those from the previous experiment.

The experimental design allowed the question of whether or not the increase in antibody production was dependent on continued exposure to the parasite to be addressed. In the sera of mice which had been given larvae only before anthelmintic the amount of antibody did not increase with time. This may be because there was no further stimulation that outstripped the antibody's handling capacity and IgG1 is long-lived (Dobson, Brindley and Sitepu, 1985). In mice given larvae both before and after ivermectin the amount increased. This suggests that the input of infective stages was necessary for the increase in antibody production.

The sera of mice given larvae only after ivermectin had more antibody than the sera of mice given larvae only before ivermectin. This is probably because the former received a greater number of larvae. This lends further support to the notion that the increase is dependent on the accumulated experience of infection. Furthermore, the plateauing of the antibody concentration at the two later times of assaying would tend to suggest that there is a limit to the production of antibody.

After anthelmintic treatment and re-exposure to the parasite, the increase was the same for both the NIH mice (from which worms were recovered) and the CBA mice (from which no worms were recovered). This leads to the supposition that the increase in the anti-adult homogenate antibody is not dependent on the presence of adult worms. If this is the case then the antibodies are directed against the antigens of the larval stages which share cross reactivity with the adult's antigens (see section 4.6.4). As discussed in section 4.6.4 this does not preclude the existence of stage specific antigens, it may just be that the assay as used does not allow the difference due to them to be detected.

5.6.4 Spleen cell counts and cellular responses

It was noted that although there was no difference in the number of spleen cells of infected and naive mice, the cell counts were lower than those of similarly aged mice in the previous experiment. Thus the lack of difference could have been, in part, due to the effects of infection with MHV. The mice in this experiment were also seen to have smaller organs than those in the previous experiment.

When cells from infected NIH mice were cultured with the various antigens prepared from *H. polygyrus*, it was only the adult homogenate that caused significant proliferation and then only with cells from mice given 50 L3/week. The cells from CBA mice proliferated to both the adult homogenate and the D6L4 ES(MT). Cells from the CBA mice given 50 L3/week were the only ones to proliferate to the adult homogenate, cells from mice given either of the doses proliferated to the D6L4 ES(MT). The response to the other antigens was masked by the high levels of non-specific proliferation and the response to the adult homogenate may reflect its weak mitogenic effect.

The response of the cells of mice given either of the doses to the D6L4 ES(MT) could have been an artefact resulting from the MHV infection. However, it could also have reflected the difference in the handling capacity of the MLN in the two strains as discussed in section 3.7.5.

It was not possible to distinguish between the proliferative response of mice which had received different treatments even though they expressed different levels of resistance. It is likely that the differences were obscured by the high levels of non-specific proliferation shown. It is possible that there is no difference as the lymphocyte activity was saturated and a further input of (antigenic) worms could not increase the response above this maximal level. Thus, this level of response may be all that is required for effective resistance. However, as the response was shown to increase with time and dose (see section 4.4) it would be expected that there would be a difference in the response of those mice given

larvae only after ivermectin as compared to those mice given larvae before and after ivermectin. It is important to note that earlier experiments have shown that the high level of non-specific reactivity is not the result of the ivermectin treatment. Other experiments (not reported here) also eliminated the batch of foetal calf serum and the preparations of the antigens as causes of the high background.

5.7 Summary

The results from this chapter agree with those from previous chapters. The difference between the two strains, noted before, is also evident when the abbreviated infections are considered. The results in this chapter indicate that the CBA strain can recognise the parasite and become partially resistant to it. This is in contrast to the NIH mice which become completely resistant to the parasite after abbreviation of an infection.

The fecundity of the worms is lower in the more resistant NIH mice. In the CBA mice which are partially resistant to the parasite the fecundity is as great as in the mice which have not previously experienced the parasite. This suggests that whilst the parasite cannot affect the host's effects on worm establishment it can reduce the effects of immunity on fecundity.

As in previous chapters the immunoglobulin changes were the same for both strains indicating that immunosuppression has little effect on the humoral response. The production of the antibody in both strains is positively associated with the host's exposure to the parasite.

Chapter Six: General Summary and Discussion

Infection with *H. polygyrus* causes a number of changes in the host's immune system. Amongst the alterations induced by infection is an increase in the number of eosinophils, neutrophils and mast cells. This study, however, chose to investigate changes in the host's T lymphocyte and humoral responses.

The cellular response was assayed using the *in vitro* lymphocyte proliferation assay. Cells were incubated with Con A to establish the assay conditions. It was observed that the magnitude of the response to the mitogen was highly variable within defined experimental conditions. Other studies that investigated antigen-specific proliferation also recorded marked heterogeneity in response. The causes of variability are discussed particularly that which is inherent in the assay. This part of the variability is due to the exponential growth of cell populations and must be accounted for in any analysis.

Differences in the ability of the two mouse strains to respond to the parasite were recorded. These differences were evident in their worm burdens and the fecundity of the worms they harboured. Both of these parameters were lower for NIH mice than for CBA mice and the parasite showed different dynamics in the two types of host. These differences are reflected in the ability of the spleen cells of the two strains to proliferate to the antigens of *H. polygyrus*: the cells of NIH mice proliferated more than the cells of CBA mice. In addition the dynamics of the responses were different. However, the humoral response was similar for the two strains.

Although T-cells may not be the ultimate effectors of immunity, and may not even be the most important factors in determining immunity, they may be an indicator of response status. The *in vitro* cellular response is not, it would seem, an indicator of past experience of infection despite there being a dose-dependence in the response. Furthermore, it cannot be used as an indicator of worm burden. What may be more useful as an *approximate* indicator of experience of infection is

the serum antibody level. In both strains of mice the amount of IgG1 increases with increasing input of larvae.

The difference between the two strains may relate to the parasite burden, and, therefore, the concentration of antigens necessary to trigger a response. This threshold may be lower in the high responder NIH strain than in the low responder CBA strain. It is unlikely that the regulation of the parasite burden is achieved by density dependent mechanisms alone. It is probable that differences in the dynamics of the parasite in the two strains are the result of differences in the degree to which the two strains acquire immunity.

The main conclusions of the study may be summarised as follows:

(a) The antigens of *H. polygyrus* can be mitogenic. The degree of mitogenicity depended on the stage from which the antigen was prepared. The non-specific properties of the antigens depended on their preparation and the cells with which they were cultured. In general, the adult homogenate was the least mitogenic of the antigens. Of the others, there was not always a difference in their non-specific properties but when there was it was generally the ES(MT)s that caused the most non-specific proliferation. The different levels of proliferation to the antigens could be due to their importance as immunogens or the anatomical location of the stage producing them.

(b) There were organ-specific reactions to the various preparations of *H. polygyrus*. The spleen (of immunised mice and mice given a single infection) was the only organ whose cells proliferated to adult homogenate. Similarly, the MLN is the only source of cells which proliferate to D6L4 ES(MT) antigen. The spleen cells of trickle infected mice which proliferated to the D6L4 ES(MT) were those of mice given the highest weekly dose. This suggests a threshold in the antigen handling

capacity of the MLN. The different responses in the two organs may be due to differences in the composition of their cell populations.

(c) The D6L4 homogenate and the D6L4 ES(MT) caused different patterns of proliferation when incubated with MLN cells of immunised mice. The different antigen-dose response patterns for the two preparations suggest a difference in their immunogenic/proliferation inducing constituents. The response to the two antigens increased with increasing time of incubation.

Two of the protocols in which the infections were abbreviated before adults developed gave rise to cells which proliferated to adult homogenate. This would suggest that there is cross-reactivity between the antigens of the adult and larval stages.

(d) The MLN cells of mice which received low doses of *H. polygyrus* for six weeks proliferated to some of the antigens of *H. polygyrus*. It was possible to distinguish between mice given different doses by their ability to proliferate to these antigens. However, cells of mice given 200 L3/week did not proliferate to a greater degree than cells of mice given 25 L3/week. Thus the relationship between proliferative response and larval dose was not linear. This non-linearity was probably the result of the dynamics of the cellular response rather than a result of true differences in *in vivo* immune functioning. The results were repeatable although the size of the response was seen to differ. This emphasises the need to consider inter-experimental variation.

(e) On repeated infection of low and high responder mice, different patterns of change in worm burden with time were observed. The pattern for NIH mice was convex, the time at which the last worm was detected being dependent on the dose given. In CBA mice the worm burden increased with time then plateaued. The size of the worm burden was dependent on the dose given. The difference in the pattern of worm burden with time for the two strains is probably related to the effectiveness of their acquired immune response and their ability to overcome the

parasite's immunosuppressive capabilities. On interrupting an infection and re-exposing the mice to the parasite no worms were recovered from the NIH mice. However, worms were recovered from CBA mice although their number was less than in mice which had not been given larvae. This indicates that CBA mice, although low responders, can develop partial resistance.

(f) The difference in the parasite burdens of the two strains also correlates with the fecundities of their worms. The fecundity of the female worms was less in NIH mice although in both strains the fecundity does not appear to be affected by the number of worms in the intestine. In NIH mice the fecundity of the individual female worms decreased as the worm burden decreased. In CBA mice given repeated doses the number of eggs per female does not decrease with time. In contrast to NIH mice the fecundity of worms in mice showing resistance (those with a lower worm burden) was not lower than that of mice showing little or no resistance (cf mice given L3 before and after ivermectin vs mice given L3 after ivermectin). This would suggest that the parasite may overcome established resistance or that the anti-fecundity effects are different to the effects causing reduced establishment/worm survival.

(g) The number of cells in the spleens of CBA mice given the highest dose of repeated low level doses of *H. polygyrus* was initially greater than in the spleens of uninfected mice. With increased length of time the spleen diminished in size. The spleens of NIH mice did not change in size. This may represent differences in the antigen handling capacity of the spleens of the two strains. The regression in size of the spleen of CBA mice could reflect an increase in the antigen handling capacity of that strain's MLN.

(h) The amount of immunoglobulin produced by the two strains to either of the two homogenates (D6L4 and adult homogenates) used was not different. This leads to the supposition that antibody cannot be the sole effector of resistance.

(i) The amount of *H. polygyrus* specific antibody produced was dose dependent. The amount also increased with the number of infections given. However, this increase was not dependent on the presence of adults and would suggest that the increase is caused by the input of larval stages, there being a large amount of cross-reactivity between the two homogenates used.

(j) The change in proliferation with time of the spleen cells of the two strains was different. The proliferation of the spleen cells of NIH mice increased with time whereas the spleen cells of CBA mice showed first an increase and then a decrease. This difference may be due to differences in ability to overcome the parasites immunosuppression and/or a switching of the relative importance of T-cell subsets. The continued increase in proliferation in NIH mice even in the absence of adult parasites would suggest that the increase in response to their antigens is not dependent on their presence. Rather the increase is due to the input of larval stages and cross-reactivity of their antigens with those of the adults.

In summary, this investigation of immune changes during a chronic parasitic infection raises many interesting points. Many of these questions are worthy of further research. Future work should be aided by the many recent technical advances that have occurred in the field of immunology.

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Appendix 1: Statistical Analysis of Results from Chapter 3

Statistical comparisons were made on log transformed data using the analysis of variance test (ANOVA). If a significant difference was found, the levels within a factor were compared pair-wise using the Student-Newman-Keuls (S.N.K.) test. On comparing one level with all the others Dunnett's test was used. The statistical methods used were those given by Sokal and Rohlf (1981).

Table A1.1 Proliferation to Concavalin A

Comparison: Response at different cell Con A concentrations

Test	Statistic	Deg. Free.	Probability
Anova	F=70.09	v1=7, v2=80	P<0.05

Comparison: Response of different mice to Con A

Test	Statistic	Deg. Free	Probability
Anova	F=6.129	v1=4, v2=108	P<0.05
S.N.K. highest vs lowest responder:	q=4.6	v=108, p=5	P<0.05
S.N.K. Highest vs 2nd lowest responder:	q=2.01	v=108, p=4	P>0.05

Comparison: Response to Con A in different assays

Test	Statistic	Deg Free	Probability
Anova	F=8.34	v1=2, v2=30	P<0.05
S.N.K. assay 1 vs assay 2:	q=5.84	v=30, p=2	P<0.05
S.N.K. assay 1 vs assay 3:	q=0.08	v=30, p=3	P>0.05

Table A1.2 Response of naive mice to H. polygyrus antigens

(a) Adult homogenate at different cell densities.

Comparison: Proliferation to medium vs proliferation to antigen

Test	Statistic	Deg. Free.	Probability
Anova	F=2.31	v1=7, v2=11	P>0.05

Comparison: proliferation to medium at different cell densities

Test	Statistic	Deg. Free. Probability	
Anova	F=4.96	v1=4, v2=11	P<0.05

(b) D6L4 homogenate and adult homogenate at one cell density

Comparison: proliferation to medium vs proliferation to D6L4

Test	Statistic	Deg. Free. Probability	
Anova	F=2.06	v1=7, v2=24	P>0.05

Comparison: proliferation to medium vs proliferation to adult

Test	Statistic	Deg. Free	Probability
Anova	F=4.02	v1=7, v2=24	P<0.05
S.N.K. medium vs 6.25 ug/ml homogenate:	q=9.87	v=25, p=3	P>0.05

Table A1.3 Response of cells of mice given one infection

Comparison: Naive vs infected response to adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=0.01	v1=1, v2=23	P>0.05

Comparison: Naive vs infected response to D6L4 homogenate

Test	Statistic	Deg. Free	Probability
Anova	F=47.97	v1=1, v2=23	P<0.05

Comparison: response to adult vs response to D6L4

Test	Statistic	Deg. Free.	Probability
Anova	F=49.62	v1=1, v2=44	P<0.05

Table A1.4 Responses of cells from immunised mice

(a) *Comparison*[1]: Response of mice immunised by S&J1 protocol

Test	Statistic	Deg. Free.	Probability
Anova for adult homog.[2]	F=0.01	v1=1, v2=24	P>0.05
Anova for adult ES(MT)	F=7.73	v1=1, v2=24	P>0.05
Anova for D6L4 homog.	F=25.26	v1=1, v2=24	P<0.05
Anova for D6L4 ES(MT)	F=9.1	v1=1, v2=24	P<0.05
Anova for L3 homog.	F=1.98	v1=1, v2=24	P>0.05

(b) *Comparison*: mice immunised by irrad[3] protocol

Anova for adult homog.	F=3.21	v1=1, v2=24	P>0.05
Anova for adult ES(MT)	F=1.19	v1=1, v2=24	P>0.05
Anova for D6L4 homog.	F=59.9	v1=1, v2=24	P<0.05
Anova for D6L4 ES(MT)	F=117.3	v1=1, v2=24	P<0.05
Anova for L3 homog.	F=3.17	v1=1, v2=24	P>0.05

(c)*Comparison*: Mice immunised by S&J3 protocol

Anova for adult homog.	F=0.48	v1=1, v2=24	P>0.05
Anova for adult ES(MT)	F=79.07	v1=1, v2=24	P<0.05
Anova for D6L4 homog.	F=377.08	v1=1, v2=24	P<0.05
Anova for D6L4 ES(MT)	F=75.8	v1=1, v2=24	P<0.05
Anova for L3 homog.	F=1.34	v1=1, v2=24	P>0.05

(d)*Comparison*: Mice immunised by Behnke protocol

Anova	F=14.83	v1=7, v2=223	P<0.05
S.N.K.[4] on adult homog.	q=2.08	v=223, p=2	P>0.05
S.N.K. on adult ES(MT)	q=3.4	v=223, p=3	P<0.05
S.N.K. on D6L4 homog.	q=7.16	v=223, p=4	P<0.05
S.N.K. on D6L4 ES(MT)	q=7.36	v=223, p=5	P<0.05

(e)*Comparison*: Response to medium of infected cells vs naive cells

Test	Statistic	Deg. Free.	Probability
T test	T=21.3	v=24	P<0.05

(f)*Comparison*: Proliferation of cells of irrad mice after different lengths of incubation.

Test	Statistic	Deg. Free.	Probability
Anova	F=7.57	v1=3, v2=12	P<0.05

1: Comparison between response of infected and naive mice.
2: homog.= homogenate
3: Response of infected mice incubated for 96 hr compared to response of naive mouse cells to the same antigen.
4: Comparison made between infected and naive mice

Table A1.5 Response of spleen cells and MLN cells

Comparison: Response in spleen vs response in MLN

Antigen	Test	Statistic	Deg. Free.	Prob.
Adult homog.	Anova	F=64.65	v1=2, v2=10	P<0.05
	S.N.K	q=10.77	v=10, p=2	P.05
D6L4 homog.	Anova	F=52.13	v1=2, v2=10	P<0.05
	S.N.K.	q=6.52	v=10, p=2	P<0.05
D6L4 ES(MT)	Anova	F=25.26	v1=2, v2=10	P<0.05
	S.N.K.	q=4.94	v=10, p=2	P<0.05
L3 homog.		Anova	F=27.39	v1=2, v2=10
	S.N.K	q=3.1	v=10, p=2	P>0.05

Comparison: Response of MLN and spleen cells from infected vs uninfected mice.

Test	Statistic	Deg. Free.	Probability
T-test	T=0.01	v=12	P>0.05

Appendix 2: Statistical Analysis of Results from Chapter 4

Table A3.1.1 Results from the first assay to determine lymphoproliferative conditions of MLN cells from trickled mice.

(a) *Comparison*: Proliferation of cells of naive mice vs infected mice

Test	Statistic	Deg. Free.	Probability
Anova on Adult homogenate	F=1.09	v1=3, v2=9	P>0.05
Anova on Adult ES(MT)	F=8.83	v1=3,v2-9	P<0.05
Anova on D6L4 homogenate	F=5.4	v1=3, v2=9	P<0.05
Anova on D6L4 ES(MT)	F=6.45	v1=3, v2=9	P<0.05
Anova on L3 homogenate	F=4.49	v1=3, v2=9	P<0.05
Anova on Con A	F=0.87	v1-3, v2=9	P>0.05

(b) *Comparison*: Response of mice given different doses to D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
S.N.K. dose 50 vs dose 25	q=2.95	v=9, p=2	P>0.05
S.N.K. dose 50 vs dose 10	q=6.15	v=9, p=3	P<0.05
S.N.K. dose 10 vs dose 200	q=1.53	v=9, p=2	P>0.05

(c) *Comparison*: Response of mice given different doses to D6L4 homog.

Test	Statistic	Deg. Free.	Probability
S.N.K. dose 50 vs dose 25	q=2.33	v=9, p=2	P>0.05
S.N.K. dose 50 vs dose 10	q=4.87	v=9, p=3	P<0.05
S.N.K dose 10 vs dose 200	q=4.78	v=9, p=2	P>0.05

(d) *Comparison*: Response of cells to the various antigens

Test	Statistic	Deg. Free.	Probability
S.N.K. D6L4 ES vs D6L4 homog.	q=4.61	v=78, p=2	P<0.05
S.N.K. D6L4 ES vs L3 homog.	q=7.27	v=78, p=3	P<0.05
S.N.K. L3 homog vs D6L4 homog.	q=2.72	v=12, p=2	P>0.05

Table A3.1.2 Results from the second assay to determine lymphoproliferative conditions of MLN cells from trickled mice.

(a) *Comparison*: Proliferation of cells of naive mice vs infected mice.

Test	Statistic	Deg. Free.	Probability
Anova on Adult homog.	F=1.16	v1=3, v2=12	P>0.05
Anova on D6L4 homog.	F=1.47	v1=3, v2=12	P>0.05
Anova on D6L4 ES(MT)	F=10.29	v1=3, v2=12	P<0.05
Anova on L3 homog.	F=7.05	v1=3, v2=12	P<0.05

(b) *Comparison*: Response of mice given different doses to D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
S.N.K. dose 50 vs dose 25	q=0.76	v=12, p=2	P>0.05
S.N.K. dose 50 vs dose 10	q=6.22	v=12, p=3	P<0.05
S.N.K. dose 10 vs dose 200	q=1.53	v=9, p=2	P>0.05

Table A2.2 Epidemiological data from repeated low level infections in NIH mice

(a) *Comparison*: Worm burdens for mice given different doses

Test	Statistic	Deg. Free.	Probability
Anova	F=20.9	v1=5, v2=40	P<0.05
S.N.K. dose 50 vs dose 2	q=4.18	v=40, p=3	P<0.05
S.N.K. dose 50 vs dose 10	q=2.9	v=40, p=2	P<0.05
S.N.K. dose 10 vs dose 2	q= 1.98	v=40, p=2	P>0.05

(b) *Comparison*: Sex ratio

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=0.53	v1=2, v2=40	P>0.05
Anova for time	F=1.94	V1=2, v2=40	P>0.05

(c) *Comparison*: Faecal Egg Output

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=2.25	v1=2, v2=40	P>0.05
Anova for time	F=9.88	v1=2, v2=40	P<0.05

(d) *Comparison*: Spleen cell counts

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=0.01	v1=3, v2=111	P>0.05
Anova for time	F=23.21	v1=3, v2=111	P<0.05

Table A2.3 Immunoglobulin levels in NIH mice given repeated low level infections

(a) *Comparison*: Total antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.63	v1=1, v2=171	P>0.05
Anova for time	F=104.73	v1=5, v2=171	P<0.05
Anova for dose	F=35.02	v1=2, v2=172	P<0.05
S.N.K. dose 2 vs dose 10	q=5.5	v=171, p=2	P<0.05
S.N.K. dose 2 vs dose 50	q=11.72	v=171, p=3	P<0.05
S.N.K. dose 10 vs dose 50	q=6.73	v=171, p=2	P<0.05

(b) *Comparison*: IgG1 levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=2.179	v1=1, v2=171	P>0.05
Anova for time	F=179.4	v1=5, v2=171	P<0.05
Anova for dose	F=9.3	v1=2, v2=172	P<0.05
S.N.K. dose 2 vs dose 10	q=5.5	v=171, p=2	P<0.05
S.N.K. dose 2 vs dose 50	q=5.89	v=171, p=3	P<0.05
S.N.K. dose 10 vs dose 50	q=1.67	v=171, p=2	P>0.05

Table A2.4 Proliferation of cells of NIH mice given repeated low level infections of *H. polygyrus*

(a) *Comparison*: Responses of cells from naive mice to the antigens of *H. polygyrus*

Antigen	Test	Statistic	Deg. Free.	Probability
	Anova	F=27.05	v1=5, v2=169	P<0.05
Adult homog	Dunnett's1	d=0.79	v=169, p=1	P>0.05
L3 homog.	"	d=10.35	v=169, p=2	P<0.05
D6L4 homog.	"	d=11.9	v=169, p=3	P<0.05

D6L4 ES(MT)	"	d=14.02	v=169, p=4	P<0.05
Adult ES(MT)	"	d=15.3	v=169, p=5	P<0.05
Adult ES(MT) vs L3 homog."		S.N.K.	q=3.08, v=169	P>0.05

(b) *Comparison*: Responses of cells from infected mice to the antigens of *H. polygyrus*

Antigen	Test	Statistic	Deg. Free.	Probability
-	Anova	F=69.07	v1=5, v2=706	P<0.05
Adult homog.	Dunnett's1	d=8.94	v=706, p=1	P<0.05
D6L4 ES(MT).	"	d=12.8	v=706, p=2	P<0.05
D6L4 homog.	"	d=13.9	v=706, p=3	P<0.05
Adult ES(MT)	"	d=15.7	v=706, p=4	P<0.05
L3 homog.	"	d=15.8	v=706, p=5	P<0.05
D6L4 ES(MT) vs L3 hom.	S.N.K.	q=4.4	v=706, p=4	P<0.05

(c) *Comparison*: response to adult vs response to other antigens

Antigen	Test	Statistic	Deg. Free.	Probability
D6L4 ES(MT)	Dunnett's	d=3.89	v=706, p=2	P<0.05
D6L4 homog.	"	d=4.9	v=706, p=3	P<0.05
Adult ES(MT)	"	d=6.76	v=706, p=4	P<0.05
L3 homog.	"	d=6.96	v=706, p=5	P<0.05

(d) *Comparison*; Change in proliferation with time

Test	Statistic	Deg. Free.	Probability
Anova	F=60.35	v1=5, v2=706	P<0.05

(e) *Comparison*: Proliferation for each antigen considered separately

(i) L3 homogenate

Test	Statistic	Deg. Free	Probability
Anova	F=2.17	v1=3, v2=96	P<0.05
S.N.K. dose 2 vs dose 10	q=0.97	v=96, p=2	P>0.05
S.N.K. dose 2 vs dose 50	q=5.04	v=96, p=3	P
<0.05			

(ii) Adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=2.3	v1=3, v2=96	P<0.05
S.N.K. dose 2 vs dose 10	q=1.78	v=96, p=2	P>0.05
S.N.K. dose 2 vs dose 50	q=6.8	v=96, p=3	P<0.05

(iii) D6L4 homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=1.25	v1=3, v2=96	P<0.05
S.N.K. dose 2 vs dose 10	q=0.79	v=96, p=2	P>0.05
S.N.K. dose 2 vs dose 50	q=5.21	v=96, p=3	P<0.05

(iv) Adult ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova	F=10.26	v1=3, v2=96	P<0.5
S.N.K. dose 0 vs dose 2	q=2.5	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=3.86	v=96, p=3	P>0.05

(v) D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova	F=3.4	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=2.7	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=2.1	v=96, p=3	P>0.05

Table A2.5 Stimulation indices for antigens cultured with cells from mice given repeated low level infections

(a) *Comparison*: SI of Adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=8.58	v1=3, v2=96	P<0.05
S.N.K. dose 2 vs dose 50	q=3.22	v=96, p=3	P>0.05

(b) *Comparison*: SI of Adult ES(MT)

Anova	F=6.09	v1=3, v2=96	P<0.05
S.N.K.dose 2 vs dose 50	q=1.33	v=96, p=3	P>0.05

(c) *Comparison*: SI of L3 homogenate

Anova	F=12.8	v1=3, v2=96	P<0.05
S.N.K. dose 2 vs dose 50	q=3.08	v=96, p=3	P>0.05

(d) *Comparison*: SI of D6L4 homogenate

Anova	F=2.67	v1=3, v2=96	P>0.05

(e) *Comparison* of D6L4 ES(MT)

Anova	F=0.36	v1=3, v2=96	P>0.05

Table A2.6 Epidemiological data from repeated low level infections in CBA mice

(a) *Comparison*: Worm burdens for mice given different doses

Test	Statistic	Deg. Free.	Probability
Anova	F=103.9	v1=5, v2=82	P<0.05
S.N.K. dose 50 vs dose 2	q=20.25	v=82, p=3	P<0.05
S.N.K. dose 50 vs dose 10	q=12.16	v=82, p=2	P<0.05
S.N.K. dose 10 vs dose 2	q= 8.2	v=82, p=2	P<0.05
S.N. K. week 6 vs week 10: q=1.37, v=68. p=3 P<0.05			

(b) *Comparison*: Sex ratio

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=1.15	v1=2, v2=82	P>0.05
Anova for time	F=0.77	v1=2, v2=82	P>0.05

(c) *Comparison*: Faecal Egg Output

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=4.43	v1=2, v2=68	P<0.05
S.N.K. dose 2 vs dose 10	q=3.42	v=68, p=2	P<0.05
S.N.K. dose 2 vs dose 50	q=0.87	v=68, p=3	P>0.05
S.N.K. dose 10 dose 50	q=2.39	v=68	P>0.05
Anova for time	F=105.34	v1=2, v2=68	P<0.05
S.N.K. wk 2 vs wk 4	q=22.25	v=68, p=3	P<0.05
S.N.K. wk 4 vs wk 12	q=1.49	v=68, p=4	P<0.05

(d) Comparison: spleen cell counts

Test	Statistic	Deg. Free.	Probability
Anova for controls	F= 3.58	v1=5, v2=24	P>0.05
Anova for time	F=12.19	v1=5, v2=111	P<0.05
Anova for dose	F=17.66	v1=3, v2=111	P<0.05
S.N.K. dose 0 vs dose 50	q=8.66	v=111, p=4	P<0.05
S.N.K. dose 0 vs dose 10	q=2.46	v=111, p=3	P>0.05
S.N.K. dose 0 vs dose 2	q=0.55	v=111, p=2	P>0.05

Table A2.7 Immunoglobulin levels in CBA mice given repeated low level infections

(a) *Comparison*: Total antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=7.56	v1=1, v2=171	P<0.05
Anova for time	F=75.6	v1=5, v2=171	P<0.05
Anova for dose	F=24.69	v1=2, v2=172	P<0.05
S.N.K. dose 2 vs dose 10	q=1.35	v=171, p=2	P>0.05
S.N.K. dose 2 vs dose 50	q=9.6	v=171, p=3	P<0.05
S.N.K. dose 10 vs dose 50	q=6.79	v=171, p=2	P<0.05

(b) *Comparison*: IgG1 levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.004	v1=1, v2=171	P>0.05
Anova for time	F=111.86	v1=5, v2=171	P<0.05
Anova for dose	F=11.25	v1=2, v2=172	P<0.05
S.N.K. dose 2 vs dose 10	q=4.69	v=171, p=2	P<0.05
S.N.K. dose 2 vs dose 50	q=8.79	v=171, p=3	P<0.05
S.N.K. dose 10 vs dose 50	q=1.67	v=171, p=2	P>0.05

Table A2.8 Proliferation of cells of CBA mice given repeated low level infections of *H. polygyrus*

(a) *Comparison*: Responses of cells from naive mice to the antigens of H. polygyrus

Antigen	Test	Statistic	Deg. Free.	Probability
-	Anova	F=18.26	v1=5, v2=169	P<0.05
Adult homog.	Dunnett's1	d=4.11	v=169, p=2	P>0.05
D6L4 homog.	"	d=6.64	v=169, p=3	P<0.05
Adult ES(MT)	"	d=12.1	v=169, p=4	P<0.05
D6L4 ES(MT)	"	d=14.95	v=169, p=5	P<0.05
L3 homog.	"	d=19.5	v=169, p=6	P<0.05
Adult homog. vs L3 homog.	S.N.K.	q=9.96	v=169, p=5	P<0.05
L3 homog. vs D6L4 honog.	S.N. K.	q=6.64	v=169, p=4	P<0.05
Adult ES vs adult hom.	S.N.K.	q=4.77	v=169, p=4	P<0.05

(b) *Comparison*: Responses of cells from infected CBA mice *H. polygyrus* antigens

Antigen	Test	Statistic	Deg. Free.	Probability
-	Anova	F=52.21	v1=5, v2=706	P<0.05
Adult homog.	Dunnett's1	d=7.35	v=706, p=1	P<0.05
D6L4 ES(MT).	"	d=12.4	v=706, p=2	P<0.05
D6L4 homog.	"	d=13.5	v=706, p=3	P<0.05
Adult ES(MT)	"	d=16.02	v=706, p=4	P<0.05
L3 homog.	"	d=16.3	v=706, p=5	P<0.05

(c) *Comparison*; Change in proliferation with time

Test	Statistic	Deg. Free.	Probability
nova	F=75.68	v1=5, v2=706	P<0.05

(d) *Comparison*: Proliferation for each antigen considered separately

(i) L3 homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=10.16	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=14.4	v=96, p=2	P>0.05
S.N.K. dose 10 vs dose 50	q=4.61	v=96, p=2	P.05

(ii) Adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=18.3	v1=3, v2=96	P.05
S.N.K. dose 0 vs dose 2	q=3.6	v=96, p=2	P>0.05
S.N.K. dose 10 vs dose 50	q=4.69	v=96, p=2	P<0.05

(iii) D6L4 homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=10.76	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=2.18	v=96, p=2	P>0.05
S.N.K. dose 2 vs dose 50	q=5.21	v=96, p=3	P<0.05
S.N.K. dose 10 vs dose 50	q=3.88	v=96, p=2	P<0.05

(iv) Adult ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova	F=9.15	v1=3, v2=96	P<0.5
S.N.K. dose 0 vs dose 2	q=0.32	v=96, p=2	P<0.05
S.N.K. dose 0 vs dose 10	q=4.14	v=96, p=3	P>0.05
S.N.K. dose 10 vs dose 50	q=1.58	v=96, p=2	P>0.05

(v) D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova	F=6.73	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=2.17	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=5.46	v=96, p=3	P>0.05
S.N.K. dose 10 vs dose 50	q=0.54	v=96, p=2	P>0.05

Table A2.9 Stimulation indices for antigens cultured with cells from mice given repeated low level infections

(a) *Comparison*: SI of adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova	F=5.87	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=0.467	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=3.13	v=96, p=3	P>0.05

(b) *Comparison*: SI of Adult ES(MT)

Anova	F=3.7	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=0.69	v=96, p=3	P>0.05
S.N.K. dose 0 vs dose 10	q=0.18	v=96, p=3	P>0.05

(c) *Comparison*: SI of L3 homogenate			
Anova	F=4.2	v1=3, v2=96	P<0.05
S.N.K. dose 0 vs dose 2	q=1.14	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=0.24	v=96, p=3	P>0.05
(d) *Comparison*: SI of D6L4 homogenate			
Anova	F=1.29	v1=3, v2=96	P>0.05
(e) *Comparison*: SI of D6L4 ES(MT)			
Anova	F=4.02	v1=3, v2=96	P>0.05
S.N.K. dose 0 vs dose 2	q=2.67	v=96, p=2	P>0.05
S.N.K. dose 0 vs dose 10	q=4.37	v=96, p=3	P<0.05
S.N.K. dose 0 vs dose 50	q=0.54	v=96, p=3	P>0.05

Appendix 3: Statistical Analysis of Results from Chapter 5

Table A3.1 Epidemiological data from repeated low level infections in NIH mice

Comparison: Worm burdens for mice given L3 ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=1.449	v1=1, v2=26	P>0.05
Anova for time	F=20.73	V1=1, v2=26	P<0.05

Comparison: Sex ratio for parasites in mice given L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=0.0002	v1=1, v2=26	P>0.05
Anova for time	F=1.78	v1=2, v2=26	P>0.05

Comparison: Faecal Egg Output

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=0.19	v1=1, v2=84	P>0.05
Anova for time	F=1.84	v1=2, v2=84	P<0.05

Comparison: Spleen cell counts

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=1.86	v1=1, v2=84	P>0.05
Anova for time	F=2.9	v1=2, v2=84	P>0.05
Anova for treatment	F=0.01	v1=2, v2=84	P>0.05

Table A3.2 Immunoglobulin levels in NIH mice given abbreviated, repeated low level infections

(a) *Comparison*: Total antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.677	v1=1, v2=172	P>0.05
Anova for dose	F=63.49	v1=1, v2=172	P<0.05
Anova for treatments1	F=44.19	v1=2, v2=172	P<0.05
S.N.K. before and after vs after	q=11.3	v=172, p=2	P<0.05
S.N.K. before and after vs before	q=11.8	v=172, p=3	P<0.05
S.N.K. before vs after	q=0.35	v=172, p=2	P>0.05

Amount of antibody from each treatment

(i) L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=66.24	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 6	q=12.6	v=55, p=2	P<0.05

(ii) L3 before and after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=14.52	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=6.2	v=55, p=2	P<0.05
S.N.K. wk 6 vs wk 9	q=0.71	v=55, p=2	P>0.05

(iii) L3 before ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=4.21	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=1.4	v=55, p=2	P>0.05
S.N.K. wk 3 vs wk 6	q=4.05	v=55, p=2	P<0.05

S.N.K. wk 6 vs wk 9	q=2.01	v=55, p=22	P>0.05

(b) *Comparison*: IgG1 antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.575	v1=1, v2=172	P>0.05
Anova for dose	F=19.08	v1=1, v2=172	P<0.05
Anova for treatments1	F=38.29	v1=2, v2=172	P<0.05
S.N.K. before and after vs after	q=6.12	v=172, p=2	P<0.05
S.N.K. before and after vs before	q=12.4	v=172, p=3	P<0.05
S.N.K. before vs after	q=8.9	v=172, p=2	P>0.05

Amount of antibody from each treatment

(i) L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=156.2	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=12.6	v=55, p=3	P<0.05

(ii) L3 before and after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=22.69	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=7.63	v=55, p=2	P<0.05
S.N.K. wk 6 vs wk 9	q=0.98	v=55, p=2	P>0.05

(iii) L3 before ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=1.4	v1=2, v2=55	P>0.05

Table A3.3 Proliferation of cells of NIH mice given repeated low level infections of *H. polygyrus*. Results expressed as counts per minute

(a) *Comparison*: Response to adult homogenate.

Test	Statistic	Deg. Free.	Probability
Anova for dose	F= 5.35	v1=2, v2=97	P<0.05
S.N.K. dose 0 vs dose 10	q=1.79	v=97, p=2	P>0.05
S.N.K. dose 0 vs dose 50	q=3.52	v=97, p=3	P<0.05
Anova for time	F=4.75	v1=2, v2=97	P<0.05
S.N.K. wk 6 vs wk 9	q=4.44	v=97, p=2	P<0.05
Anova for treatments	F=1.63	v1=2, v2=97	P>0.05

(b) *Comparison*: Response to D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=5.79	v1=2, v2=97	P<0.05
S.N.K. dose 0 vs dose 10	q=4.71	v=97, p=2	P>0.05
S.N.K. dose 0 vs dose 50	q=3.56	v=97, p=3	P<0.05
Anova for time	F=5.21	v1=2, v2=97	P<0.05
S.N.K. wk 6 vs wk 9	q=3.57	v=97, p=2	P<0.05
Anova for treatments	F=7.87	v1=2, v2=97	P<0.05
S.N.K. before vs after	q=4.7	v=97, p=2	P<0.05
S.N.K. before & after vs before:	q=4.4	v=97, p=3	P<0.05
S.N.K. after vs before	q=0.13	v=97, p=2	P>0.05

(c) *Comparison* responses to other antigens

Test	Statistic	Deg. Free.	Probability
Anova for Adult ES(MT)	F=2.28	v1=2, v2=97	P>0.05
Anova for D6L4 homogenate	F=1.07	v1=2, v2=97	P>0.05

Anova for L3 homogenate	F=0.94	v1=2, v2=97	P>0.05

Table A3.4 Proliferation of cells of NIH mice given repeated low level infections of *H. polygyrus*. Results expressed as stimulation indices

(a) *Comparison*: response to adult homogenate

Test	Statistic	Deg. Free.	Probability
Anova on dose	F=3.35	v1=2, v2=97	P<0.05
S.N.K. dose 0 vs dose 10	q=3.22	v=97, p=2	P<0.05
S.N.K. dose 0 vs dose 50	q=4.12	v=97, p=3	P<0.05
Anova on treatment	F=1.163	v1=2, v2=97	P>0.05

(b) *Comparison*: Response to D6L4 ES(MT)

Test	Statistic	Deg. Free.	Probability
Anova on dose	F= 1.63	v1=2, v2=97	P<0.05
S.N.K. dose 0 vs dose 10	q=5.42	v=97, p=2	P<0.05
S.N.K. dose 0 vs dose 50	q=4.49	v=97, p=3	P<0.05

Table A3.5 Epidemiological data from repeated low level infections in CBA mice

Comparison: Worm burden

Test	Statistic	Deg. Free.	Probability
Anova for treatment	F=49.56	v1=1, v2=50	P<0.05
(i) *Comparison*: In mice given L3 after ivermectin			
Anova for time	F=7.39	v1=1, v2=26	P<0.05
S.N.K. wk 3 vs wk 9	q=4.27	v=26, p=3	P<0.05
Anova for dose	F=22.51	v1=1, v2=26	P<0.05
(i) *Comparison*: In mice given L3 before & after ivermectin			
Anova for time	F=0.47	v1=1, v2=22	P>0.05
Anova for dose	F=0.41	v1=2, v2=22	P<0.05

Comparison: Sex ratio for parasites in mice given L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=1.64	v1=1, v2=49	P>0.05
Anova for time	F=1.53	v1=2, v2=49	P>0.05
Anova for treatment	F=13.81	v1=2, v2=49	P<0.05

Comparison: Faecal Egg Output

Test	Statistic	Deg. Free.	Probability
Anova for doses	F=2.83	v1=1, v2=79	P>0.05
Anova for time	F=0.29	v1=2, v2=79	P>0.05
Anova for treatment	F=9.68	v1=1, v2=79	P>0.05

Comparison: Spleen cell counts

Test	Statistic	Deg. Free.	Probability
Anova for dose	F=1.86	v1=1, v2=84	P>0.05
Anova for time	F=2.9	v1=2, v2=84	P>0.05
Anova for treatment	F=0.01	v1=2, v2=84	P>0.05

Table A3.6 Immunoglobulin levels in CBA mice given abbreviated, repeated low level infections

(a) *Comparison*: Total antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.5	v1=1, v2=159	P>0.05
Anova for dose	F=41.31	v1=1, v2=159	P<0.05
Anova for treatments1	F=116.2	v1=2, v2=159	P<0.05
S.N.K. before and after vs after	q=9.19	v=159, p=2	P<0.05
S.N.K. before and after vs before	q=12.1	v=159, p=3	P<0.05
S.N.K. before vs after	q=3.36	v=159, p=2	P>0.05

Amount of antibody from each treatment

(i) L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=56.45	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=12.6	v=55, p=3	P<0.05
S.N.K. wk 6 vs wk 9	q=1.97	v=55, p=2	P>0.05

(ii) L3 before and after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=0.52	v1=2, v2=49	P>0.05

(iii) L3 before ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=0.341	v1=2, v2=47	P<0.05

(b) *Comparison*: IgG1 antibody levels

Test	Statistic	Deg. Free.	Probability
Anova on antibody to D6L4 or adult	F=0.50	v1=1, v2=159	P>0.05
Anova for dose	F=33.71	v1=1, v2=159	P<0.05
Anova for treatments1	F=62.22	v1=2, v2=172	P<0.05
S.N.K. before and after vs after	q=12.51	v=159, p=2	P<0.05
S.N.K. before and after vs before	q=15.9	v=159, p=3	P<0.05
S.N.K. before vs after	q=3.36	v=159, p=2	P<0.05

Amount of antibody from each treatment

(i) L3 after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=125.74	v1=2, v2=55	P<0.05
S.N.K. wk 3 vs wk 9	q=17.2	v=55, p=3	P<0.05
S.N.K. wk 6 vs wk 9	q=3.8	v=55, p=2	P<0.05

(ii) L3 before and after ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=3.87	v1=2, v2=49	P<0.05
S.N.K. wk 3 vs wk 9	q=3.53	v=49, p=2	P<0.05

(iii) L3 before ivermectin

Test	Statistic	Deg. Free.	Probability
Anova for time	F=0.34	v1=2, v2=47	P>0.05

Table A3.7 Proliferation of cells of CBA mice given repeated low level infections of H. polygyrus. Results expressed as counts per minute

(a) *Comparison*: Response to adult homogenate.

Test	Statistic	Deg. Free.	Probability
Anova for dose	F= 12.76	v1=2, v2=86	P<0.05
S.N.K. dose 0 vs dose 10	q=0.61	v=86, p=2	P>0.05
S.N.K. dose 0 vs dose 50	q=4.91	v=86, p=3	P<0.05
Anova for time	F=8.1	v1=2, v2=86	P<0.05
S.N.K. wk 6 vs wk 9	q=4.58	v=86, p=2	P<0.05
Anova for treatments	F=0.97	v1=2, v2=86	P>0.05

(b) *Comparison* Response to other antigens

For all other antigens, where any significance was found, it was the cells from mice given no larvae that proliferated most of all.

Appendix 4a Serological reagents and equipment

(a) Buffers

PBS	.85 g NaCl, 1.28 g Na_2HPO_4,
	.0.156 g $NaH_2PO_4.2H_2O$, 1 l dist. H_20..
Incubation Buffer	.100 ml PBS, 0.05 ml Tween 20 (Sigma Ltd, U.K.).
Coating Buffer	.1.59 g Na_2CO_3, 8.9 g $NaHCO_3$, 1 l dist. H_2O, pH 9.6.
Washing Buffer	.9 g NaCl, 0.5 ml Tween 20, 1 l dist. H_2O.
Substrate Buffer	.7.19g Na_2HPO_4, 5.19g citric acid, 1 l dist H_2O.
Stock substrate	.100 mg orthophenylamine diamine, 10 ml methanol.
Substrate solution	.100 ml substrate buffer, 1 ml stock substrate,
	.10ul 30% H_2O_2.

Unless otherwise stated all reagents were obtained from BDH Ltd., U.K...

(b)Equipment

Linbro ELISA plates	.Flow Laboratories, Harefield, Middx...

Appendix 4b *In vitro* lymphocyte proliferation assay equipment and reagents

(a) Constituents of cell growth medium (for 100 ml)

1.0 ml	.100 mM sodium pyruvate
1.0 ml	.200 mM L-glutamine
1.0 ml	.100x solution of non-essential amino acids
1.0 ml	.Hepes buffer
1.5 ml	.7.5% sodium bicarbonate
1.0 ml	.5000 units/ml penicillin/streptomycin
0.2 ml	.10 mg/ml genatmycin
93.3 ml	.RPMI 1640

All reagents were obtained from Gibco Ltd., U.K.

(b) Hypotonic lysis solution (for 1 l) (Enriquez *et al., 1986)*

NH_4Cl	8.19 g
$KHCO_3$	1 g
EDTA	0.037 g
dist. H_2O	1 l

(c) Equipment

Nunc 96f, tissue culture plates	Gibco Ltd., U.K.
200 ml tissue culture flasks	Celcult, Sterilin Ltd., Feltham, U.K.
Multimash 200 cell harvester	Dynatech Ltd., U.K.
934 AH glass fibre filter paper	Whatman Ltd., U.K.
LP3 tubes	Luckham Ltd., U.K.
13.5 ml conical bottom tubes	Sterilin Ltd., Feltham, U.K.
7 ml bijou bottle	Sterilin Ltd., Feltham, U.K.

GENERAL DISCUSSION

On infection of the mouse with the intestinal parasite *Heligmosomoides polygyrus*, a number of physiological and immunological changes occur. The immunological changes are apparent in the cellular and humoral compartments of the immune system. They have been described in a number of papers and their relevance in protection has been discussed in the introductory chapter.

In this study, in an attempt to approximate naturally occurring patterns of infection, two strains of mice were given repeated low level (trickle) doses of the parasite. The two strains of mice exhibited different response dynamics. The changes in their parasite numbers were compared to their immune responses in an attempt to identify changes that correlated with resistance.

In a number of experiments, performed to establish lymphocyte proliferation assay conditions, strong indications of organ specificity were noted. It was observed that in the majority of cases the cells from the mesenteric lymph node responded only to the D6L4 products. On direct comparison of the responses of the cells from the MLN and cells from the spleen, this specificity of response was confirmed. It was also apparent that cells capable of responding to the adult homogenate were obtainable only from the spleen. It should be stressed that although a response could be detected it was not necessarily indicative of resistance.

Within the discussion of chapter three, a number of hypotheses were proposed to account for the organ specific responsiveness. One hypothesis is that there are differences in the cellular composition of the organs. If the cells have different antigen presenting or T-cell populations then differences in the patterns of antigen presentation or recognition may result. These differences in the two organs' populations may then be apparent as differences in *in vitro* reactivity of their cells. The difference in responses to the antigens could, however, be a result of the anatomical location of the stages producing the antigens.

It has been widely demonstrated that systemic immune responses can be suppressed after oral feeding of an antigen (see Bienenstock and Befus, 1983; Pen, Turner and Strobel, 1988). It is thought that this depression occurs as antigen presentation within the gut associated lymphoid tissue leads to the generation of antigen specific T-suppressor cells. These cells are thought to migrate to the MLN (and subsequently to the spleen) where they depress the proliferation of antigen-specific lymphocytes. *In vitro*, their effect can be seen as a depression of antigen-specific proliferative responses.

Adult *H. polygyrus* resides in the lumen of the intestine and its antigens are shed into this lumen. Thus the mechanism of induction of oral tolerance may be applicable to this system. If it is, it may explain the lack of an observable *in vitro* response of MLN cells to the adult's products. In support of this hypothesis, the different route of uptake of the D6L4 antigens would be less likely to result in the generation of suppressor cells. The occurrence of cells capable of responding to the D6L4's antigens in the MLN, but not the spleen, may reflect the MLN's position in the hierarchy of the secondary lymphoid system. However, it has been reported that cells generated in the GALT can depress the response in the spleen as well as the MLN (see Pen et al., 1988). Depression in the spleen occurs after depression in the MLN. Thus it may be that if the cellular response was assayed at a later time, no response (or a one significantly smaller than that observed) would have been noted. The fact that the response to the adult homogenate of the spleen cells from trickle infected mice homogenate continued to increase with time would suggest that this depression is unlikely to occur. It is possible that antigen-specific suppression did occur in the spleen and that the response, although not ablated, is lowered. Alternatively, the cells may be localised only in the MLN, as suggested by Enriquez et al. (1986). However, they were not able to suppress the in vitro response of spleen cells by the transfer of MLN cells.

It should be noted that the response to the adult homogenate occurred when cells from mice, whose infection had been terminated before adults developed, were cultured with it. When cells from NIN mice which had lost their parasites after repeated infection were cultured with the adult homogenate, they also proliferated to it. The latter response indicates the existence of memory cells, whereas the former case suggests that there must be cross-reaction between the antigens of the stages. If antigen presentation in the gut does lead to the generation of adult-antigen specific suppressor cells and if these cells localise within the MLN then they must also suppress the reaction to the cross-reactive larval product. Proliferation to the D6L4 product, and the adult products, would only be observable where these cells have not localised i.e. in the spleen.

Reaction of spleen cells to the D6L4 antigens was seen when the response was assayed during a trickle infection, although the cells which responded were generally from those mice (especially CBA mice) which received the larger doses of the parasite. This would suggest that cells in the spleen, whose role in antigen processing is probably secondary to that of the MLN, only process antigen when the handling capacity of the MLN is exceeded.

In the course of the experiments it was noted that there were differences in the patterns of the response of cells to the D6L4 and adult antigen preparations. When cells were cultured with the homogenates a definite peak was seen at a given antigen concentration whereas, in comparisin, the response to the ES(MT)s plateaued at the highest antigen concentrations. It is possible that these patterns of response reflect differences in the proliferation inducing constituents of the preparations. The single peak of response may be because within the preparation there is only one component that causes proliferation (alternatively there may be many constituents but all induce the same pattern of response). The lack of a definite peak of proliferation in the response to the ES(MT)s may be because they have more than one component capable of inducing proliferation. If the peak of

response for each constituent occurs at different concentrations and the proliferation that each constituent induces at a given concentration of antigen is additive then no definite peak in the proliferative response would be seen. The same phenomenon could also occur if the time at which maximal proliferation occurred for each constituent was different, even if the actual concentration at which this occurred was the same for each constituent.

The biological significance of this phenomenon is not certain. If not an artefact of the preparation of the antigens, the range of concentrations over which proliferation occurs may reflect the changes in the composition of the ES(MT) during larval development (Ey, 1988). The patterns of reactivity may also relate to the importance of ES(MT) as an immunogen and the broad range of reactions reflecting responses to a number of important parasite molecules.

It would be possible to test these hypotheses and those concerning the observed cross-reactivity by using a technique known as a T-cell western. Although not reported in here, this technique has been developed in this laboratory for use in assessing lympho-proliferative responses during nematode infection. In the assay, components of an antigen separated electrophoretically are tested for their ability to induce proliferation. This technique could also be used to test the hypothesis that the range of antigens recognised by T-cells is greater in high responders than low responders, especially relevant as this phenomenon of recognition differences has been reported for the humoral response (Wakelin, 1985).

The level of response to each antigen may reflect the immunogenicity of the stages which may in turn relate to their inherent properties as well as their relative exposure to the immune system.

On repeated infection of the two strains, markedly different patterns in dynamics of their parasite populations were noted. In the high responder NIH strain, the worm burden peaked after two weeks whereas in the low responder CBA strain, the worm burden was seen to plateau much later. The persistence of

The resistance of the mice could be expressed as reduced establishment of the larvae, a retardation in the development time of the larvae or a reduction in the survival of the adults. The use of radio-labelled larvae would allow the importance of each possibility to be determined.

The lack of any larval-dose related effects on individual female fecundity would also suggest that density-dependence is not affecting egg production. However, it may have been that, due to the low infectivity of the larvae, the numbers of worms that established were too low for density-dependence mechanisms to come into play. A lack of any density-dependent effect was also noted when the egg production of parasites in CBA mice, which had been infected at different times relative to anthelmintic treatment, was examined. Furthermore, although the mice which had received larvae before and after ivermectin had lower worm burdens than the mice which had received larvae only after ivermectin, the fecundities of the females in the mice from the two groups were not different. This would suggest that the anti-fecundity effects are different to the anti-establishment/survival effects. These effects may be under separate genetic control or the processes that lead to each may be influenced to different extents by the parasite. Furthermore, although a reduction in fecundity and worm expulsion occur at the same time in NIH mice it is likely that the link is only temporal.

These differences in worm burden and egg-production in the two strains are not reflected in the humoral responses. This would indicate that antibody is not the sole effector of resistance. However, there was a relationship between dose and antibody production: the larger the weekly dose the greater the production of antibody and there was a positive correlation between cumulative dose and measured antibody levels. This would suggest that antibody production is related to stimulation. Experiments in which mice were infected with larvae at different times relative to ivermectin treatment support the notion that antibody production is related to stimulation, in this case the input of larvae/antigen. It is hypothesised

the parasite in CBA mice and the observation that the dynamics of loss of the parasite from the NIH mice do not resemble the loss of parasites such as *T. spiralis* suggests that both hosts are susceptible to the immunosuppressive effects of the parasite -albeit to different extents.

The lower worm burdens in the NIH mice, as compared to those in the CBA mice, would suggest that the strain is less susceptible to the immunosuppressive effects of the parasite. It could be that the CBA strain has a lower threshold of susceptibility to the immunomodulatory factors of the parasite.

The difference in parasite numbers could also be because a certain number of worms (or their products) is necessary before the mechanisms that result in effective resistance are stimulated i.e. the critical effector mass of Bell and Liu (1988). It is likely that the ability to recognise/ respond to antigen is dependent on the host genetic background. It is possible that this threshold is higher in CBA mice than it is in NIH mice. It could also be that the rate at which resistance develops in CBA mice is lower than in NIH mice and thus more worms develop before establishment/worm survival is curtailed.

That resistance can develop is, in part, indicated by the plateauing of the worm burdens in CBA mice. However, it must be noted that simple immigration (infection) models show that a plateau in worm burdens can result, under trickle conditions, even in the absence of an effective immune response. The observation that mice which had been given parasites before and after ivermectin had less worms than those mice which had been given larvae only after ivermectin also supports the supposition that resistance can develop. The development of resistance may have resulted from the removal of the adults by anthelmintic treatment or, alternatively, it may have been a function of past experience of the parasite. The use of an uninterrupted trickle started at the same time as the interrupted infection regime would have allowed more information to be obtained, but logistic constraints meant that it could not be performed.

that as well as playing a part in worm rejection, one of the functions of antibody is the removal of antigen. It may be possible to correlate antibody production with resistance but this would probably require the use of certain antigens (e.g. those from the cuticular surface or in the ES(MT)) rather than the ill-defined mixtures used in this study.

The proliferation of spleen cells from the two strains of trickled mice is larval dose-dependent, the larger the dose of larvae given the greater the amount of proliferation. This may be because with increasing dose the number of effectors, be they cells or antibodies, must also increase and the production of these effectors is ultimately under the control of T-cells.

A difference between the two strains was noted when their in vitro lympho-proliferative responses were considered. Under the same assay conditions, the responses of the NIH's spleen cells to the antigens of H. polygyrus were greater than those of the CBA mice's spleen cells. This difference could reflect differences in the suppression of the anti-parasite repones, due to differences in the susceptibility of the two strains to the parasite's immunomodulation (e.g. generation of suppressor cells due to inappropriate presentation as a result of infection with the parasite).

In addition to the level of proliferation being different, the change in proliferation with time was not the same. The proliferation shown for the NIH spleen cells increased with time, whereas the pattern for the CBA cells was convex. These responses should be compared to the humoral responses, which increased in both strains. It would, therefore, seem that the humoral and cellular arms of the immune response are under separate control. This is in accordance with the work of Mossmann and Coffman (1987). They provide evidence for two types of helper clones: Th1 which produces IL2 and gamma interferon and plays a part, in vitro, in mediating delayed type hypersensitivity responses; and Th2 which produces IL4 and IL5, aiding IgE and IgG1 production. It may be that

prolonged infection with H. polygyrus causes a down regulation in Th1 function whilst not affecting Th2 production, although how this would be achieved is not certain. Nevertheless, it would be interesting to determine whether interleukin production (and, therefore, T helper function) alters during an infection and if the production correlates with responder status.

In summary, differences were apparent in the responses of the two strains to the parasite and the population dynamics of the parasites within the two hosts. The parasite numbers and fecundities were higher in the CBA strain than in the NIH strain. These differences were reflected in some, but not all, measures of the immune response. It was noted that the in vitro lympho-proliferative response of the spleen cells of CBA mice was lower than the response of spleen cells from NIH mice. It was postulated that these differences were the result of genetically determined differences in antigen recognition and, possibly, the ability to overcome the parasite's immunosuppressive effects. The patterns of change in lympho-proliferative response with time were different between the two strains and it was suggested that this was due to differences in the function of regulatory T-cells in each strain. The marked similarity of the humoral response in the two strains suggests that antibodies are not the sole determinants of resistance. It also leads to the suppositon that the humoral and cellular responses are under separate control.

It was also observed that there was organ-specific reactivity to the antigens of different stages. It was suggested that this was the resukt of the localisation of specific suppressor cells or, alternatively differences in the cellular composition of the two organs. Differences were also seen in the patterns of response to the homogenates and the ES(MT)s and it was hypothesised that this was a result of differences in the composition of the two types of antigens.